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Original Article

Over-expression of lysine-specific demethylase 1 predicts tumor progression and poor prognosis in human esophageal cancer

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Abstract: Lysine specific demethylase 1 (LSD1), the first characterized histone demethylase, roles importantly in epigenetic regulation of carcinogenesis and cancer progression. In the present study, we examined LSD1 expression in 103 cases of esophageal cancer tissues, and further investigated its relationship to patient's clinical parameters and post-operative prognosis. We found that the positive LSD1 immunochemical staining was predominantly observed on the nuclei and cytoplasm of esophageal cancer cells, while negative or very weak in adjacent normal tissues. The intensity of LSD1 immunostaining was significantly correlated to the tumor size ($P = 0.013$), nodal metastasis ($P = 0.002$), distant metastasis ($P = 0.025$), and TNM stage ($P = 0.010$), whereas it was not correlated to patient's gender, age and tumor invasion depth. The overall survival rate of patients with low LSD1 expression was better than those with high LSD1 expression ($P = 0.014$). We also showed that the tumor size ($P = 0.003$) as well as the TNM stage ($P = 0.007$) was a useful prognostic predictor for esophageal cancer. However, when the gender, age, tumor size, TNM stage and LSD1 expression level were involved in the multivariate proportional hazards regression analysis in a Cox's model, we showed that the tumor size ($P = 0.013$) and the TNM stage ($P = 0.032$) could be used as independent risk factors to predict patient's postoperative prognosis, but LSD1 expression level as well as other factors could not independently predict patient's outcome. Thus, our results indicated that LSD1 was involved in cancer progression and metastasis in human esophageal cancer, and could be a potential prognostic predictor for this malignancy.

Keywords: LSD1, immunohistochemistry, prognosis, esophageal cancer

Introduction

Esophageal cancer is one of the most common types of human malignancies, and it ranks as the sixth cause of cancer deaths in the past year [1]. Esophageal cancer could be divided into two major types, adenocarcinomas and squamous cell carcinomas according to the histological classification [2-4]. And the esophageal squamous cell carcinoma constitutes more than 90% of all esophageal cancer cases worldwide [2, 5]. As of now, numerous types and combinations of surgery, chemotherapy and radiotherapy, as well as other therapeutic strategies, have been used over recent decades to the treatment of esophageal cancer, but those patients usually undergo local recurrence or distant metastasis after curative operation, due to the aggressive nature of this cancer type [2-4, 6]. Therefore, it is of great importance for us to investigate the molecular mechanism of the initiation and progression, and further find out the novel therapeutic targets for this cancer.

Lysine specific demethylase 1 (LSD1) was the first characterized histone demethylase, which could specifically remove H3K4 me1/2 through flavin adenine dinucleotide dependent oxidative reaction [7]. LSD1 is highly conserved from fission yeast to mammals, and consists of three protein domains, an N-terminal Swi3p/Rsc8p/Moira structural domain, a central protruding tower domain, and a C-terminal amine oxidase domain [8, 9]. It has been demonstrated that the over-expression of LSD1 could promote pro-

Table 1. Correlation between clinical parameters and LSD1 expression level in esophageal cancer tissues

Clinical parameters	Cases	LSD1 immunostaining			*P*-value
		H-score ≤ 235	*H-score* > 235	χ^2	
Gender					
Male	75	65 (86.7%)	10 (13.3%)	0.016	0.900
Female	28	24 (85.7%)	4 (14.3%)		
Age (years)					
< 60	63	55 (87.3%)	8 (12.7%)	0.110	0.740
≥ 60	40	34 (85.0%)	6 (15.0%)		
Tumor size (cm)					
< 3.5	38	37 (97.4%)	1 (0.6%)	6.159	**0.013**
≥ 3.5	65	52 (83.9%)	13 (16.1%)		
Tumor invasion depth (T)					
$T_1 + T_2$	59	53 (89.8%)	6 (10.2%)	1.378	0.241
$T_3 + T_4$	44	36 (81.8%)	8 (18.2%)		
Nodal metastasis (N)					
Yes	49	37 (75.5%)	12 (24.5%)	9.450	**0.002**
No	54	52 (96.3%)	2 (3.7%)		
Distant metastasis (M)					
Yes	16	11 (68.8%)	5 (31.2%)	5.029	**0.025**
No	87	78 (89.7%)	9 (10.3%)		
TNM stage*					
I	13	13 (100.0%)	0 (0.0%)	6.621	**0.010**
II	56	50 (89.3%)	6 (10.7%)		
III	18	15 (83.3%)	3 (16.7%)		
IV	16	11 (68.8%)	5 (31.2%)		

Values in bold signify $P < 0.05$. *Chi-square test for trend.

liferation, migration, and invasion in various cancer cells, and the abnormal expression of LSD1 in human cancer tissues significantly associated with cancer recurrence and poor prognosis [10, 11]. Moreover, Ding *et al.* [12] also demonstrated that the positive expression of LSD1 and negative expression of E-cadherin correlated with metastasis and poor prognosis of colon cancer patients, suggesting that the over-expression of LSD1 might contribute to poor outcomes of cancer patients via regulating the epithelial mesenchymal transition, which plays an essential role in the initial step of cancer metastasis.

