

Original Article

Immunohistochemical analysis of oxidative stress-related molecules and radiosensitivity of human esophageal cancer

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Abstract: Radiation therapy exerts antitumor effects by increasing the levels of reactive oxygen species (ROS) and oxidative stress in cancer cells. In this study, we evaluated the role of the oxidative stress-related molecules CAT, MnSOD, MPO, and eNOS in increasing the sensitivity of human esophageal cancer to radiation therapy. In addition, we evaluated the feasibility of using these biomarkers for selecting patients for radiation therapy at initial diagnosis. The study included 142 patients with esophageal cancer who underwent preoperative radiotherapy (36-70 Gy), followed by surgical resection of the cancer. Based on their pathological response to radiotherapy, the patients were classified into 2 groups: sensitive to radiation (SR group, no residual tumor cells in surgical specimens, 49 patients) and resistant to radiation (RR group, viable residual tumor cells in surgical specimens, 93 patients). CAT, MnSOD, MPO, and eNOS expression were determined by immunohistochemical analysis in biopsied tumor specimens obtained during the surgery. Expression of CAT, which neutralizes ROS, was low in 75.3% (37/49) SR patients and in 55.1% (51/93) RR patients. eNOS, which generates ROS, was expressed in 69.72% of patients with esophageal cancer, with high expression in 65.3% (32/49) and 41.9% (39/93) of SR and RR patients, respectively. No statistically significant differences were observed in MnSOD and MPO expression between SR and RR patients. We found that the CAT low expression and the eNOS high expression were significantly associated with radiosensitivity ($P = 0.026$ and $P = 0.013$, respectively). Moreover, CAT and eNOS expression are significant independent markers for predicting radiotherapy sensitivity.

Keywords: Catalase, endothelial nitric oxide synthase, radiation sensitivity, esophageal cancer, oxidative stress-related molecules, immunohistochemical staining

Introduction

Esophageal squamous cell carcinoma (ESCC), with a poor prognosis, is one of the fastest growing cancers worldwide [1] and remains a highly fatal disease despite the availability of aggressive therapy [2]. Concurrent radiotherapy is a standard therapeutic strategy for treating patients with ESCC who have a locally advanced disease [3]. However, clinical outcomes of patients with ESCC are heterogeneous. Treatment of patients with ESCC who have the same clinical stage with same multi-therapeutic strategies, including surgery, chemotherapy, and radiotherapy, often exerts dif-

ferent therapeutic effects, probably because of differences in the expression of key molecules in these patients [4].

Radiation therapy exerts antitumor effects and induces tumor cell death, partly by increasing the levels of reactive oxygen species (ROS) and oxidative stress in tumor cells [5]. Manganese superoxide dismutase (MnSOD) and catalase (CAT) play a crucial role in endogenous defense mechanisms against oxidative stress by converting superoxide radicals to H_2O_2 [6] and H_2O_2 to H_2O and O_2 , respectively [7]. In contrast, enzymes such as myeloperoxidase (MPO) and endothelial nitric oxide synthase (eNOS) gener-

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Table 1. Demographic and clinical characteristics of the study patients

Characteristics	UICC stage		Lymph node metastasis		
	I/II	III/IV	Yes	No	
Age (years)					
Mean	58.5	56.3	60.2	59.3	57.5
Range	39-79	39-72	41-79	40-79	39-75
Sex					
Male	90	38	52	47	43
Female	52	23	29	31	21
Tumor location					
Upper		17	23	22	18
Middle		24	32	32	24
Lower		20	26	24	22
Tumor length (cm)					
< 5		39	24	25	38
≥ 5		22	57	53	26
Histological grade					
Well		19	24	9	34
Moderate		23	27	31	19
Poor		19	30	38	11
Radiation effect					
RR		27	66	59	34
SR		34	15	19	30

ate ROS [8, 9]. Individual variability in the expression of oxidative stress-related genes may account for differences in the radiosensitivity of patients with ESCC.

