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High expression of lysine-specific demethylase 1 correlates with poor prognosis of patients with esophageal squamous cell carcinoma ☆

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ABSTRACT

Recent studies have elucidated the role of lysine-specific demethylase 1 (LSD1), a member of the histone demethylases, in epigenetic regulation of tumor suppressing/promoting genes and neoplastic growth. However, the expression of LSD1 in patients with esophageal squamous cell carcinoma (ESCC) is still unknown. Here, we reported that LSD1 expression was elevated in cancerous tissue and correlated with lymph node metastasis and poorer overall survival in patients with ESCC. Compared to EC109 cells, LSD1 expression was unregulated in aggressive cancer cell lines KYSE450 and KYSE150. Knockdown of LSD1 using lentivirus delivery of LSD1-specific shRNA abrogated the migration and invasion of ESCC cells *in vitro*. Further, a LSD1 inhibitor, tranylcypromine, suppressed H3K4me2 demethylation and attenuated cellular motility and invasiveness in a dose-dependent manner. Taken together, these data suggested that LSD1 was a potential prognostic maker and may be a molecular target for inhibiting invasion and metastasis in ESCC.

1. Introduction

Esophageal cancer is the eighth most common cancer worldwide [1,2], with advanced esophageal cancer being predictive of significantly poorer prognosis [3]. The 5-year survival rate for esophageal squamous cell carcinoma (ESCC) is only 5–12.3% in Europe [4,5]. Therefore, novel prognostic and molecular targets for therapeutic intervention are rapidly needed for patients with ESCC.

A wide range of genetic and epigenetic modifications have been shown to play a pivotal role in the development and tumorigenesis of esophageal cancers. These epigenetic changes are associated with DNA methylation and histone modifications [6–9]. Understanding epigenetic changes may help to identify a novel cancer-related network that may represent attractive targets for ESCC treatment and provide new insights into the biological characteristics of ESCC. Chromatin with histone tailing is defined as a critical regulator of gene transcription [6,8]. Histone demethylase lysine specific demethylase 1 (LSD1) was the first histone demethylase that was discovered, and is a nuclear homolog of amine oxidases. LSD1 removes methyl groups from mono- and dimethylated Lysine (Lys) 4 of histone H3 (H3K4me1/2), and Lys9 of histone H3 (H3K9me1/2) [10]. A recent study uncovered the role of LSD1 in cell phase transition, suggesting that its over-expression may promote tumorigenesis [11]. The expression of LSD1 has been associated with tumor recurrence during therapy in various human cancers, implicating LSD1 as a tumor promoter. It is of note that LSD1 is involved in embryonic differentiation [12], proliferation of pluripotent stem cells [13], HIV infection [14], as well as in the development and metastasis of cancers [15,16]. Furthermore, LSD1 is highly expressed in prostate cancer [17,18], bladder cancer [15], breast cancer [16,19], hepatoma [3], non-small-cell lung cancer [20] and hematopoietic tumors [21]. All of these studies imply that LSD1 may be associated with the pathogenesis of ESCC, however the expression and significance of LSD1 in ESCC is obscure.

In our study, we investigated the expression of LSD1 in ESCC tissues. Then, we tested the role of LSD1 in inducing tumor cell invasion in ESCC cells *via* genetic elimination or pharmacological inhibition. Our results provided a novel insight that LSD1 may

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serve as a prognostic indicator and potential molecular target in the pathogenesis of ESCC.

2. Materials and methods

2.1. Patients and tissue microarray

This study enrolled a total of 134 histopathologically-confirmed ESCC patients who underwent a resection of esophageal carcinoma in the Institute of Surgery Research, Daping Hospital affiliated to the Third Military Medical University between 2002 and 2007. All patients received no previous chemotherapy or radiotherapy before surgery. A multi-disciplinary team, including an oncosurgeon, an oncologist, and a radiologist, determined the therapeutic regimen. The clinicopathological information and patients' medical history were documented during postoperative follow-up. The histologically-confirmed ESCC tissues were investigated by micro-array. With the exception of cancerous tissues, we also obtained 23 cases of esophageal precancerous lesions, and 29 cases of esophageal normal mucosal tissue. We obtained written consent from all participants. Our study was approved by ethical review board of the Third Military Medical University.

2.2. Immunohistochemistry (IHC)

Paraffin tissue sections (4 μm) were incubated overnight with an antibody against LSD1 (1:400; Abcam, USA). The slides were incubated for 30 min with goat anti-rabbit immunoglobulin (E0432, Abcam) after being washed with Tris-buffered NaCl solution for 30 min. The percentage of positive cells was determined by counting 500 cells within five high-resolution fields. Immunohistochemical staining was evaluated using the semi-quantitative Remmele scoring system [22], which links the IHC staining intensity (SI) with the percentage of positive cells (PP). SI was scored according to the following criteria: (0) no positive nuclei, (1) all the positive nuclei display weak staining, (2) the most stained nuclei display moderately positive, (3) the nuclei display intensive staining. PP was scored between 0 and 4 (0: no positive cells, 1: less than 10% nuclei display intense staining, 2: 11–50% nuclei, 3: 51–80%, 4: more than 80%). The IHC score was finally calculated by SI × PP. According to the IHC score, all patients were classified into two groups: low expression level (0–4) and high expression level (4–12).

