

Original Article

Epigenetic modulation by lysine-specific demethylase 1 induces gastric cancer metastasis

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Abstract: Objectives: Increasing evidence demonstrate that lysine-specific demethylase 1 (LSD1) functions as a pro-oncogene and exerts vital roles in cancer cell proliferation and metastasis. We herein sought to investigate the role and mechanisms of LSD1 in human gastric cancer (GC) metastasis. Methods: The expression level of LSD1 in GC tissue samples was firstly examined. By utilizing siRNA-mediated knockdown or chemical inhibitors (tranylcypromine), we investigated the role of LSD1 in migration/invasion and epithelial-to-mesenchymal transition (EMT) processes of GC cells. We further analyzed the epigenetic mechanisms concerning its ability of histone H3 lysine 4 (H3K4) demethylation. Correlation between LSD1 and CDH1 mRNA expression was finally analyzed in GC cell lines and tissues. Results: We found that LSD1 was overexpressed in GC tissues, especially increased in those at advanced clinical stages and with positive cervical node metastasis. LSD1 deletion by either siRNA or pharmacological inhibition both resulted in impaired migration, invasion and EMT. Furthermore, we found that LSD1 directly interacted with the promoter of CDH1 and reduced the dimethyl H3K4 level of this region. Finally, we proved a significantly negative correlation between LSD1 and CDH1 mRNA levels in GC cell lines and tissues. Conclusion: Our results revealed the oncogenic roles of LSD1 by promoting GC metastasis and identified LSD1 as a key epigenetic factor to downregulate the expression of CDH1. The LSD1 inhibitors might benefit for suppressing the metastasis of GC.

Keywords: Gastric cancer, LSD1, epigenetic modification, metastasis, CDH1

Introduction

Epigenetic gene regulation, especially the dynamic posttranslational modifications of histones, plays vital roles in maintaining cell identity and reprogramming cell phenotypes in response to environmental stresses [1, 2]. DNA methylation and covalent modifications of the histones constitute the two major types of epigenetic regulation, which actively contribute to cancer initiation and progression [3-5].

Lysine-specific demethylase 1 (LSD1, also named KDM1A) was identified as the first histone demethylase that demethylates histone H3K4 and H3K9, and represses gene transcription [6]. Additionally, through interaction with sequence-specific DNA-binding transcription factors, LSD1 is anchored to specific genomic loci to regulate target genes essential for stem cell function and animal development [7-9]. Notably, overexpression of LSD1 has been ob-

served in many types of human cancers, and its high expression is associated with tumor progression, recurrence, and bad prognosis [10-12]. Numerous reports supported that LSD1 could promote cell proliferation and invasion in lung, colon and ovarian cancer [13-15]. In gastric cancer (GC), there're only two reports discussing the role of LSD1 in cancer development: 1) Triazole-dithiocarbamate based selective LSD1 inactivators inhibit GC cell growth, invasion, and migration [16]; 2) Interference of LSD1 inhibits cellular invasion and proliferation in vivo in GC [17]. Although the latter paper has addressed the possible mechanisms of LSD1 in GC (The expression of TGF- β 1, VEGF, Bcl-2, β -catenin, p-ERK and p-Smad 2/3 proteins were inhibited in LSD1 knockdown cells [17]), the direct regulation evidence is still lacking.

Epithelial-to-mesenchymal transition (EMT) is a cellular reprogramming process that is defined by the loss of epithelial characteristics

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and acquisition of a more migratory and invasive mesenchymal phenotype [18]. E-cadherin, coded by *CDH1* gene, is an epithelial marker, and functions as a suppressor of cancer cell migration/invasion by regulating cell-cell adhesion [19, 20]. As is known, transcription factor like Snail, can induce EMT by suppressing *CDH1* transcription [21]. Recent studies revealed that LSD1 is required in Snail-mediated transcription repression of *CDH1* in EMT: Snail directly represses *CDH1* expression through the recruitment of LSD1 and subsequent LSD1-mediated H3K4 demethylation [22-24]. However, little is known about whether LSD1-*CDH1* axis contributes to gastric cancer metastasis.

In this study, we attempted to investigate the expression and function of LSD1 in GC, especially the role of LSD1 in the pro-metastasis processes (migration, invasion and EMT). We also aimed to elucidate the mechanism of GC metastasis regulated by LSD1.