Single nucleotide polymorphisms in oxidative stress-related genes are suggested to predict clinical responses of patients with breast cancer [10] and head and neck cancer to radiotherapy [11]. Sun et al. showed that MnSOD regulates the radiosensitivity of esophageal cancer cells bidirectionally [12]. However, it is unclear whether expression levels of oxidative stress-related genes in patients with esophageal cancer can serve as biomarkers of radiosensitivity and treatment outcomes.

In this study, we determined the expression levels of CAT, MnSOD, MPO, and eNOS in pretreatment biopsy samples of esophageal cancer and analyzed their correlation with the clinicopathological characteristics, survival, and disease-free survival of patients with ESCC. Our results suggest that expression levels of CAT and MnSOD are useful biomarkers for predict-

ing radiosensitivity and clinical outcomes in patients with esophageal cancer.

Materials and methods

Patients

We recruited 142 patients with ESCC who underwent radiotherapy, followed by esophagectomy, at the radiotherapy department of Jiangsu Cancer Hospital (Nanjing, China) between 2009 and 2013. The treatment schedules of these patients were decided based on their clinicopathological characteristics. All of the patients were Chinese. Informed consent was obtained from all of the patients according to criteria specified by the Jiangsu Cancer Hospital. The study protocol was approved by the ethics committee of the institution. All of the patients fulfilled the following criteria: (1) preoperatively staged with locally advanced (cT3, cN0+, cM0) disease according to the International Union Against Cancer tumor-node-metastasis classification; (2) no distant metastasis (except to the celiac lymph nodes and the supraclavicular); (3) no current or previous treatment with chemotherapy (to avoid potential confounding); and (4) no other severe medical disease. The clinicopathological characteristics of the patients are listed in **Table 1**. Patients with stage IV disease were not considered as candidates for definitive radiotherapy and therefore were excluded from the study. Follow-up information was obtained from office charts, hospital records, and telephonic interviews.

Radiotherapy

All of the patients underwent external beam radiotherapy with a 6-MeV high-energy linear accelerator. The initial treatment targeted primary tumors and enlarged lymph nodes. Three-dimensional treatment was performed under the guidance of computed tomography (CT) scans. Each patient received a median radiation dose of 42.9 Gy (range, 36-70 Gy; 2 Gy/fraction for 5 days per week), with a 3- or 4-field technique [13].

Surgery

After radiotherapy, each patient underwent esophagectomy and systematic mediastinal lymphadenectomy through video-assisted thoracoscopic surgery or limited thoracotomy on

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the right side, depending on the surgical approach used [14]. An experienced pathologist who was blinded to patient outcomes examined each resected specimen to evaluate the response to radiotherapy and expression of the oxidative stress-related proteins CAT, MnSOD, MPO, and eNOS. All of the patients were routinely admitted to the intensive care unit and placed on prophylactic mechanical ventilation after the surgery. Patients with no residual cancer cells in the resected specimen were classified as sensitive to radiation (SR, 49 patients), whereas those with residual viable cancer cells in the resected specimens were classified as resistant to radiation (RR, 93 patients).

Immunohistochemical staining

Expression levels of CAT, MnSOD, MPO, and eNOS in the resected specimens of 142 patients with ESCC were assessed by immunohistochemical staining, as described previously. Briefly, 5-mm paraffinized sections of the resected specimens were deparaffinized with 3 changes of xylene and dehydrated using a graded alcohol series. Next, endogenous peroxidase activity in these sections was blocked using 0.3% H₂O₂ in methanol. Furthermore, the sections were heated in 0.01 M citrate buffer (pH 6.0) for 45 min at 95°C for antigen retrieval. The sections were then incubated with 10% normal goat or rabbit serum albumin (blocking buffer) in PBS for 60 min to reduce nonspecific background staining. The sections were then drained off and incubated overnight at 4°C with working dilutions of primary antibodies against MnSOD, eNOS, CAT, and MPO (PA1-125, PA3-031A, PA5-23246, and PA5-16672, respectively; Pierce Antibody Products, Thermo Scientific, USA), according to the manufacturer's instructions. After 5 rinses with PBS, the sections were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Maixin-Bio, Fujian, China) for 1 h at 37°C. Immunostaining was visualized by staining the slides with diaminobenzidine (Maixin-Bio, Fujian, China) and by counterstaining with Meyer's hematoxylin, according to the manufacturer's instructions.

