Article

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KDM6B Promotes the Development, Progression and Metastasis of Oesophageal Cancer through Demethylation of Histones in the LDHA Promoter Region

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Background: Esophageal squamous cell carcinoma (ESCC) is a strongly metastatic and fatal cancer. Currently, the mechanisms underlying the pathogenesis of ESCC are poorly understood. Lysine-specific demethylase 6B (KDM6B) plays a key role in the occurrence and development of various human diseases, such as cancers, immune diseases and developmental diseases. Studies have confirmed that KDM6B may exhibit both tumour-suppressive and oncogenic activities in different cancer types. However, the expression and role of KDM6B in ESCC remain unclear.
Methods: KDM6B expression was analysed using The Cancer Genome Atlas (TCGA) database, and the correlation between KDM6B mRNA expression and prognosis was analysed. The expression of KDM6B in Eca109 and TE11 ESCC cells and ESCC tissues was evaluated using immunohistochemical (IHC) and Quantitative Real-time PCR (RT–qPCR) analyses. *In vitro* assays were further performed to reveal the functions and characterize the upstream and downstream regulation of KDM6B expression.
Results: KDM6B was overexpressed in stage T3/T4 as well as N2/N3 ESCC tissues relative to stage T1/T2 and N1/N2 ESCC tissues, respectively, and this overexpression was linked to worse prognosis of ESCC. Inhibiting KDM6B expression significantly impaired the proliferation and metastasis of ESCC cells. Lactate dehydrogenase isoform A (LDHA) plays a critical role in tumour aerobic glycolysis. The expression of LDHA was inhibited in ESCC cells to assess its role in ESCC proliferation. In this study, KDM6B knockdown suppressed not only LDHA expression but also ESCC cell proliferation.
Conclusions: Based on these results, we speculate that KDM6B might be a novel therapeutic target for ESCC.

Keywords: ESCC; KDM6B; LDHA; proliferation; metastasis

Introduction

Esophageal squamous cell carcinoma (ESCC) is the 8th commonest malignancy worldwide affecting more than 450,000 people [1], particularly in China [2]. Despite recent advances in ESCC treatment approaches, the clinical course of ESCC is grave [3]. Understanding the mechanisms driving ESCC tumorigenesis can reveal more accurate early diagnostic markers and treatments for ESCC.

Epigenetic changes participate in cancer initiation and progression [4]. DNA methylation and deacetylation are the two major epigenetic modifications [5]. DNA methylation often involves histone modifications, which interfere with the binding of transcription factors to gene promoter regions [5,6]. For instance, methylation of H3K27 upregulates the transcription of genes [7]. H3K27 expression has been implicated in the development of different cancers, including kidney [8], lung [9], pancreatic [10], oesophageal [11] and head and neck [12] cancer. Targeting enhancer of zeste homolog 2 (EZH2) mediated methylation of H3K27 inhibits the proliferation and migration of synovial sarcoma *in vitro* [13]. Lysine-specific demethylase 6B (KDM6B) is an autosomal H3K27 demethylase overexpressed in numerous tumours [14]. KDM6B may either suppress or promote the progression of tumours. In particular, activation of KDM6B enhances the malignant properties of hypoxic tumours [15]. Overexpression of KDM6B promotes the metastasis of prostate cancer [16] and the invasion, recurrence, and metastasis of breast carcinomas [17]. However, the role of KDM6B in ESCC is largely unknown.

Lactate dehydrogenase isoform A (LDHA) a key HIF-1α target, catalyses the reduction of pyruvate to lactate and maintains cell survival under hypoxic conditions by compensating for the reduction in oxidative mitochondrial functions. LDHA expression is elevated in a variety of tumour cells and plays an important role in tumour development

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and maintenance. LDHA is highly expressed in cancer cells and regarded as a biomarker for multiple malignancies, including lymphoma, prostate cancer, renal cell carcinoma, melanoma and ESCC. It has been confirmed that upregulated KDM6B facilitates tumour metastasis in OS by modulating LDHA expression. However, the regulatory mechanism of LDHA in ESCC is unknown.

