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Lysyl oxidase impacts disease outcomes and correlates with global DNA hypomethylation in esophageal cancer

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Abstract

Abnormal function of human body enzymes and epigenetic alterations such as DNA methylation have been shown to lead to human carcinogenesis. Lysyl oxidase (LOX) enzyme has attracted attention due to its involvement in tumor progression in various cancers. The purpose of this study was to clarify the clinical importance of LOX expression and its epigenetic regulation in the pathogenesis of esophageal squamous cell carcinoma (ESCC). Using a database of 284 ESCCs, we examined LOX expression and its prognostic characteristics. The functional role of LOX was assessed by in vitro growth, migration, and invasion assays. The relationship between LOX expression, global DNA hypomethylation (ie, LINE-1 methylation), and *LOX* promoter methylation was evaluated by using mRNA expression arrays and pyrosequencing technology. High LOX expression cases had a significantly shorter overall survival and cancer-specific survival (log-rank, $P < .001$). The prognostic effect of LOX expression was not significantly modified by other clinical variables. Silencing and enzymatic inhibition of LOX suppressed growth and reduced the invasion and migration ability of ESCC cell lines along with the downregulation of AKT and MMP2. An integrated gene analysis in tissues and cell lines revealed that *LOX* was the most highly upregulated gene in LINE-1 hypomethylated tumors. In vitro, LOX expression was upregulated following DNA demethylation. *LOX* promoter methylation was not associated with LOX expression. Conclusively LOX expression was associated with poor prognosis in ESCC and was regulated epigenetically by genome-wide hypomethylation. It could serve as a prognostic biomarker in ESCC patients, and therapeutically targeting LOX could reverse the progression of esophageal cancer.

KEYWORDS

esophageal cancer, hypomethylation, LINE-1, LOX, prognosis

Abbreviations: 5-AZA, 5-aza-2′-deoxycytidine; ACTB, β-actin; BAPN, β-aminopropionitrile; ESCC, esophageal squamous cell carcinoma; GEPIA, Gene Expression Profiling Interactive Analysis; LINE-1, long interspersed nucleotide element-1; LOX, lysyl oxidase; OS, overall survival; p-AKT, phosphorylated AKT; qRT-PCR, quantitative RT-PCR; siLOX, siRNA for LOX.

1 | INTRODUCTION

Esophageal cancer affects more than 450 000 people globally annually.[1] Despite outstanding progress in treatment approaches, the outcome of ESCC patients is still unfavorable, even after complete tumor resection.[2,3] The exact pathogenesis of ESCC remains unclear; a deeper understanding of pathogenesis and development along with finding new molecular markers associated with ESCC could lead to the identification of novel therapeutic strategies for the regulation of this fatal disease.

Abnormal enzymatic function has been hugely implicated in human carcinogenesis. Lysyl oxidase is an essential extracellular copper proenzyme that catalyzes the cross-linkage of collagen and elastin in the ECM.[4] In recent years, the LOX molecule has attracted attention due to its involvement in the progression of various cancers. It is synthesized as a 50-kDa inactive proenzyme (pro-LOX) and then cleaved extracellularly to give the mature 32-kDa enzyme (LOX) and an 18-kDa LOX propeptide.[5,6] Recent publications support that high LOX expression typically indicates a poor prognosis in colon, breast, prostate, and lung cancer patients.[7-9] Lysyl oxidase has been reported to have intracellular functions such as regulation of cell differentiation, migration, and gene transcription.[10,11] In the tumor microenvironment, LOX also reportedly promotes tumor cell invasion.[7,12,13] Lysyl oxidase-mediated function occurs through activation and regulation of various molecules. Previous reports in colorectal cancer have shown that LOX drives angiogenesis by AKT activation,[14] proliferation, and metastasis through SRC activation.[15] Moreover, another report showed that LOX might play a role in the metastasis of nonsmall-cell lung cancer by promoting MMP2/MMP9 expression.[16] Expression of LOX is regulated at transcriptional and posttranscriptional levels.[17] Even though various regulators of LOX are known, including transforming growth factor-β1,[18] hypoxia inducible factor-1,[7] and bone morphogenetic protein-1,[19] the epigenetic regulation of LOX is still unclear.