Expression levels of CAT, MnSOD, MPO, and eNOS in all the resected specimens of 142 patients with ESCC were assessed by a pathologist who was blinded to the clinical informa-

tion of the patients. Immunoreactivity of the specimens was graded into the following 4 groups based on the frequency of positively stained cells [15, 16]: grade 0 (negative), no specific staining or < 5% positive staining; grade 1, ≥ 5% to < 30% positive staining; grade 2, ≥ 30% to < 70% positive staining; and grade 3, ≥ 70% positive staining. Grades 0 and 1 were considered as low expression, and grades 2 and 3 were considered as high expression. In addition, 5 random fields of each section were examined at 200× magnification.

Follow-up evaluations

Follow-up evaluations were performed every 3-4 months after the surgery until the end of the study or death of patients. Follow-up evaluations such as clinical examination, esophagography, CT scan, chest radiography, abdominal ultrasonography, and bone scanning (when necessary) were performed to detect tumor recurrence and/or metastasis. Esophagogastroduodenoscopy and tumor biopsy were performed when indicated.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). IHC staining and multiple clinicopathological characteristics were compared using Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

Patient characteristics and distribution of CAT, MnSOD, MPO, and eNOS in patients with ESCC

The study included 142 patients with primary ESCC (**Figure 1**). The clinicopathological characteristics of these patients are listed in **Table 1**. The mean age of these patients was 58.5 years (range, 39-79 years), and the male-to-female ratio was approximately 0.63. Immunohistochemical staining showed that MPO was expressed in 111/142 (78.17%) patients, eNOS was expressed in 99/142 (69.72%) patients, CAT was expressed in 64/142 (45.07%) patients, and MnSOD was expressed in 43/142 (30.28%) patients. MPO was expressed in the cytoplasm of tumor cells. Specimens from 27 (19.01%), 48 (33.8%), and 36 (25.35%) patients were classified as grades 1, 2, and 3, respectively, based on MPO expression. eNOS was expressed in epithelial cells in ESCC