In the present study, we examined LSD1 expression in 103 cases of esophageal cancer tissues by using immunohistochemistry, and further investigated its relationship to patient's clinical parameters and post-operative prognosis, in order to explore its value of clinical application in diagnosis and therapeutics against this malignancy.

Patients and methods

Patients and tissue samples

Esophageal cancer samples were collected from 103 patients who underwent surgical resection between January 2001 and March 2005 in the hospital (75 men and 28 women, median age at diagnosis was 58 years). No patients received preoperative chemotherapy or radiotherapy. All cases were confirmed as the esophageal squamous cell carcinoma (ESCC), and the tumor-node-metastasis (TNM) stages were assigned according to the American Joint Committee on Cancer Criteria [13]. Patients' clinical parameters are shown in the **Table 1**, and patients' survival intervals were dated to the end of Nov 2013. In addition, 5 cases of normal esophagus tissues from the non-malignant portion were collected and used as controls. The present study was approved by the ethics committee of the hospital.

Immunohistochemistry procedures

Immunohistochemical staining was performed by using the Dako EnVision™ technique according to the manufacturer's instructions (Dako, Glostrup, Denmark). In brief, formalin-fixed, paraffin-embedded tissues were cut into 3 μm-thick sections, and were then dewaxed in xylene, rehydrated and graded ethanol solutions. Antigen was retrieved by heating the tissue sections at 100°C for 30 min under EDTA solution. Sections were cooled and immersed in presence of 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity, and then rinsed in PBS for 5 min, blocked with 3% BSA at room temperature for 20 min, and then incubated with primary polyclonal antibody against LSD1 (diluted in 1:300, Novus Biologicals, Littleton, CO, USA) at 4°C overnight.