Therefore, in the current study, KDM6B expression in ESCC and the biological roles of KDM6B in the development of ESCC were investigated. In addition, the relationship between KDM6B and LDHA in ESCC tumours was investigated. We found KDM6B protein overexpression in ESCC cell lines and tissues. Overall, our findings indicated that KDM6B enhanced the malignant properties of ESCC. Moreover, KDM6B knockdown suppressed not only LDHA expression but also ESCC cell proliferation. These findings enrich our understanding of the pathologic role of KDM6B in ESCC.

Materials and Methods

Human ESCC Cell Lines

The human Eca109 and TE11 ESCC cell lines were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were grown at 37 °C in high-glucose DMEM supplemented with 10% FBS and a 1% mixture of streptomycin and penicillin. Cells were incubated in 5% CO_2. All the cells have been tested for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (Lonza, L07-218). Cells tested mycoplasma free. Eca109 and TE11 was confirmed by short tandem repeat (STR) and karyotype analyses; their luminal versus basal-like classification and isogenicity were demonstrated by gene expression profiling. These cells were also authenticated by STR analysis. All cells are mycoplasma-freeCells were identified with STR profiles.

Cell Transfection

For transient transfection experiments, 24 hours prior to transfection CHO cells were seeded at a cell density of 300,000 or 1,200,000 cells respectively per well of a 6 well plate (ThermoFisher Scientific). Each well was then transfected with LgBIT-p53 and either SmBIT-Mdm4 or SmBIT-Mdm2 plasmids in a ratio of 1:3 or 1:1 respectively by using Lipofectamine 3000 (ThermoFisher Scientific) according to manufacturer's instructions. After a 24 hour incubation, medium was removed and cells were washed with PBS saline. Transfected Eca109 and TE11 cells were trypsinised and re-suspended in Opti-MEM media with 0% FCS. Cells were then spun down at 1000 rpm for 5 minutes at room temperature. si-KDM6B (50 nM) and mimic-LDHA (50 nM) were transfected into Eca109 and TE11 cells with Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA). Transfection lasted 8 h. The sequence of si-KDM6B was 5′GCTGATGACAAGAGGCTG GTA3′, and the sequence of the LDHA mimic was 5′AGGCTAGCGAATACTGCACGTA3′. All primers were synthesized by Nanjing Genscript Biotechnology Co., Ltd.

Table 1. The specific primers for the genes.

Primer Name	Sequence (5′ to 3′)
KDM6B	Forward: TCCAATGAGACAGGGCACAC
	Reverse: CTTTCACAGCCAATTCCGGC
LDHA	Forward: GAAGCTGAACGTGCACATGATGA
	Reverse: GTAGGGACAGAGTCTTCACCACT
GAPDH	Forward: CCACTCCTCCACCTTTG
	Reverse: CACCACCCTGTTGCTG

KDM6B, Lysine-specific demethylase 6B; LDHA, Lactate dehydrogenase isoform A.

Cell Proliferation Assay

The impact of KDM6B on the proliferative capacity of Eca109 and TE11 ESCC cells was assessed using a Cell Counting Kit (CCK)-8 (Beyotime, Shanghai, China). In brief, cells (5×10^3 cells/well) were cultured at 37 °C with 10 μL of CCK-8 solution for 0, 24, 48 or 72 h. The absorbance of the cells was measured at 450 nM using a 96-well plate reader (BMG Labtech, Aylesbury, UK). Each experiment was repeated 3 times.

Quantitative Real-time PCR

Total RNA from cell lines was extracted using TRIzol (Thermo Scientific, USA) or a Gene RNA Purification Kit (Thermo Scientific, USA), and reverse transcribed into cDNA using random hexamers and reverse transcriptase (Invitrogen, Paisley, UK). cDNA was amplified using SYBR Green PCR Master Mix and the Light Cycler 480 SYBR I Master system (Roche, Basel, Switzerland). GAPDH was used as the internal control. The amplification conditions were as follows: 94 °C for 10 min, followed by 40 cycles of denaturation for 15 s at 94 °C and extension for 1 min at 60 °C. The expression levels of genes relative to that of GAPDH were calculated based on the $2^{-\Delta\Delta Ct}$ method. The specific primers used for amplification of the selected genes are listed in Table 1.