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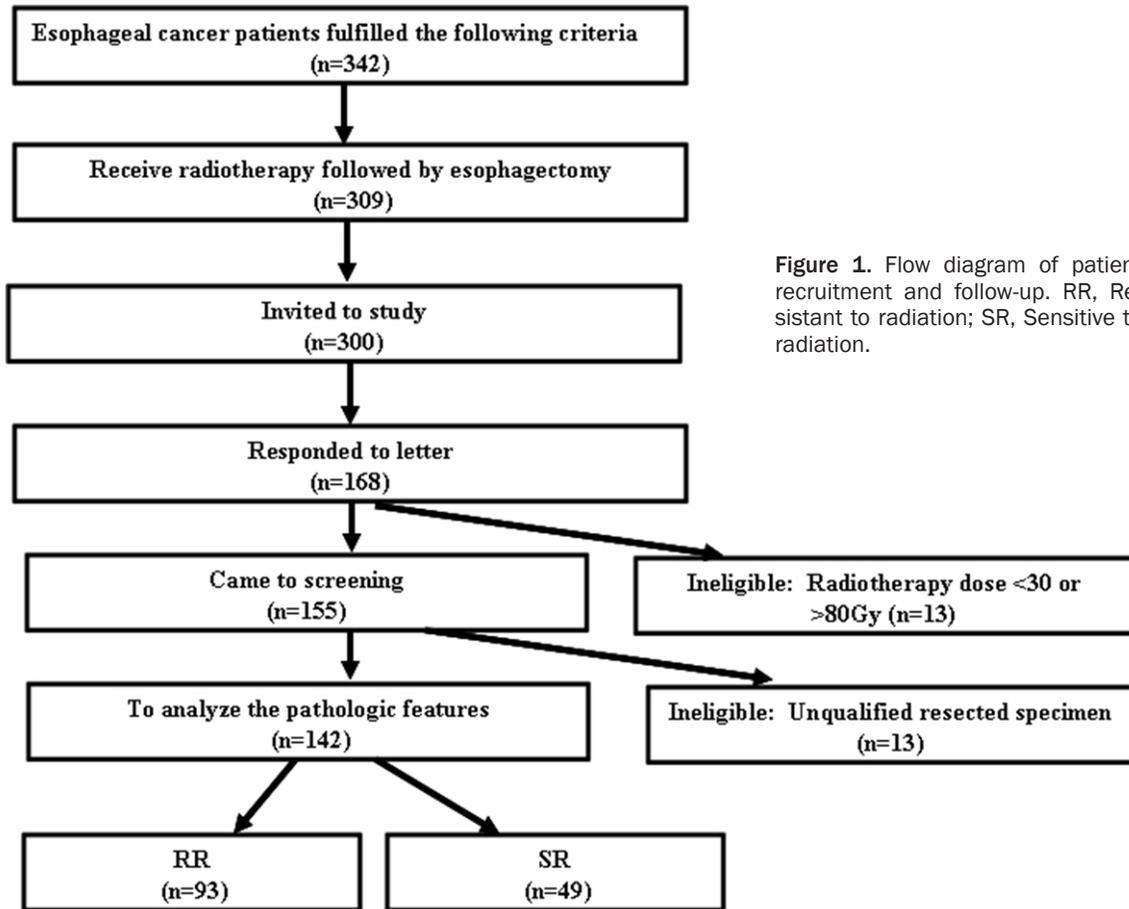


Figure 1. Flow diagram of patient recruitment and follow-up. RR, Resistant to radiation; SR, Sensitive to radiation.

specimens. Specimens from 28 (19.72%), 43 (30.28%), and 28 (19.72%) patients were classified as grades 1, 2, and 3, respectively, based on eNOS expression. CAT was mainly expressed in the cytoplasm of tumor cells and cells of tumor blood vessels. Specimens from 19 (13.38%), 28 (19.72%), and 17 (11.97%) patients were classified as grades 1, 2, and 3, respectively, based on CAT expression. MnSOD was expressed in the cytoplasm of tumor cells in specimens from 43, 23, and 9 patients. These specimens were classified as grades 1, 2, and 3, respectively (data not shown). All of the 4 oxidative stress-related molecules were expressed in 43 (30.28%) patients. Representative images of the immunostained slides are shown in **Figure 2**.

Correlation between immunohistochemical profiles and clinicopathological characteristics of patients with ESCC

Next, we examined the correlation between expression of oxidative stress-related molecules

(CAT, MnSOD, MPO, and eNOS) and clinicopathological characteristics of patients with ESCC. The relationship between clinicopathological characteristics of the patients and expression levels of oxidative stress-related molecules is summarized in **Table 2** (scored as low expression and high expression). Immunohistochemical analysis of MPO expression in 61 patients with stage I/II ESCC showed high expression in 29 (47.5%) patients and low expression in 32 (52.5%) patients. Among 81 patients with stage III/IV ESCC, MPO expression was high in 55 (67.9%) patients and low in 26 (32.1%) patients. Immunohistochemical analysis of eNOS expression in 78 patients with tumor metastasis showed low expression in 32 (41.0%) patients and high expression in 46 (59.0%) patients. In 64 patients without tumor metastasis, eNOS expression was high in 25 (39.1%) patients and low in 39 (60.9%) patients. Interestingly, eNOS and CAT expression was significantly correlated with radiosensitivity ($P = 0.013$ and 0.023 , respectively). However, CAT, MnSOD, MPO, and