Materials and methods

Patients and tissues

A number of 40 gastric cancer patients hospitalized in Jingmen No.1 People's Hospital, Hubei Province from January 2016 to December 2016 were used in this study. The clinical stage and the cervical node metastatic status of patients were evaluated as per the TNM Classification of Malignant Tumors. The gastric cancer tissue and the paired normal tissue were both collected during surgery, followed by frozen in liquid nitrogen immediately and finally stored at -80°C until used. Informed consents were obtained from all 40 patients conformed to the guideline of the ethics committee of Jingmen No.1 People's Hospital, Hubei Province.

Cell line and cell culture

Cell lines used in this study were purchased from the American Type Culture Collection (ATCC). These cells include gastric cancer cell lines MKN-45, MKN-28, KATO III, SGC-7901, AGS and human normal gastric epithelial cell line GES-1. Cells were cultured with 1640 medium or DMEM medium (Invitrogen, Life Technology) with 10% fetal bovine serum (FBS, Life Technology) and 1% penicillin/streptomycin

(Invitrogen, Life Technology). All of the cell lines were grown in 5% CO_2 at 37°C in incubators.

Short interfering RNAs and transfections

LSD1 siRNAs with corresponding controls were purchased from Santa Cruz. MKN-45 cells were seeded in six-well plates and transfections were performed using the Lipofectamine 3000 reagent (Invitrogen, Life Technology) according to the manufacturer's protocol.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen), and cDNA synthesis was performed using the oligo(dT)_n and First Strand cDNA Synthesis Kit (Promega). Quantitative real-time PCR (qPCR) was performed by using SYBR mix (TOYOBO, Japan) on the ABI7900 PCR system. GAPDH was used for as internal controls. The relative expression of genes was computed by comparative cycle threshold ($2^{-\Delta\Delta\text{CT}}$) method. The primer sequences were as below: LSD1: F: 5'-CAAGTGTC AATTTGTTCCGGG-3'; R: 5'-TTCTTTGGGCTGAGGTA CTG-3'; GAPDH: F: 5'-GCAAAATCCATGGCACCGTC-3'; R: 5'-TCGCCCCACTTGATTTGG-3'.

Antibodies and reagents

The LSD1 and GAPDH antibodies were purchased from Santa Cruz. The H3K4m2, E-cadherin, vimentin and snail antibodies were purchased from Cell Signaling Technology. The LSD1 inhibitor tranilcyproline (TCP) was obtained from Biomol International (Plymouth Meeting, PA, USA).

Western blotting

Total protein was extracted by RIPA buffer from Beyotime Company. Protein concentration was determined using the BCA Protein Assay (Beyotime). 40 mg of total protein were separated on 8% or 10% SDS-PAGE gels and transferred to PVDF membranes (Bio-Rad). The membranes were blocked with 5% milk for 1 hour and immuno probed with certain antibodies against LSD1 E-cadherin (Santa Cruz Biotechnology, Inc. 200 ug/mL), vimentin (Santa Cruz Biotechnology, Inc. 200 ug/mL), snail (Santa Cruz Biotechnology, Inc. 200 ug/mL) and GAPDH (Santa Cruz Biotechnology, Inc.