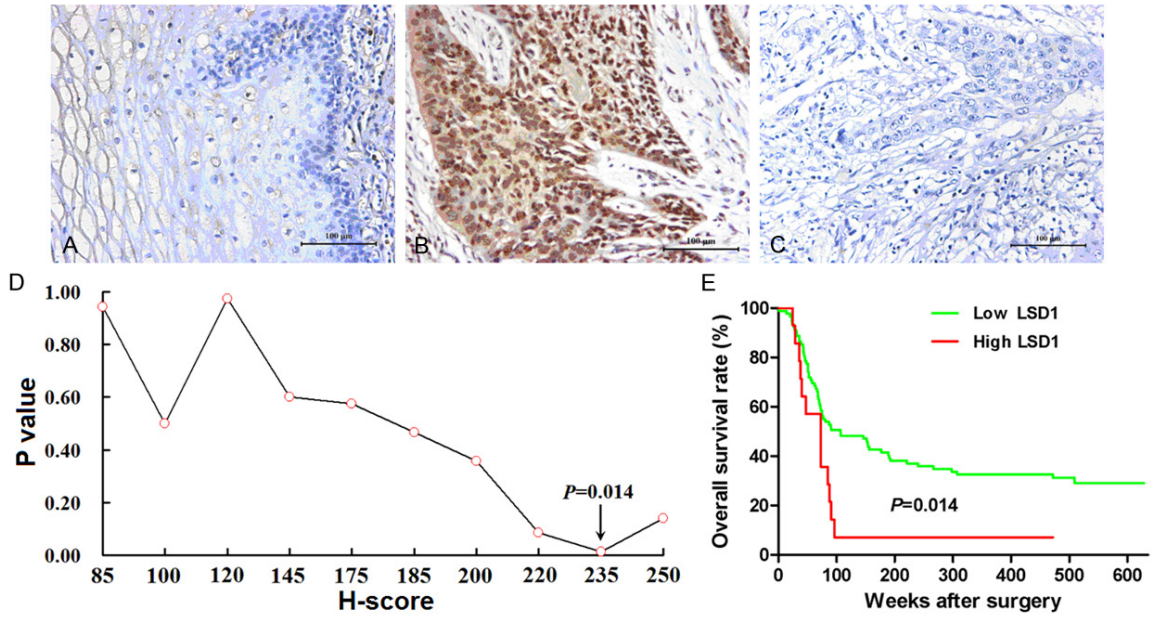

Figure 1. LSD1 expression in human esophageal cancer and its prognostic value. A. LSD1 was negatively or weakly expressed on normal esophagus tissues. B. LSD1 was positively expressed on the nuclei and the cytoplasm in esophageal cancer cells. C. Negative control. D. The minimum *P*-value seek was performed by using the log-rank survival analysis, and when the cut-off value of *H-score* = 235 was selected, the overall survival rate of patients with lower LSD1 expression (*H-score* ≤ 235) was better than those with higher LSD1 expression (*H-score* > 235) (*P* = 0.014, Hazard Ratio = 2.12, 95% CI: 1.15-3.89. E. The survival curve was shown when the cut-off value *H-score* = 235 was selected.

A negative control was performed by omitting the primary antibodies. The sections were then incubated with HRP-labeled goat anti mouse/rabbit secondary antibody (Ready to use, Dako, Glostrup, Denmark) for 30 min at 37°C. Diaminobenzene was used as the chromogen and hematoxylin as the nuclear counterstain. The sections were dehydrated, cleared and mounted.

Evaluation of LSD1 expression in esophageal cancer tissues

All slides were examined independently by two pathologists who were not informed patients' clinical data. The LSD1 immunostaining intensities were assessed according to the *H-score* method described by our previous reports [3, 4, 14]: *H-score* = (% tumor cells unstained × 0) + (% tumor cells stained weak × 1) + (% tumor cells stained moderate × 2) + (% tumor cells stained strong × 3). The *H-scores* ranged from 0 (100% negative tumor cells) to 300 (100% strong staining tumor cells). Results from the two pathologists were averaged and used in the statistical analysis. In the present study we ranks intensity of the immunochemical staining as, low intensity (*H-score* ≤ 235) and high intensity (*H-score* > 235), the cut-off value = 235 was selected by using the minimum *P*-value seek in the log-rank survival analysis, which was conducted according to the method from the literatures and our previous studies [3, 4, 14-16].

Statistical analyses

Statistical analysis was performed using the GraphPad Prism 5.0 software package (GraphPad Software, Inc., San Diego, USA). Paired Student's *t*-test, the Wilcoxon signed rank test or the survival analysis were used where appropriate. The Cox model was analyzed by the SPSS13.0 software package. A *p*-value of < 0.05 was deemed significant.

Results

Immunochemical staining of LSD1 in esophageal tissues

As shown in **Figure 1**, positive LSD1 immunochemical staining was predominantly observed on the nuclei and cytoplasm of esophageal

Table 2. Prognostic factors in Cox's proportional hazards model

Clinical parameters	Univariate			Multivariate		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
Gender						
Female/Male	1.24	0.76-2.04	0.393	1.26	0.76-2.08	0.378
Age (years)						
≥ 60/< 60	1.11	0.70-1.76	0.658	1.05	0.65-1.70	0.847
Tumor size (diameter, cm)						
≥ 3.5/< 3.5	2.17	1.31-3.61	**0.003**	1.98	1.16-3.37	**0.013**
TNM stage						
TNM_{3+4}/ TNM_{1+2}	1.91	1.19-3.06	**0.007**	1.74	1.05-2.88	**0.032**
LSD1 immunostaining						
High LSD/Low LSD	2.12	1.15-3.89	**0.014**	1.34	0.69-2.60	0.397

Values in bold signify $P < 0.05$.

cancer cells (**Figure 1B**), while none or very weak staining was found in normal esophageal tissues (**Figure 1A**). We could found LSD1 immunostaining could be found in all the specimens of esophageal cancer, and according to the evaluation of LSD1 immunostaining intensity by *H-score* method, we found and characterized that, 89 out of 103 (86.4%) cases of esophageal cancer patients with low intensity (*H-score* ≤ 235), and 14 out of 103 (13.6%) cases of esophageal cancer patients with high intensity (*H-score* > 235).The cut-off value of *H-score* = 235 was selected according to the minimum *P*-value seek in the log-rank survival analysis (**Figure 1D** and **1E**), which has been described in the literatures and our previous studies [3, 4, 14-16].