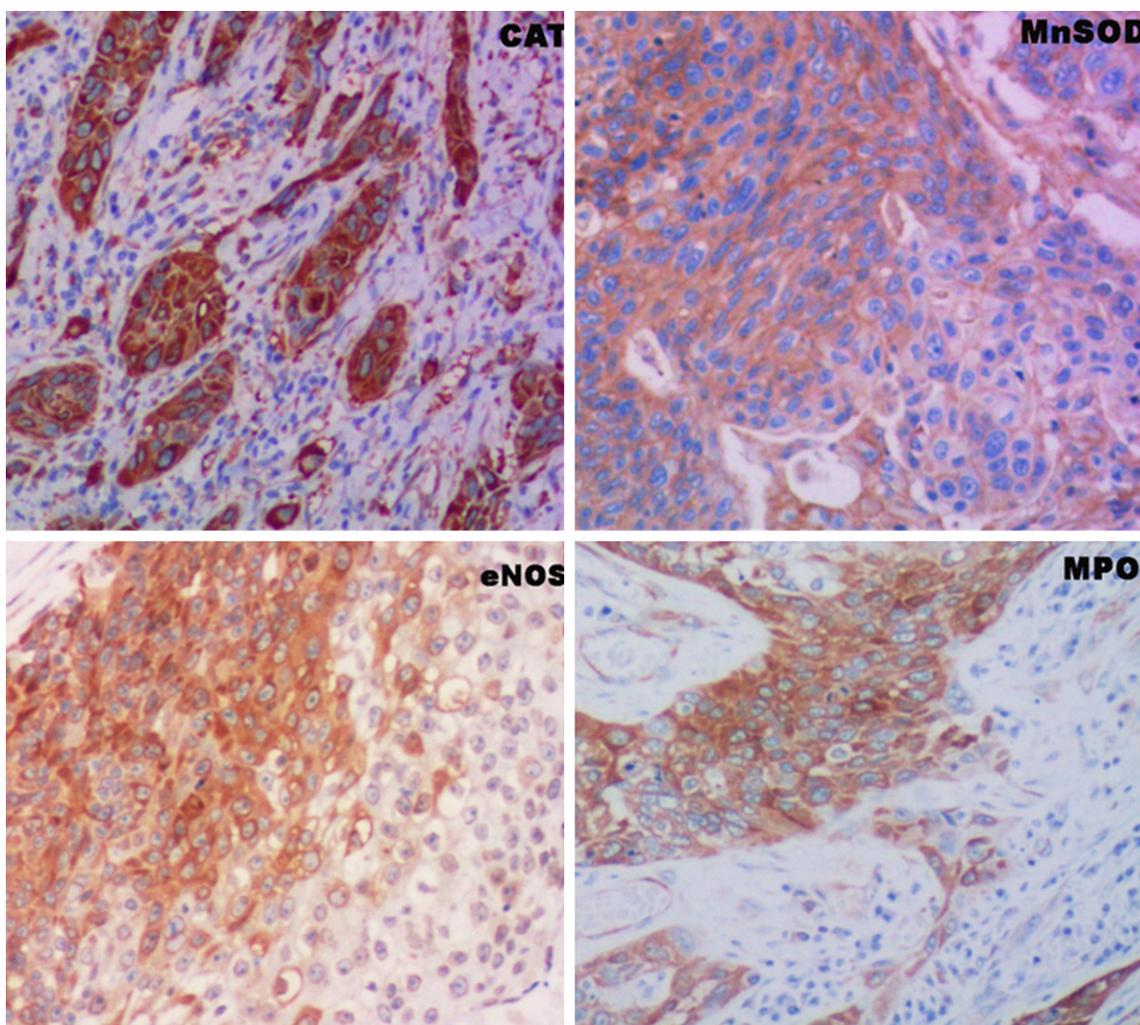


Figure 2. Representative images of positive immunohistochemical staining for CAT, MnSOD, eNOS, and MPO in human esophageal cancer tissues. Images were taken using the Olympus BX50 microscope (magnification, $\times 100$).

eNOS expression was not significantly correlated with age and histological grade (Table 3).