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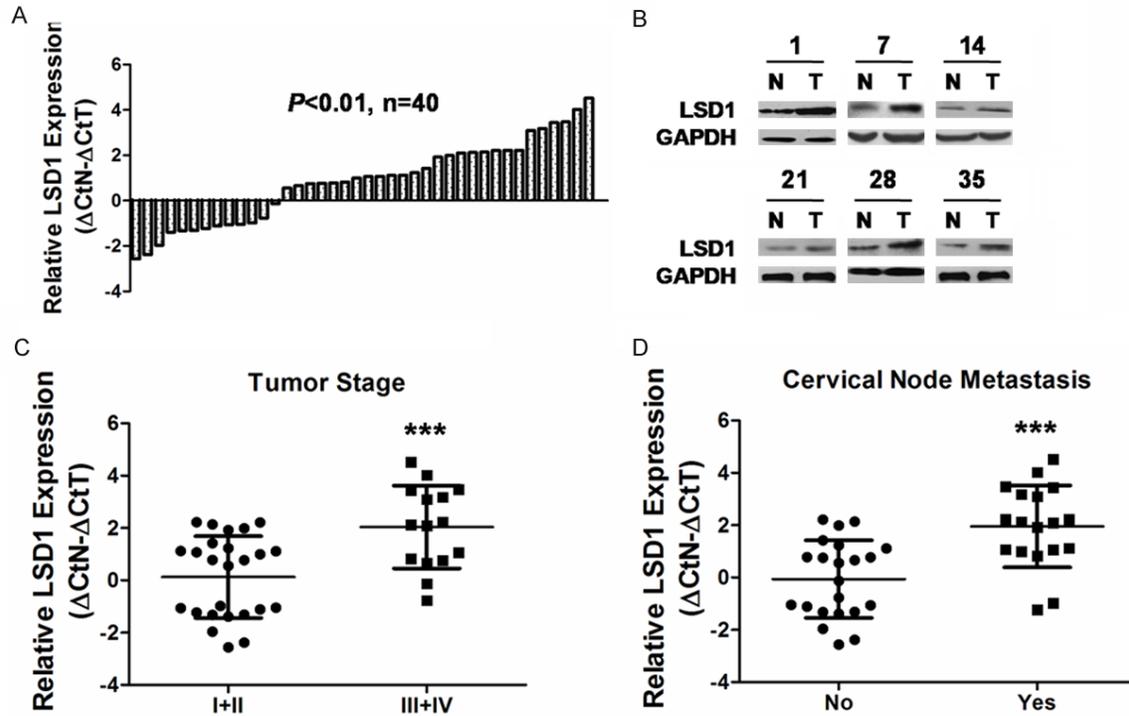


Figure 1. LSD1 is overexpressed and associated with aggressive clinicopathological features in GC tissues. A. The LSD1 mRNA level was upregulated in 50.0% (20 of 40) of GC tissues, compared to paired normal tissues (N=40, $P < 0.01$). The relative expression of genes was computed by comparative cycle threshold ($2^{-\Delta\Delta Ct}$). ΔCtN : Ct value of GAPDH was subtracted from Ct value of LSD1 of paired normal tissue. ΔCtT : Ct value of GAPDH was subtracted from that of LSD1 of GC tissue. Bar value ($\Delta CtN - \Delta CtT$) represented the difference between LSD1 mRNA level of GC tissues and paired normal tissues. Bar value ≤ -1 indicated that the expression of LSD1 was decreased in GC tissues. Bar value ≥ 1 indicated that the expression of LSD1 was increased in GC tissues. B. The LSD1 protein level was upregulated in 100.0% (6 of 6) of the selected GC tissues, compared to paired normal tissues. C. Average relative expression of LSD1 mRNA levels in different clinical stages (I+II or III+IV) of paired gastric tissues. D. Average relative expression of LSD1 mRNA levels in cervical node metastatic and non-cervical node metastatic paired gastric tissues. ***: $P < 0.001$.

200 $\mu\text{g/mL}$), respectively, overnight at 4°C . Immunodetection was performed after incubation with the corresponding secondary antibodies in PBST for 1 hour at room temperature. ECL reagents (Millipore) were used to reveal the positive bands. ChemiDoc XRS system (Bio-Rad) was used to perform digital images and densitometry analysis of the positive bands by using Quantity One program.

Wound healing assay

MKN-45 cells were seeded in a 6-well plate and allowed to grow until reaching 100% confluence. A wound was then generated by scratching a straight line using a 200 μl pipette tip. The cells were washed twice with PBS and cultured in full medium for another 48 hours. The migration of MKN-45 cells into denuded areas was monitored and visualized using a

40 \times magnification phase contrast microscope. Accurate wound measurements were performed at 0 and 48 hour to calculate the migration rate as per the following formula: percentage of wound healing = $[(\text{wound length at 0 hour}) - (\text{wound length at 48 hour})] / (\text{wound length at 0 hour}) \times 100\%$.

Transwell assay

Transwell chambers were coated with Matrigel matrix (BD) as per the manufacturer's protocol. MKN-45 cells were seeded on top of the Matrigel in the upper chamber, and the lower chamber was filled with culture medium containing FBS. Cells that invade through the Matrigel-coated membrane after 36 hours were fixed with paraformaldehyde, followed by staining with crystal violet and photographing under a microscope.