Epigenetic alterations, such as DNA methylation, has been hugely implicated in human carcinogenesis. Global DNA hypomethylation could contribute to tumorigenesis and cancer progression by promoting genomic instability, reactivating endogenous parasitic sequences, and inducing the expression of oncogenes.[20] Long interspersed nuclear element-1 retrotransposons are widespread repetitive elements in the human genome, composing approximately 17% of the entire DNA content.[21] Hypomethylation of LINE-1 promoter has been associated with genomic instability.[22] Our group previously reported that LINE-1 hypomethylation correlates with poor outcome in esophageal cancer,[23] gastric cancer,[24] and liver cancer.[25] In addition, we suggested that LINE-1 hypomethylation in ESCC contributes to the acquisition of malignant tumor behavior through the genomic gain of oncogenes such as *CDK6*.[26]

Despite the involvement of LOX in tumor progression in other types of cancers, the importance of LOX enzyme in esophageal cancer progression and its epigenetic regulation has yet to be fully elucidated. Thus, we undertook this study to examine the association between LOX expression and the clinical profiles of ESCC patients and to study the role of LOX in the aggressiveness of esophageal cancer in vitro. Because DNA hypomethylation has been linked to oncogene expression and poor prognosis in esophageal cancer, we investigated whether LOX expression was regulated epigenetically by DNA hypomethylation.

2 | MATERIALS AND METHODS

2.1 | Study subjects

Surgical specimens were obtained from 284 patients with ESCC who underwent surgical resection at Kumamoto University Hospital (Kumamoto, Japan) between April 2005 and June 2013. A total of 117 patients received preoperative treatment. The clinicopathological characteristics are presented in Table 1. Overall survival was defined as the time between the date of surgery and the date of death, and disease-free survival as the time between the date of surgery and recurrence. Cancer-specific survival was defined as the time from the date of surgery to the date of death attributable to esophageal cancer. The protocol for this study was approved by the institutional review board. We acquired written informed consent from each enrolled patient before collecting tumor specimens for this study.

2.2 | Pyrosequencing to measure LINE-1 and LOX promoter methylation

DNA was collected from cultured cell lines and tissue specimens with a QIAamp DNA Mini Kit (Qiagen). Genomic DNA was converted with sodium bisulfite using an EpiTect Bisulfite kit (Qiagen). We undertook PCR and pyrosequencing for LINE-1 as previously described[27,28] with a PyroMark kit (Qiagen). For the *LOX* promoter, we used commercially available primers for LOX (HS_02_PM PyroMark CpG assay No. 978745; Qiagen) according to manufacturer's protocol.

2.3 | Immunohistochemical staining

Immunohistochemistry was carried out in formalin-fixed, paraffin-embedded tissue sectioned at 4 μm, dewaxed, deparaffinized, and rehydrated through a graded series of ethanol treatments. After rehydration, antigen retrieval was carried out using Proteinase K Ready-to-use (Dako) according to the manufacturer's instructions. Endogenous peroxidases were blocked by 3% hydrogen peroxidase treatment for 30 minutes. The sections were then incubated with the rabbit anti-LOX polyclonal Ab (ab31238) (Abcam) at a dilution of 1:100 at 4°C overnight. For negative controls, this primary Ab was replaced with PBS. Detection was carried out with a biotin-free HRP-labeled polymer of the Envision Plus detection system (Dako). The sections were developed in 3,3-diaminobenzidine and counterstained with Mayer's hematoxylin.

Lysyl oxidase immunoreactivity was evaluated by a researcher (R.K.) who was blinded to the other data; it included both staining intensity and the percentage of LOX-positive tumor cells. The extent

TABLE 1 Lysyl oxidase (LOX) expression and clinicopathological features of 284 patients with esophageal squamous cell carcinoma