Transwell Migration and Invasion Assays

The effect of KDM6B on the migration and invasion of ESCC cells was assessed using Transwell migration and invasion assays with a polycarbonate membrane (Corning, NY, USA) containing 8.0-mm pores. Cells were cultured for 24 h in serum-free DMEM in the upper chamber, and lower chamber contained DMEM in supplemented with 10% FBS. The cells in the lower chamber were fixed and counted. The cells that passed through the membrane secreted proteases that degraded the Matrigel matrix (BD-

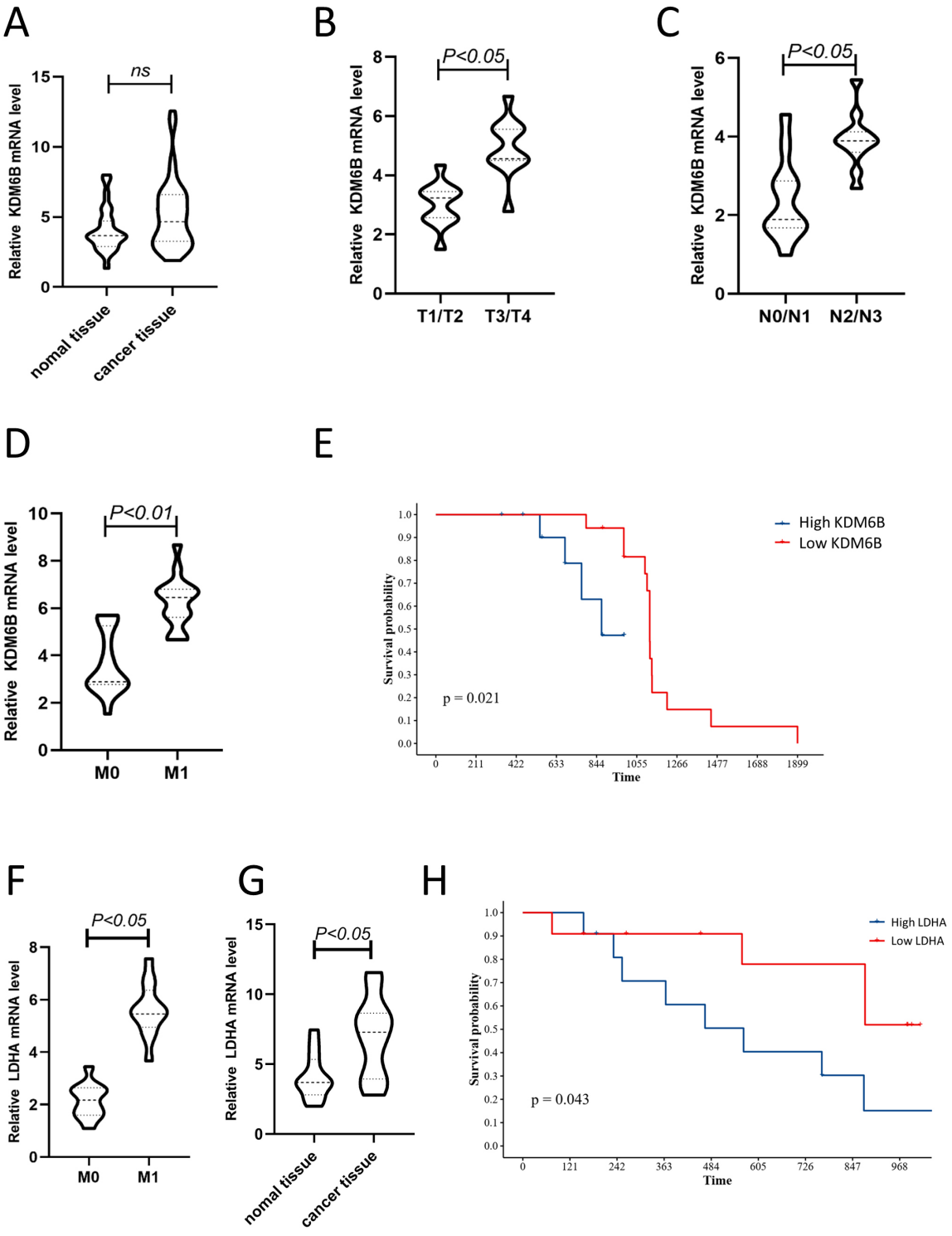

Fig. 1. KDM6B level in metastatic ESCC. Upregulation of KDM6B correlated with poor prognosis in ESCC. (A) KDM6B mRNA expression in ESCC tissues and matched normal tissues. (B) Expression