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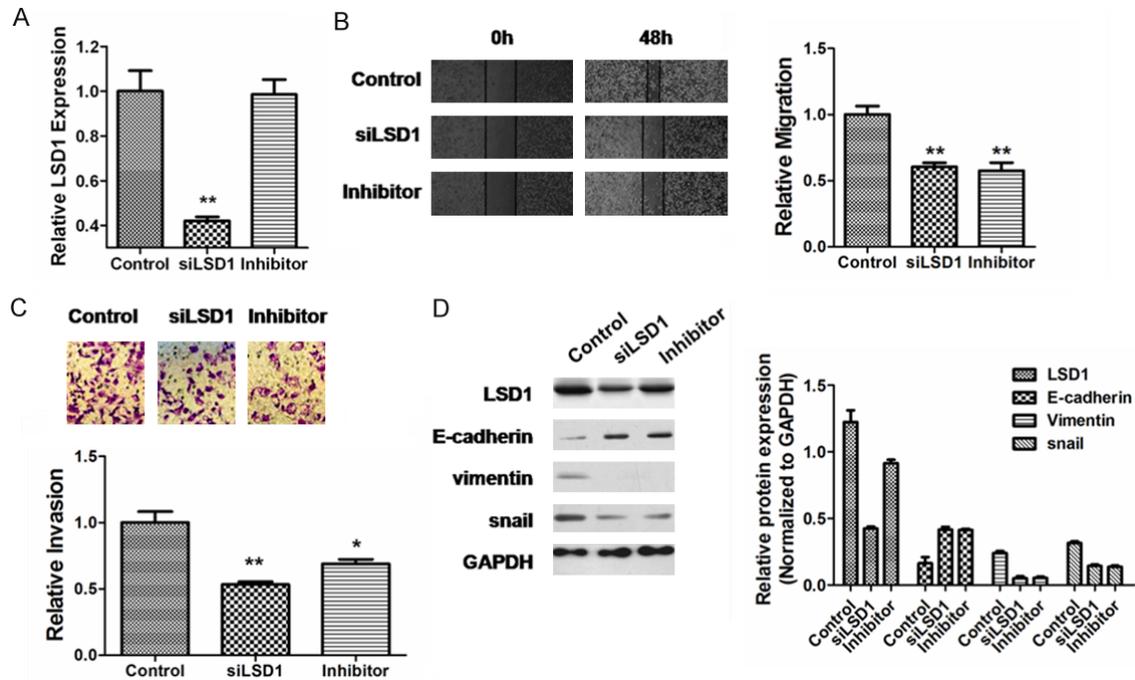


Figure 2. LSD1 is required for gastric cell migration, invasion and EMT. A. QPCR showed the knockdown effect of LSD1 by siRNA mixture in MKN-45 cells. B. Wound healing assay demonstrated that siLSD1 and tranilcypromine effectively inhibited the migration ability of MKN-45 cells. C. Invasion assay demonstrated that siLSD1 and tranilcypromine effectively inhibited invasion ability of MKN-45 cells. D. Western blotting showed the change of EMT markers in MKN-45 cells while knocking down LSD1 or treated with tranilcypromine. *: $P < 0.05$, **: $P < 0.01$.

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed using Protein A/G beads (Santa Cruz). Cells were exposed to 1% formaldehyde to crosslink proteins, and 1.0_107 cells were used for each ChIP assay. The antibodies against H3K4m2 and LSD1 were from Santa Cruz. qPCR for quantitative ChIP was performed on the ABI7900 PCR system. The CDH1 promoter primer sequences for ChIP assay were as follows: F: 5'-AGTCCCACAA-CAGCATAGGG-3', R: 5'-TTCTGAACTCAGGCGATC-CT-3'.

Sheared genomic DNA was used as input and for the normalization.

Statistical analysis

Statistical analysis was performed using SAS software (version 9.2). Statistical analysis was performed with paired or grouped Student's t-test. The correlation between LSD1 and CDH1 was performed by Pearson correlation test. Protein quantification was performed using Image J software (version 1.6). Values represent

mean \pm SD of three independent experiments. A P values < 0.05 was considered to be statistically significant.