Clinicopathological features	Total N	LOX expression		*P* value
		Low	High	
All cases	284	107	177	
Mean age, y, ± SD		64.7 ± 7.88	66.18 ± 9.69	.880
Sex				
Male	251	93	158	.550
Female	33	14	19	
Tumor location				
Upper	101	30	71	.120
Middle	134	56	78	
Lower	59	21	28	
Tumor depth				
T1	141	79	62	<.001
T2	38	12	26	
T3	96	15	81	
T4	6	1	8	
Lymph node metastases				
Negative	152	69	83	.004
Positive	132	38	94	
Distant metastases				
Negative	280	106	174	.600
Positive	4	1	3	
Stage				
I, II	198	90	108	<.001
III, IV	86	17	69	
Lymphatic invasion				
Negative	201	82	119	.090
Positive	83	25	58	
Venous invasion				
Negative	144	69	75	.001
Positive	140	38	102	

of staining was categorized semiquantitatively, based on the percentage of positive tumor cells: 0, 5% positive cells; 1, 6%-25% positive cells; 2, 26%-50% positive cells; 3, 51%-75% positive cells; and 4, >75% positive cells. The intensities of cytoplasmic and membrane staining were also determined semiquantitatively as follows: 0, negative; 1, weakly positive; 2, moderately positive; and 3, strongly positive. The scores of sections were defined as "extent of staining X intensity". Scores obtained ranged from 1 to 6 and a cut-off value of 3 was used to group into LOX-low and LOX-high expressing tumors.

2.4 | Cell lines and cell culture

Human ESCC cell lines (TE-1, TE-4, TE-6, TE-8, TE-9, TE-10, TE-11, TE-14, TE-15 and KYSE-30) were obtained from the Japanese Collection of Research Bio Resources Cell Bank and Riken Bio Resource Center Cell Bank. The cell lines were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS in a 5% CO_2 atmosphere.

2.5 | DNA and RNA extraction

Genomic DNA and total RNA were extracted from snap-frozen tissue and cell lines using a QIAamp Mini Kit (Qiagen) and an miRNeasy Mini Kit (Qiagen), respectively.

2.6 | Quantitative RT-PCR

Total RNA extraction, cDNA synthesis, and qRT-PCR were carried out as reported previously.[26] All primers were synthesized by GeneNet and probes for qRT-PCR were designed with the Universal Probe Library (Roche, Basel, Switzerland) following the manufacturer's recommendations.

The primers and probes for qRT-PCR were as follows: *LOX* forward, 5′-TGGGAATGGCACAGTTGTC-3′, reverse, 5′- AAACTTGCTTTGTGGCC TTC-3′, and universal probe #82; *ACTB* forward, 5′-ATTGGCAATG AGCGGTTC-3′, reverse, 5′-CGTGGATGCCACAGGACT-3′, and

universal probe #11; *MMP2* forward, 5′-ATAACCTGGATGCCGTCGT-3′, reverse, 5′-AGGCACCCTTGAAGAAGTAGC-3′, and universal probe #70; and *MMP9* forward, 5′-GAACCAATCTCACCGACAGG-3′, reverse, 5′-GCCACCCGAGTGTAACCATA-3′, and universal probe # 6.

2.7 | Western blot analysis

Protein samples were boiled for 5 minutes with loading buffer, electrophoresed in SDS-polyacrylamide gel, and then transferred to a nitrocellulose membrane. Membranes were incubated overnight with primary Ab, LOX (1:1000 dilution; Abcam), AKT (1:1000; Cell Signaling Technology), p-AKT (1:1000; Cell Signaling Technology), and ACTB (1:1000, Cell Signaling), followed by secondary Ab for 1 hour at room temperature. Specific proteins in each lysate were detected by enhanced chemiluminescence with ECL Plus (Amersham) according to the manufacturer's instructions.

2.8 | Treatment with 5-AZA

Cells were seeded in a 100-mm and 6-well dish for 24 hours. To demethylate methylated CpG sites, cells were continuously treated with 100 nmol/L 5-AZA (Wako) over the next 72 hours with medium replacement every 24 hours.

2.9 | Design of siRNA

The siRNA and nontargeting siRNA (negative siRNA) were purchased from Ambion (Life Technologies): siLOX#1 (ID s8254) and siLOX#3 (ID s8256). TE-11 cells were prepared at 50%-60% confluence in 6-well dishes. Transfection was carried out using Lipofectamine RNAiMAX reagent (Life Technologies) according to the manufacturer's recommendations.

2.10 | Pharmacological inhibition of LOX

We inhibited catalytically active LOX enzyme using the irreversible inhibitor BAPN (Sigma-Aldrich). The cells were treated with 500 μmol/L and 1000 μmol/L BAPN for assays.