Correlation of CAT, MnSOD, MPO, and eNOS expression with the radiosensitivity of patients with ESCC

The correlation of clinicopathological characteristics (UICC stage and lymph node metastasis) and CAT, MnSOD, MPO, and eNOS expression with the radiosensitivity of RR (n = 93) and SR patients (n = 49) was determined based on the presence of residual cancer cells in resected tumor specimens obtained after radiotherapy.

High eNOS expression was observed in 39/93 (41.9%) RR patients and 32/49 (65.3%) SR

patients, and high CAT expression was observed in 23/93 (24.7%) RR patients and 22/49 (44.9%) SR patients. These results suggest that CAT and eNOS expression was strongly associated with the sensitivity of patients with ESCC to radiation therapy.

Coexpression of CAT and eNOS could be used for predicting sensitivity to radiotherapy because no correlation was observed between CAT and eNOS expression (Table 3). Furthermore, high coexpression of CAT and eNOS was observed in 16/49 (32.7%) SR patients but only in 1/93 (1.1%) RR patients (Table 4). Multivariate logistic regression analysis, which included CAT, MnSOD, MPO, and eNOS indices, indicated that CAT and eNOS were significant

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Table 2. Analysis of the expression of oxidative stress-related molecules in biopsy specimens

Parameters	eNOS expression		MPO expression		MnSOD expression		CAT expression	
	Low (%)	High (%)	Low (%)	High (%)	Low (%)	High (%)	Low (%)	High (%)
Sex								
Male	42 (46.7%)	48 (53.3%)	37 (41.1%)	53 (58.9%)	68 (75.6%)	22 (24.4%)	61 (67.8%)	29 (32.2%)
Female	29 (55.8%)	23 (44.2%)	21 (40.4%)	31 (59.6%)	42 (80.8%)	10 (19.2%)	36 (69.2%)	16 (30.8%)
p	0.384		0.926		0.612		0.994	
Grade								
Well/Moderate	49 (52.7%)	44 (47.3%)	38 (40.9%)	55 (59.1%)	70 (75.3%)	23 (24.7%)	62 (66.7%)	31 (33.3%)
Poor	22 (44.9%)	27 (55.1%)	20 (40.8%)	29 (59.2%)	40 (81.6%)	9 (18.4%)	35 (71.4%)	14 (28.6%)
p	0.48		0.861		0.515		0.696	
UICC stage								
I/II	35 (57.4%)	26 (42.6%)	32 (52.5%)	29 (47.5%)	45 (73.8%)	16 (26.2%)	40 (65.6%)	21 (34.4%)
III/IV	36 (44.4%)	45 (55.6%)	26 (32.1%)	55 (67.9%)	65 (80.2%)	16 (19.8%)	57 (70.4%)	24 (29.6%)
P	0.175		0.023		0.477		0.67	
Metastasis								
Yes	32 (41.0%)	46 (59.0%)	31 (39.7%)	47 (60.3%)	62 (79.5%)	16 (20.5%)	54 (69.2%)	24 (30.8%)
No	39 (60.9%)	25 (39.1%)	27 (42.2%)	37 (57.8%)	48 (75%)	16 (25%)	43 (67.2%)	21 (32.8%)
P	0.028		0.902		0.664		0.937	
Radiation effect								
RR	54 (58.1%)	39 (41.9%)	43 (46.2%)	50 (53.8%)	73 (78.5%)	20 (21.5%)	51 (55.1%)	42 (44.9%)
SR	17 (34.7%)	32 (65.3%)	15 (30.6%)	34 (69.4%)	37 (75.5%)	12 (24.5%)	37 (75.3%)	12 (24.7%)
P	0.013		0.105		0.847		0.026	

IHC staining and multiple clinicopathological characteristics were compared using Fisher's exact test.

Table 3. Relationship between CAT and eNOS expression

	eNOS expression grades				Total
	0	1	2	3	
CAT expression grade 0	8	5	8	2	23
1	24	6	11	7	48
2	11	14	9	3	37
3	9	20	3	2	34
Total	52	45	31	14	

Spearman's rank correlation test, P = 0.765, multivariate logistic regression analysis.