of KDM6B mRNA in stage T1, T2, T3, and T4 ESCC tissues. (C) Expression of KDM6B mRNA in stage N0, N1, N2, and N3 ESCC tissues. (D) KDM6B mRNA expression in metastatic (M1) and nonmetastatic (M0) ESCC tissues. (E) Kaplan–Meier analysis of the relationship between KDM6B expression and overall survival in ESCC patients and the relationship between LDHA expression and ESCC prognosis. (E) LDHA mRNA expression in ESCC and matched normal tissues. (F) LDHA mRNA expression in metastatic (M1) ESCC and nonmetastatic (M0) ESCC tissues. (G) Kaplan–Meier survival analysis of the relationship between LDHA expression and OS in ESCC patients. $p < 0.05$, $p < 0.01$.

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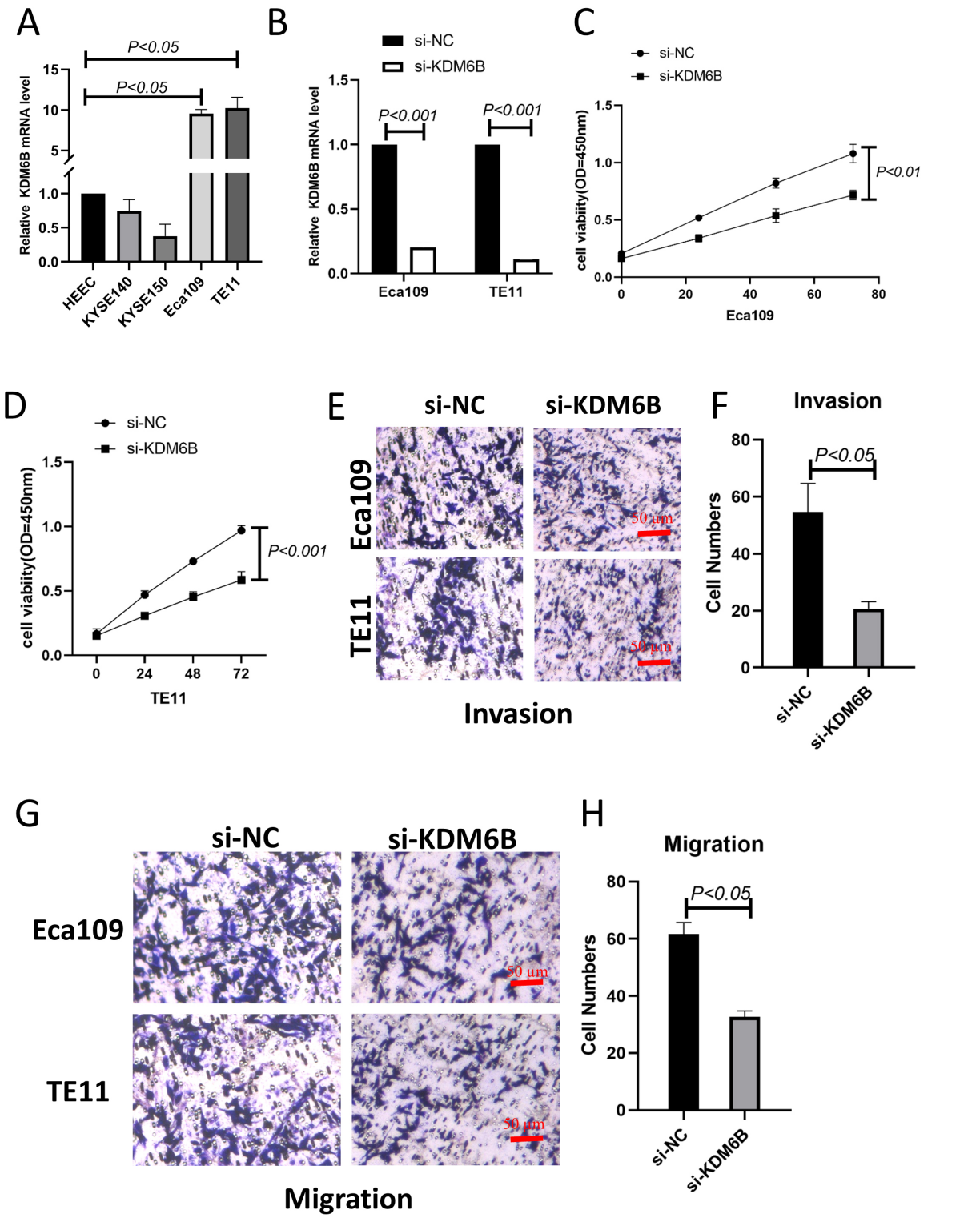