2.11 | Cell proliferation assay

A CCK-8 assay was used to analyze cell proliferation. Cells were seeded in 96-well plates at a density of 3×10^3 cells/well at 37°C. After 24 hours, the cells were transfected with siRNAs. The LOX enzymatic activity was inhibited by treating cells with 500 μmol/L and 1000 μmol/L BAPN (a competitive irreversible inhibitor of active LOX). Cell proliferation was assessed using a CCK-8 kit (Dojindo Laboratories) 48 hours posttransfection and after treatment with BAPN.

2.12 | Transwell invasion assay

The siRNA-transfected cells and untreated controls were trypsinized with 0.25% trypsin containing EDTA. The cells were then suspended, counted, and resuspended in FBS-free RPMI-1640, and 5×10^4 cells were seeded in invasion chambers (Corning Bio Coat Matrigel Invasion Chambers with 8.0-mm polyethylene terephthalate membranes). The chambers were then placed in wells of a 24-well plate containing 700 μL medium with 10% FBS and incubated at 37°C in 5% CO_2. After 12 hours, a cotton swab was used to remove nonmigrated cells, and the migrated cells were then fixed with methanol and stained with toluidine blue. Cell counting was carried out in 5 random fields for each chamber. Cells were treated with siRNA negative control and 2 siRNAs for LOX (siLOX#1 and siLOX#3), and then incubated on BD Matrigel-coated Transwell chambers for 12 hours. Following incubation, transchamber (invasion) cells were fixed and stained.

2.13 | Real-time imaging for cell migration

Real-time imaging of esophageal cancer cells was undertaken in 6-well plates coated with 200 μL Corning Matrigel Matrix (Corning) diluted 2-fold with culture medium. The siRNA-transfected/BAPN-treated cells were seeded on Matrigel-coated 6-well plates at 5×10^4 cells/well. After a 24-hour incubation at 37°C in 5% CO_2, the cells were imaged using a Keyence BZ-X700 all-in-one fluorescence microscope equipped with a CO_2- and temperature-controlled chamber and a time-lapse tracking system. Images were captured every 10 minutes for 24 hours using BZ-X Viewer software (Keyence) and were then converted into movie files using BZ-X Analyzer software (Keyence). Cell migration was analyzed from movies using the video editing analysis software VW-H2MA (Keyence). The tracking data were subsequently processed with Excel (Microsoft) to create X-Y coordinate plots and velocity and distance measurements.

2.14 | Microarray analysis

Total RNA was isolated from frozen sections of 10 esophageal cancer biopsy specimens using an RNeasy Mini Kit (Qiagen). Gene expression microarray analysis was carried out using Sure Print G3 Human GE Microarray 8 60K version 2.0 (Agilent Technologies) according to the manufacturer's protocol. For comparison, we used microarray data of 10 esophageal cancer cell lines (similar to those we used in vitro) downloaded from a public database (GSE63941). DNA methylation status of the specimens and cell lines was determined by pyrosequencing previously.

2.15 | In silico analysis

We used a Web-based tool for GEPIA to analyze associations between *LOX*, other genes, and clinical outcome.[29]

2.16 | Statistical methods

All statistical calculations were carried out with JMP version 13 (SAS Institute) and Excel for Mac 2011 (Microsoft). Survival time

distribution in the survival analysis was assessed by the Kaplan-Meier method using log-rank tests. We constructed a multivariate model to compute the hazard ratio based on LOX expression including performance status (0 vs 1–), tumor location (upper vs lower), tumor stage (I and II vs III and IV), tumor depth (deep vs superficial), lymphatic invasion (positive vs negative), and venous invasion (positive vs negative). Interactions were assessed by including the cross-product of LOX expression status and another variable of interest in a Cox model.

3 | RESULTS

3.1 | Lysyl oxidase expression and patient prognosis

In silico analysis using a public database (GSE33810) of ESCC tissue microarray suggested that LOX expression is higher in tumor than in normal epithelium (Figure 1A). Likewise, measurement of RNA from 17 samples of matched normal epithelium and tumor in esophageal cancer specimens revealed LOX expression was significantly higher in the cancerous part than in normal epithelium (Figure 1B). The LOX protein expression was evaluated by immunohistochemistry and showed that, in cancer tissues, LOX was localized in the cytoplasm of cancer cells (Figure 1C). In vitro we determined LOX expression in esophageal cancer cell lines by real-time PCR and western blot analysis; LOX was expressed in all the cell lines, with TE-6, TE-11, and TE-14 showing the highest and TE-1 and KYSE-30 the lowest LOX expression, as shown in Figures 1D and S1A. Next, we assessed LOX immunohistochemical expression status in ESCC patients and survival analysis. High LOX expression cases had a significantly shorter OS and cancer-specific survival (log-rank, $P < .001$ and $P < .001$, respectively) (Figure 1E). Moreover, high LOX expression cases had a worse prognosis in disease-free survival (Figure S1B). We then examined whether the influence of LOX expression on OS was affected by any clinicopathological