LSD1 immunochemical staining in relation to patients' clinical data and prognoses

As shown in **Table 1**, the intensity of LSD1 immunostaining was significantly correlated to the tumor size ($P = 0.013$), nodal metastasis ($P = 0.002$), distant metastasis ($P = 0.025$), and TNM stage ($P = 0.010$), whereas it was not correlated to patient's gender, age and tumor invasion depth. When the cutoff value of *H-score* = 235 was selected, the overall survival rate of patients with low LSD1 expression was better than those with high LSD1 expression ($P = 0.014$, Hazard Ratio = 2.12, 95% CI: 1.15-3.89, **Figure 1D** and **1E**). We also showed that the tumor size ($P = 0.003$) as well as the TNM stage ($P = 0.007$) was also a useful prognostic predictor for esophageal cancer (**Table 2**). However, when the gender, age, tumor size, TNM stage and LSD1 expression level were involved in the multivariate proportional hazards regression analysis in a Cox's model, we showed that the tumor size ($P = 0.013$) and the TNM stage ($P = 0.032$) could be used as independent risk factors to predict patient's postoperative prognosis, while LSD1 expression level as well as other factors could not independently predict esophageal cancer patient's outcome (**Table 2**).

Discussion

Epigenetic alteration is a current hallmark in cancer biology. The epigenetic mechanisms role importantly in carcinogenesis and cancer progression, which include DNA methylation, histone modifying enzymes and their histone modifications [17]. As of now, many attractive targets from the epigenetic research of cancer biology have been developed as novel prognostic biomarkers, and further explored for drug development [18]. It has been demonstrated that some histone-modifying enzymes, such as lysine-specific demethylase 1 (LSD1) and histone deacetylase 2 (HDAC2), are involved in the initiation and progression of many human cancers [19]. In the present study, we examined LSD1 expression in esophageal cancer tissues by using immunohistochemistry, and further investigated its relationship to patient's clinical parameters and post-operative prognosis.

Our immunohistochemistry study results showed that positive LSD1 immunochemical staining could be found in all the specimens of esophageal cancer, while it was negative or very weak in adjacent normal esophagus tissues. Based on the classification of low/high expression levels of LSD1 in esophageal can-

cer tissues, we found that the LSD1 expression in esophageal cancer was significantly correlated to the tumor size, nodal metastasis, distant metastasis, and TNM stage, whereas it was not correlated to patient's gender, age and tumor invasion depth. Shinya *et al.* [20] suggested that the over-expression of LSD1 contributes to human carcinogenesis through chromatin regulation in various cancers. Yu *et al.* [21] also showed that LSD1 expression in human esophageal cancer significantly associated with nodal metastasis status. In human hepatocellular carcinoma, over-expression of LSD1 significantly associated with tumor stage and tumor grade [11]. And in human colon cancer, the LSD1 expression level significantly associated with tumor stage and distant metastasis [12]. Thus, in combination with the results from other groups, our results suggested that LSD1 expression in esophageal cancer was involved in the initiation and progression of this malignancy, particularly contributed to the nodal metastasis and the distant metastasis.

The prognostic value of LSD1 has been a well-concerning focus in human cancers. Yuan *et al.* [22] reported that the high expression of LSD1 associates with cancer cell proliferation and unfavorable prognosis in tongue cancer. Over-expression of LSD1 in hepatocellular carcinoma tissues could also predict poorer outcome of the patients [11]. Ding *et al.* [12] showed that the colon cancer patients with lower LSD1 expression favor better post-operative prognoses than the patients with higher LSD1 expression. In our present study, we found that the LSD1 expression level as well as tumor size, tumor stage could predict patients' survival by using the log-rank survival analysis, and the esophageal cancer patients with lower LSD1 expression represented better prognoses than the patients with higher LSD1 expression. However, in the COX model analysis, we could only found that tumor size and TNM stage could be used as the independent risk factors for predicting esophageal cancer patients' survival, while the LSD1 expression level couldn't. In future, an expanded sample of esophageal cancer was needed to further investigate its application in predicting prognosis as an independent risk factor in this malignancy.

It is needed to be concerned that LSD1 roles importantly in the alteration of cancer cell phenotype, especially in the regulation of epithelial-to-mesenchymal transition (EMT) [23]. In human colon cancer, the LSD1 expression was negatively associated with E-cadherin expression, suggesting an essential role of LSD1 in regulating the EMT of cancer cells [12]. Lin *et al.* [24] demonstrated that the transcription factor Snai1 could interact with and recruit LSD1 to epithelial gene promoters, and in the absence of LSD1, Snai1 failed to repress E-cadherin, indicating that the LSD1 could be a potential therapeutic target for prevention of EMT-associated tumor invasion. We have previously demonstrated that the EMT status in human esophageal cancer significantly associated with invasion, metastasis and prognosis (data not shown). In future, the detailed mechanism of LSD1 in regulating the EMT of esophageal cancer cells might help us to further reveal the aggressive nature of this malignancy.

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Disclosure of conflict of interest

None.

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