Fig. 2. Effects of KDM6B on the proliferation, invasion and migration of ESCC cells. (A) KDM6B expression in ESCC cell lines was determined using Quantitative Real-time PCR (RT–qPCR). (B) Knockdown efficiency of KDM6B expression in Eca109 and TE11 cells using si-KDM6B. (C) Effect of KDM6B knockdown on the proliferation of Eca109 cells *in vitro*. (D) The effect of KDM6B knockdown on the proliferation of TE11 cells *in vitro*. (E,F) Transwell assay to determine the effect of KDM6B inhibition on the invasion of ESCC cells. (G,H) Effect of KDM6B inhibition on the migration of Eca109 and TE11 cells. Eca109 and TE11 cells were transfected with si-KDM6B before the experiment. $p < 0.05$, $p < 0.01$, $p < 0.001$. *Scale* = *50* μm. The sample size is 3.

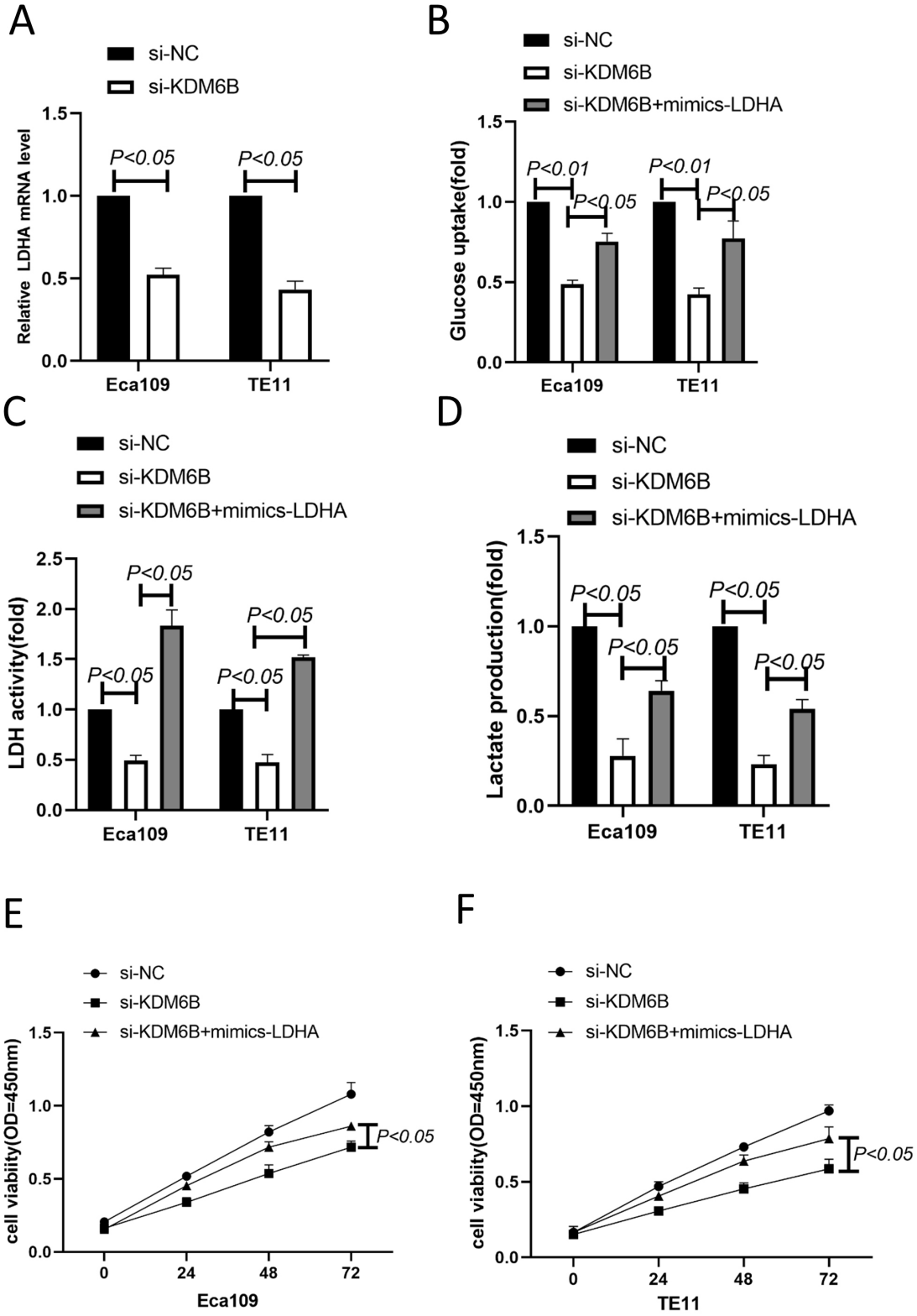

Fig. 3. Effect of KDM6B knockdown on glycolysis in ESCC cell lines. Overexpression of LDHA reversed the inhibitory effect of KDM6B knockdown on the proliferation of Eca109 and TE11 cells. (A) The relationship between KDM6B and LDHA expression in ESCC cell lines. (B–D) Glucose uptake, LDH activity, and lactate concentrations in si-KDM6B Eca109 and TE11 cells relative to control (siNC) cells. Effect of LDHA overexpression on the inhibitory effect of KDM6B knockdown on the proliferation of (E) Eca109 and (F) TE11 cells. $p < 0.05$, $p < 0.01$. The sample size is 3.

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Table 2. Association between KDM6B expression and the clinicopathological characteristics of the ESCC patients.

Characteristics	Number of cases 89	Expression of KDM6B	*p value*
Age (y)			
≥60	27	23.46 ± 8.98	
<60	62	27.87 ± 7.67	0.3212
Gender			
Male	45	18.09 ± 1.56	
Female	44	19.23 ± 1.78	0.5678
T classification			
T1/T2	23	11.56 ± 1.45	
T3/T4	66	25.56 ± 2.08	0.0012
N classification			
N0/N1	39	15.57 ± 2.89	
N2/N3	49	24.79 ± 2.67	0.0022
Lymph node Metastasis			
M0	18	10.54 ± 1.08	
M1	71	27.45 ± 4.78	00000

Science, Sparks, MD). The cells were observed using a light microscope (magnification, ×100). Five different fields were observed and photographed. The relative cell migration and invasion rates were counted through the number of the migrated or invaded cells/the number of the inoculated cells in the same field.