Result

Overexpressed LSD1 is associated with aggressive clinicopathological features in GC patients

Although up-regulated LSD1 expression has been reported in several malignant tumors, its expression level in GC tissues is currently unclear. For this, we firstly evaluated the abundance of LSD1 in GC tissue samples. The results of qPCR showed that LSD1 was significantly increased in GC tissues (Figure 1A). We also found that LSD1 protein was pronouncedly elevated in all six GC samples than that in the pair-matched adjacent noncancerous tissues (Figure 1B). To characterize the clinical significance of LSD1 upregulation in GC, we classified the above mentioned 40 GC tissues into several groups according to different clinical stages and cervical node metastasis status. As shown in Figure 1C and 1D, LSD1 mRNA was found to

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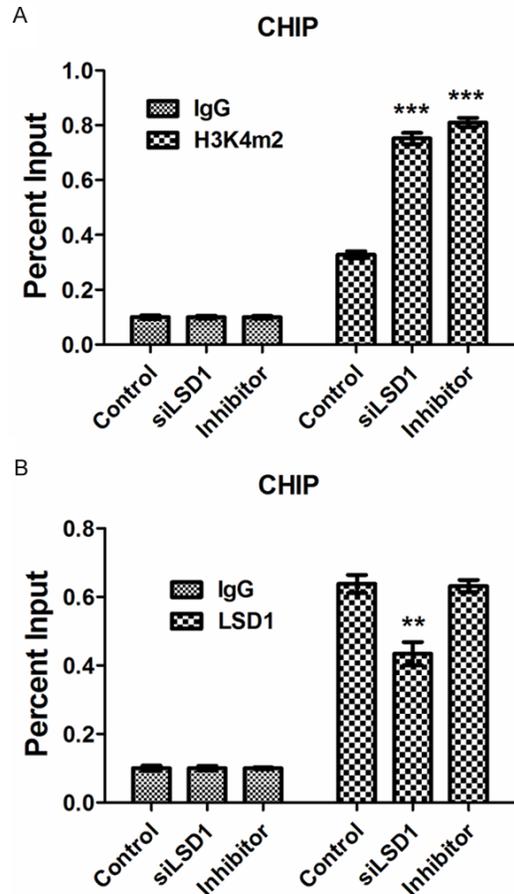


Figure 3. LSD1 regulates EMT via demethylation of CDH1 gene. A. The level of H3K4m2 at the promoter of the CDH1 gene in LSD1-silenced MKN-45 cells and tranylcypromine-treated cells was significantly higher than that in control cells. B. The enrichment of LSD1 at the proximal promoter of CDH1 (immunoprecipitated with LSD1 antibody) was significantly lower in LSD1-silenced MKN-45 cells than in control cells and tranylcypromine-treated cells. **: $P < 0.01$, ***: $P < 0.001$.

be more increased in the GC tissues of advanced tumor stages and positive cervical node metastasis. These findings indicated that overexpression of LSD1 was associated with aggressive clinicopathological features, and suggested its pro-metastasis role in GC.

LSD1 is required for cell migration, invasion and EMT in GC cells

Given the proposed oncogenic functions of LSD1 in multiple cancers and our abovementioned findings of highly expression of LSD1 in GC, we next sought to dissect the role of LSD1 in GC metastasis in vitro, via loss-of-func-

tion approach and pharmacological inhibition. We performed knockdown experiments using siRNAs targeting LSD1, and treated GC cells with LSD1 chemical inhibitors (tranylcypromine), to achieve the inhibition of endogenous LSD1 levels or activity (Figure 2A). Subsequently, the relevant phenotypic changes of GC cells by LSD1 inhibition were evaluated. We found that LSD1 inhibition induced markedly reduced capacities of cell migration and invasion, as revealed by wound healing and transwell invasion assays (Figure 2B and 2C). Next, we examined the levels of EMT markers in the LSD1-inhibited cells. In line with the above phenotypic alterations induced by LSD1 inhibition, the expression of EMT markers like vimentin and Snail diminished, whereas E-cadherin raised in LSD1-silenced cells (Figure 2D).