Table 4. Relationship between CAT/eNOS expression pattern and radiosensitivity

	CAT expression		High		Total
	Low	High	Low	High	
SR	8	12	13	16	49
RR	35	16	41	1	93
Total	43	28	54	17	

independent biomarkers for predicting the sensitivity of patients with ESCC to radiotherapy (Spearman's rank correlation test, P = 0.765).

Discussion

In this study, we evaluated the role of the oxidative stress-related molecules CAT, MnSOD, MPO, and eNOS in predicting the sensitivity of patients with esophageal cancer to radiation therapy by performing immunohistochemical staining. We observed that the CAT and eNOS expression levels were significant, independent predictors of radiosensitivity. Although numerous studies have reported the effect of genetic variants in candidate genes encoding oxidative stress-related molecules in response to radiotherapy [10, 11, 17], this is the first in vivo study to show that expression levels of CAT and eNOS are markers for predicting radiosensitivity.

Oxidant-antioxidant balance regulates the growth and invasion of tumor cells. CAT and MnSOD neutralize ROS, whereas MPO and eNOS generate ROS [18, 19]. Svensk et al. suggested that MnSOD levels were elevated in patients with lung carcinomas and that this increase was more prominent in patients with squamous cell lung carcinomas than in those with other histological types of lung carcinomas [20]. However, in our study, immunohistochemical analysis showed high expression of

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MnSOD and CAT in only a small number of patients with ESCC (22.54% and 31.69%, respectively), indicating a non-significant correlation of these markers with sex, grade, UICC stage, metastasis, and radiation effect. Some studies have suggested that downregulation of antioxidant enzymes (CAT and MnSOD) is associated with the carcinogenesis and progression in esophageal cancer.

Ambrosone et al. detected high MPO activity in patients with esophageal [21], gynecological [22], and colorectal cancers [23], and suggested that MPO genotype was associated with the efficacy of chemoradiotherapy and that certain genotypes improved the survival of patients with breast cancer [24]. Consistently, we observed that patients with stage III/IV ESCC showed higher expression of MPO than patients with stage I/II ESCC.

eNOS is involved in tumor angiogenesis and acceleration of tumor growth [25]. Kian-Huat Lim et al. observed that eNOS (or NOS3) is a downstream target of activated Ras and Akt, which are required for tumor growth and maintenance [26]. However, other studies have shown that high expression of eNOS may be cytotoxic and may promote cancer cell apoptosis [27]. Moreover, the distribution and functions of different eNOS isoforms in human esophageal cancer are not completely understood. In the present study, immunohistochemical staining showed that patients with metastasis had high eNOS expression (59% vs. 39.1%) and that these patients were radiosensitive compared with control patients who did not show metastasis. Although our results showed that expression levels of both CAT and eNOS were biomarkers of radiosensitivity of patients with esophageal cancers, these molecules may act as independent biomarkers of radiosensitivity.

Clinical features may also influence radiosensitivity. However, the patients in the present study were not significantly different regard to age, sex, UICC stage, tumor location and length; however, patients without lymph node metastasis were more sensitive than those with lymph node metastasis (81.5% VS 18.5%).

In conclusion, the results of the present study suggest that detection of CAT and eNOS in biopsied tumor samples is an important predic-

tor of the response of esophageal cancer to radiotherapy. Furthermore, our results suggest that compounds that elevate ROS levels, such as radiosensitizers, should be used in patients showing low eNOS or high CAT expression. Thus, our findings may contribute to the clinical application of these biomarkers and in the development of assays for determining the prognosis of ESCC in the future.

Disclosure of conflict of interest

None.

Authors' contribution

Minghua Ji designed the experiments, performed the histological analyses, interpreted and analyzed the data, and drafted the manuscript. Kewei Huang, Jing Han, and Wenwu Zha drafted the manuscript and helped to perform all of the experiments. Jinming Yu were involved in all the aspects of the study, including experimental design, analysis and interpretation of data, and manuscript writing. All the authors have read and approved the final manuscript.

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