Analysis of ESCC Data from The Cancer Genome Atlas (TCGA)

The RNA-sequencing and clinical data of 76 patients with ESCC in the TCGA database (https://cancergenome.nih.gov/) were used for survival analysis. Detailed overall survival (OS) data and disease-free survival (DFS) data were available for all 76 patients with ESCC. The follow-up period was 0 to 64 months. The median patient follow-up time was 12 months.

Statistical Analysis

All analyses were performed and the results visualized using SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA). Data from two groups were compared using Student's *t*-test or the Mann-Whitney U test. Comparison among multiple sets of measurement data was performed using one-way analysis of variance. The Spearman rank correlation coefficients between the mRNA expression levels of KDM6B and LDHA were calculated. Comparisons of categorical data were performed by the chi-squared test. The relationships between KDM6B and LDHA levels and patient survival were determined by the Kaplan-Meier method with the log-rank test. p values < 0.05 were considered statistically significant.

Results

KDM6B mRNA Overexpression Promotes Metastasis of ESCC Cells

Based on data in The Cancer Genome Atlas (TCGA) database, the mRNA expression of KDM6B did not differ significantly between oesophageal cancer and normal oesophageal tissues ($p > 0.05$) (Fig. 1A). Further analysis revealed that KDM6B mRNA levels were significantly higher in the stage T3/T4 ESCC ($p < 0.05$) (Fig. 1B) and N2/N3 groups ($p < 0.05$) (Fig. 1C) than in the stage T1/T2 and N2/N2 groups, respectively. Notably, KDM6B mRNA overexpression was associated with lymph node metastasis of ESCC ($p < 0.01$) (Fig. 1D). Kaplan–Meier survival analysis revealed that KDM6B overexpression was associated with poor OS in ESCC patients ($p < 0.05$) (Fig. 1E). The main reasons for missing data related to high KDM6B expression were to participant dropout or noncompliance. The clinicopathological features of ESCC patients in the TCGA database are shown in Table 2. Elevated LDHA expression was correlated with clinical outcomes in ESCC. Moreover, based on data in The Cancer Genome Atlas (TCGA) database, we found that LDHA expression was also significantly higher in metastatic ESCC tissues than in non-metastatic ESCC tissues ($p < 0.05$) (Fig. 1F). LDHA was overexpressed in oesophageal cancer tissues compared with normal oesophageal tissues ($p < 0.05$) (Fig. 1G). Kaplan–Meier analysis revealed that upregulation of LDHA correlated with shorter OS times in patients with ESCC ($p <$ 0.05) (Fig. 1H).

Suppressing KDM6B Expression Inhibits the Proliferation, Invasion and Migration of ESCC Cells

To explore the potential roles of KDM6B in ESCC, we first examined the expression of this protein in ESCC cells (KYSE140, KYSE150, Eca109, TE11) using RT–PCR ($p <$

0.05) (Fig. 2A). Genetic knockdown of KDM6B in Eca109 and TE11 cells was performed using siRNAs (Fig. 2B). We found inhibition of KDM6B expression impaired the proliferation of Eca109 and TE11 cells ($p < 0.01$) (Fig. 2C,D). In addition, the Transwell assays revealed that inhibition of KDM6B expression modulated the invasion ($p < 0.05$) (Fig. 2E,F) and migration capacities of Eca109 and TE11 cells ($p < 0.05$) (Fig. 2G,H). Collectively, these findings show that KDM6B enhances the invasion and migration capacities of Eca109 and TE11 cells.

LDHA Partially Reversed the Modulation of ESCC Proliferation Induced by Inhibition of KDM6B Expression

LDHA maintains glycolytic flux in cancer cells by preferentially converting accumulating pyruvate to lactate in these cells. We found that inhibiting KDM6B expression reduced LDHA mRNA expression in Eca109 and TE11 cells ($p < 0.05$) (Fig. 3A). Then, indexes of aerobic glycolysis in si-KDM6B-transfected ESCC cells were evaluated. We found that KDM6B knockdown reduced lactate activity, the LDH level, and glucose utilization in Eca109 and TE11 cells. However, overexpression of LDHA reversed these changes ($p < 0.05$) (Fig. 3B–D). Moreover, overexpression of LDHA partially reversed the suppressive effects of KDM6B silencing on the proliferation of Eca109 ($p < 0.05$) (Fig. 3E) and TE11 ($p < 0.05$) (Fig. 3F) cells.