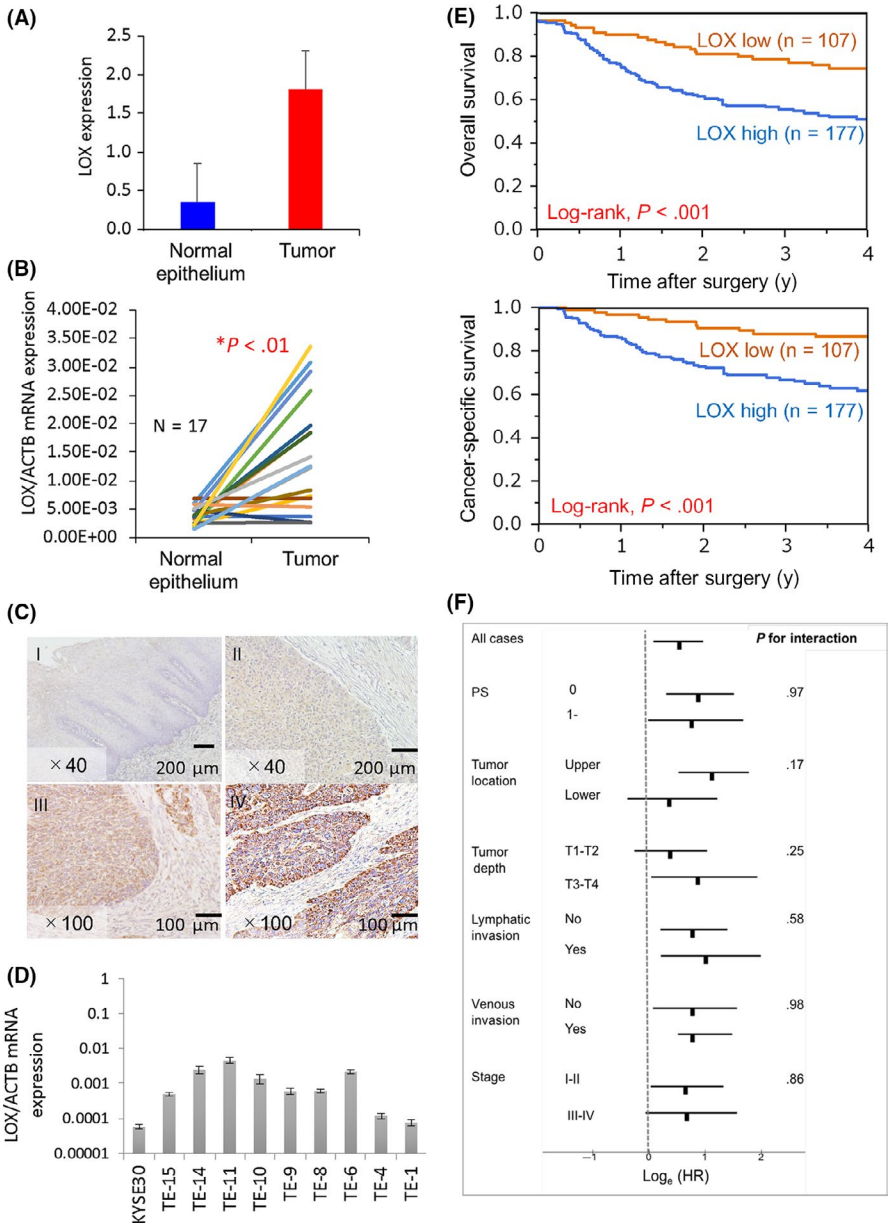

FIGURE 1 Lysyl oxidase (LOX) expression in esophageal squamous cell carcinoma (ESCC) and association with prognosis. A, mRNA expression array analysis using a public database of 10 ESCC tissue samples (GSE33810). B, LOX expression in 17 samples of matched normal epithelium and tumor showing high LOX expression in the tumor part compared to normal epithelium. C, Expression of LOX in ESCC determined by immunohistochemistry. (I) LOX was barely detected in the basal layer of normal epithelium. (II-IV) Examples of (II) mild expression, (III) moderate expression, and (IV) strong expression. Scale bars = 200 μm and 100 μm for ×40 and ×100 magnification, respectively. D, LOX mRNA expression in esophageal cancer cell lines. E, Overall and cancer-specific survival curves for groups with low and high LOX expression. F, Forest plot showing survival analyses of interaction between LOX expression and other variables. Log_e hazard ratio (HR) plots of overall survival rate are shown. ACTB, β-actin; PS, performance status

variables and found that the effect of LOX expression was not significantly modified by the parameters we examined (Figure 1F). We could confirm that the *LOX*-high expression group showed shorter OS than the *LOX*-low expression group (log-rank, $P = .043$) using GEPIA[29] (Figure S1C). Taken together, our findings indicate that LOX is highly expressed in ESCC and is associated with poor patient outcome, thus LOX can serve as a prognostic biomarker.

3.2 | Lysyl oxidase expression and clinicopathological profiles

Table 1 summarizes the clinicopathological profiles of the 284 patients we studied. Most of the tumors (177; 62.3%) showed high LOX expression, whereas 107 (37.7%) showed low LOX expression. Interestingly, there was a significant difference in tumor depth ($P < .001$), lymph node metastasis ($P = .004$), tumor stage ($P < .001$), lymphatic invasion ($P < .001$), and venous invasion ($P < .001$) between low and high LOX expression groups. In univariate analysis, high LOX expression was a significant prognostic factor for poor OS along with tumor depth, lymph node metastasis, tumor stage, lymphatic invasion, and venous invasion. Multivariate analysis of these 6 factors revealed that high LOX expression was an independent prognostic factor for poor OS ($P = .037$), as were tumor depth ($P = .027$) and tumor stage ($P = .021$) (Table S1).