LSD1 regulates EMT via demethylation of CDH1 gene

Since it has been characterized by other scholars that LSD1 is required for Snail-mediated EMT and transcriptional repression of E-cadherin in mesenchymal cancer cells [22-24], we speculated that LSD1 could promote GC metastasis in vitro by demethylation of CDH1 gene, thus downregulating its expression. To confirm this speculation, ChIP assay was performed using anti-LSD1 and anti-H3K4m2 antibodies in MKN-45 cells, LSD1-silenced MKN-45 cells and tranylcypromine-treated MKN-45 cells (transfected with siLSD1 or treated with tranylcypromine for 48 hours). QPCR analysis revealed that the enrichment of H3K4m2 at the promoter of the CDH1 gene was significantly higher in the LSD1-inhibited cells (Figure 3A). We also found that LSD1 is present at the proximal promoter of CDH1 in all the three groups, and was significantly higher in non-treated and tranylcypromine-treated MKN-45 cells than that in LSD1-silenced cells (Figure 3B), which was inconsistent with invasiveness inhibition of tranylcypromine-treated MKN-45 cells in vitro (Figure 2C). This might be explained by that tranylcypromine cannot downregulate the LSD1 protein level, but suppress its enzymatic activity at the proximal promoter of CDH1. Collectively, we concluded that LSD1 decreases the H3K4m2 level at CDH1 promoter, downregulates its expression, and consequently contributes to GC cell metastasis. Reduction of the