Discussion

Histone DNA methylation has recently been identified as an important transcriptional regulation mechanism [18]. H3K27 methylation silences the expression of numerous genes involved in critical developmental processes [19]. KDM6B, also called Jumonji domain-containing 3 (Jmjd3), is a histone demethylase that specifically catalyses the removal of trimethylation from H3K27. In addition, KDM6B suppresses the effects of H3K27 during normal development, tissue differentiation and carcinogenesis [20]. KDM6B has been implicated in the development of numerous malignant tumours [21]. Functionally, KDM6B promotes the proliferation and metastasis of numerous types of cancer cells [22]. However, the biological role of KDM6B in ESCC has not been elucidated.

Previous studies revealing the effects of inhibiting KDM6B activity indicate that KDM6B-based targeted therapy is emerging as a promising therapeutic approach for various tumours. Studies have shown that overexpression of KDM6B promotes metastasis and poor prognosis in ESCC [23]. However, the function and molecular mechanism of KDM6B in ESCC remains unclear.

To investigate the mechanism of KDM6B in ESCC, we analysed the expression of KDM6B using the TCGA database. By analysis of the publicly available TCGA database, we found that KDM6B upregulation promoted the metastasis of ESCC cells to lymph nodes. In addition, KDM6B mRNA levels were higher in stage T3/T4 than in stage T1/T2 ESCC. These findings suggest that KDM6B expression enhances the malignant properties of ESCC cells, which contributes to the poor prognosis of this cancer. KDM6B mediates drug resistance and promotes the proliferation and invasion of cancer cells [24]. For example, KDM6B induces the expression of p16INK4A, a marker for cervical cancer, which enhances the proliferative activity of cancer cells [25]. We also investigated whether KDM6B plays a vital role in the migration and invasion of ESCC cells. We silenced KDM6B with siRNA in the ESCC cell lines Eca109 and TE11. Inhibition of KDM6B expression dramatically modulated the migration and invasion of Eca109 and TE11 cells.

To further analyse the downstream molecular mechanism of KDM6B in ESCC, we first clarified the relationship between KDM6B and LDHA. A positive correlation between KDM6B and LDHA expression was observed in the ESCC dataset from the TCGA database. Additionally, as shown by TCGA analysis, higher LDHA expression was correlated with a lower survival rate. Moreover, research has shown that KDM6B promotes the metastasis of lung osteosarcoma through demethylation of histones in the promoter region of LDHA. We also found that LDHA expression was elevated in ESCC tumour samples and promoted the metastasis of tumour cells to lymph nodes. Overall, upregulation of LDHA correlated with worse clinical outcomes in ESCC.

LDHA catalyses the reversible conversion of lactate to pyruvate during glycolysis [26]. In addition, LDHA is implicated in tumorigenesis and tumour development [27]. Research has shown that LDHA promotes glycolysis and the progression of HCC [28]. Here, we sought to verify the relationship between KDM6B and LDHA in ESCC, and our study demonstrated that silencing KDM6B reduced the LDHA level and, consequently suppressed glycolysis. Glycolysis is the main source of energy for tumour progression. Furthermore, overexpression of LDHA partially reversed the inhibition of ESCC cell proliferation mediated by KDM6B silencing. The above evidence indicates that KDM6B might be involved in promoting ESCC development, and the interaction between KDM6B and the LDHA pathway needs to be revealed.

Conclusions

KDM6B and LDHA are overexpressed in metastatic ESCC tissues. Our work delineates the role of KDM6B and LDHA in ESCC pathogenesis and validates KDM6B and LDHA as promising therapeutic target in ESCC.

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