2.3. Cell culture and protein extraction

Esophageal cancer cell lines, KYSE150, KYSE450 and EC109, were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). At the indicated time points, total cell protein was extracted using cell lysis buffer (Beyotime Biotechnology, China).

2.4. Knock-down of LSD1 via shRNA-delivered by lentivirus

KYSE450 cells were cultured at a concentration of 3×10^5 cells/well for 24 h in RPMI 1640 medium supplemented with 10% FCS. According to the protocol, lentivirus with green fluorescent protein (GFP; Sunbio, China) was added to the 6-well plates. In addition, the lentiviral transfection enhancer (Sunbio, China) was applied at a concentration of 5 μg/ml. The medium was refreshed after 12-h. Notably, puromycin, a cytotoxic agent, eliminated the unstably infected cells. The shRNA sequences we used in this study are listed below: Control: TTC TCC GAA CGT GTC ACG T; LSD1-shRNA1: GCA GCT CGA CAG TTA CAA A; LSD1-shRNA2: CCA CCT GAC AGT AAG GAA T.

2.5. Western blotting

Cellular proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransfered to a nitrocellulose membrane (Bio-Rad, USA). Western blotting was carried out by sequential incubation in 5% non-fat milk blocking buffer at room temperature for 60 min, using the following antibodies: LSD1 (Abcam, USA), 1:2000; H3K4me1 (Millipore, USA), 1:2000; H3K4me2 (Millipore, USA), 1:500; H3K9me1 (Millipore, USA), 1:1000; H3K9me2 (Millipore, USA), 1:2000. Following overnight incubation at 4 °C, the secondary antibodies were added and incubated for 60 min at 4 °C. HRP-GAPDH (Kangchen, China) was used as the loading control.

2.6. RNA isolation, reverse transcription and quantitative reverse transcription (RT)-PCR

Total RNA from cell culture was extracted using the Trizol reagent, according to the manufacturer's protocol (Takara, Japan). The RNA samples were digested with DNase I (Takara, Japan), then reverse-transcribed into cDNAs and sequence amplified by Quantitative RT-PCR (Eco™ Real-Time PCR system, USA). According to the manufacturer's protocol, qRT-PCR was performed using the FastStart Universal SYBR Green Master Mix kit (Roche, USA). Relative mRNA levels of LSD1 were normalized to levels of the housekeeping gene, GAPDH, and results were calculated using the $2^{-\Delta\Delta Ct}$ method. All samples were measured in triplicate. The following primers were used: GAPDH, forward: 5′-GAA GGT GAA GGT CGG AGT CA-3′; reverse 5′-TTG AGG TCA ATG AAG GGG TC-3′. LSD1, forward: 5′-TTC TGG AGG GTA TGG AGA CG-3′; reverse: 5′-CCT TCT GGG TCT GTT GTG GT-3′.

2.7. Cell migration and invasion assay

Using a scratch assay, the cultured cells were incubated for 24 h in 6-well plates (Costar, USA) with RPMI 1640 plus 10% FCS until 90–100% confluence. A 1 mm-wide linear scratch was applied across the each well to evaluate cell migration. Subsequently, 2 ml of RPMI 1640 medium without FCS was introduced to repress cell proliferation after washing. Tranylcypromine (Sigma–Aldrich, USA) was added at a final concentration of 0 μM, 50 μM and 250 μM in the treatment groups. 48 h after application of the scratch, the width of the scratch was measured by CorelDRAW 9 (Corel Software Company, Canada).

Cell invasiveness was evaluated using a Transwell chamber assay (Costar, USA). Chamber membranes (8 μm, BD Falcon) were pre-coated with 6 μl matrigel at 4 °C overnight, and seeded with 1×10^5 cells. RPMI 1640 with 2% FCS supplement was added to the upper chamber and 600 μl of RPMI 1640 (containing 20% FCS) was added to the lower chamber. Cells were incubated for 48 h with or without treatment. The cells on the top of membranes were removed, and the cells that penetrated the membrane were fixed in ethanol, followed by crystal violet staining. The number of cells on the opposite side of the membrane was counted under the microscope in four random fields of vision.

2.8. Statistical analysis

Quantitative data is expressed as mean ± SD in figures, and multiple comparisons between the groups was performed using SNK method. Using SPSS 13.0 (SPSS Software Company, USA), one-way ANOVA and student's *t*-test were performed to determine the significance of the relationship between LSD1 expression and the clinicopathological factors in patients with ESCC, including sex, age, and pathological differentiation, infiltration, lymphatic metastasis, and the overall survival. A Kaplan–Meier survival curve