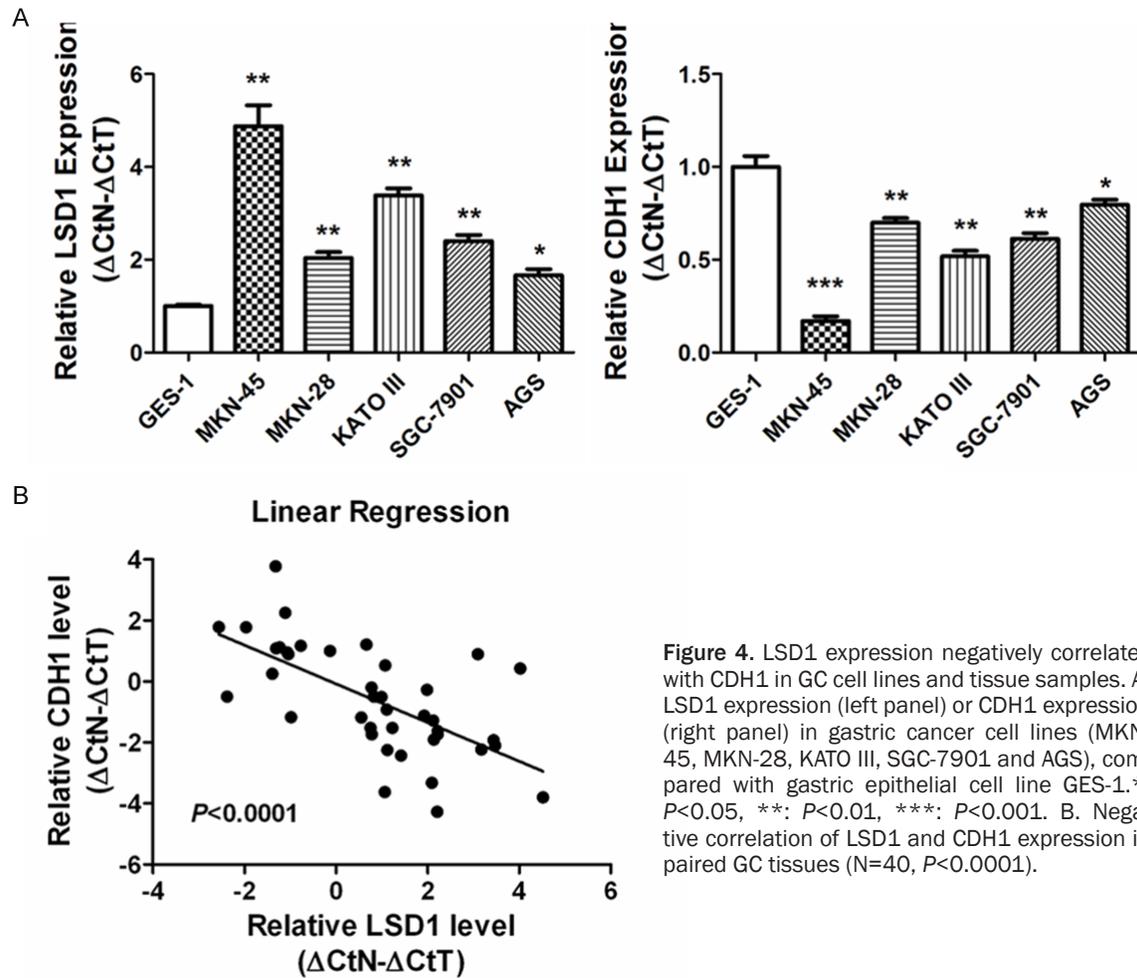


Figure 4. LSD1 expression negatively correlates with CDH1 in GC cell lines and tissue samples. A. LSD1 expression (left panel) or CDH1 expression (right panel) in gastric cancer cell lines (MKN-45, MKN-28, KATO III, SGC-7901 and AGS), compared with gastric epithelial cell line GES-1.*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. B. Negative correlation of LSD1 and CDH1 expression in paired GC tissues (N=40, $P < 0.0001$).

LSD1 protein level or inhibition its enzymatic activity can both prevent metastasis of GC cells.

LSD1 expression negatively correlates with CDH1 in GC samples

Since we have observed the downregulation of CDH1 by LSD1, we then sought to evaluate the expression correlation of the two molecules. We examined their expression in a few GC cell lines by qPCR. The results showed that the mRNA levels of LSD1 in MKN-45 cell line were significantly higher than those in the MKN-28, KATO III, SGC-7901, AGS and GES-1 (control) cell lines. However, the level of CDH1 in MKN-45 cell line was evidently lower than the other five cell lines (**Figure 4A**). Using the abovementioned 40 pairs of GC tissue samples, we found that CDH1 expression was negatively linearly correlated with the LSD1 mRNA

expression ($P < 0.0001$, **Figure 4B**). This finding further supports the direct repression of CDH1 expression by LSD1, which finally leads to GC cell metastasis promotion.

Discussion

GC is one of the malignant digestive tract tumors which seriously threaten human health, accounting for a major cause of cancer-related mortality in Asian [25]. Although promising treatment technologies have been developed, gastrectomy remains the most effective therapy option. Difficulty in early diagnosis and lacking of proper therapy strategies all give rise to the poor prognosis of GC patients, especially for those in advanced stages. Scientific research during the past decades highlights the significance of epigenetic dysregulation as one of the key hallmarks in human cancer [5]. A broad scope of genetic and epigenetic modi-

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fications plays an important role in the development and tumorigenesis of human cancers, the latter of which are mainly composed of DNA methylation and histone modification.

Metastasis is the major cause of mortality of GC patients. Therefore, it is important to understand the internal mechanisms that endow GC cells with metastatic potential. A few studies have demonstrated that LSD1 induces EMT and contributes to cancer cell migration/invasion [26, 27]. Therefore, we herein investigate the role of LSD1 in GC cell migration, invasion and EMT using LSD1-siRNA and chemical inhibitor-treated GC cells as models. Our findings revealed that both LSD1 siRNA-mediated knockdown and inhibitor treatment led to inhibition of GC cell metastasis *in vitro*. In accordance with this, we demonstrated that inhibition of LSD1 induced increase of CDH1 mRNA production via enriching the H3K4me2 levels at the promoter of the CDH1 gene. Furthermore, higher LSD1 expression correlated with lower CDH1 mRNA in GC tissues. We noticed that the available LSD1 inhibitors displayed high potency on GC metastasis resistance without changing the LSD1 expression. Nevertheless, more efforts are warranted to develop the LSD1 inhibitor with greater specificity and potency, as well as less side-effect. Also, comprehensive studies concentrating on the *in vivo* effects of LSD1 inhibitors are needed to fully recognize their advantages.

In summary, our data supply a molecular basis of LSD1 as a metastasis enhancer via demethylation of CDH1 in GC cells. Our findings further establish that targeting LSD1 by chemical inhibitors is a viable therapeutic strategy against GC metastasis.

Disclosure of conflict of interest

None.

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