3.3 | Lysyl oxidase functional analysis using proliferation and migration assay in vitro

We suppressed LOX expression in 2 high LOX expressing cell lines (TE-6 and TE-11) by treatment with 2 siRNAs (siLOX#1 and siLOX#3) and observed a significant downregulation of LOX expression compared with the negative control (Figure 2A). The effect of LOX on cell proliferation was assessed by using CCK-8 growth assay in TE-11, following either LOX knockdown or enzymatic LOX inhibition by BAPN. Interestingly, LOX knockdown suppressed cell proliferation 48 hours after transfection ($P < .001$) (Figure 2B). Similarly, LOX inhibition resulted in suppression of cell proliferation compared to the untreated controls ($P < .05$ and $P < .001$, respectively) (Figure 2C). We further revealed that there was a decrease in the expression of p-AKT following LOX knockdown (Figure 2D). Cell migration is a crucial step for cancer metastasis. We showed that both LOX knockdown (Figure 2E) and enzymatic inhibition (Figure 2F) significantly reduced cell migration($P < .001$) as there was a significant decrease in the distance moved by the tracked cells within Matrigel-coated wells in a 6-well plate within the 24-hour monitoring period by real-time (time-lapse) imaging assay. Therefore, LOX plays a key role in the proliferation and migration of esophageal cancer cells, and thus potentiates ESCC progression.

3.4 | Lysyl oxidase functional analysis using invasion assay in vitro

To study the effect of LOX on esophageal cancer cell behavior, we examined the invasion ability of TE-11 in vitro. As it is shown in Figure 3A, cells showed strong invasion behavior in siRNA negative control-treated cells in comparison to siLOX-treated cells. Quantification of invasive cell numbers revealed 110 cells per field in siRNA negative control-treated cells, which was reduced to 30 and 50 cells per field after LOX silencing with siLOX#1 and siLOX#3, respectively. We confirmed that there was no significant difference in cell viability among the siRNA negative control-treated and siLOX-treated groups (Figure 3B). Comparably, using LOX inhibitor, we found a reduction in the number of cells that invaded through the inserts ($P < .05$) (Figure 3C). There was no change in cell viability in the different treatment groups (Figure 3D). We checked the downstream effect of LOX knockdown and found a reduction in the expression of MMP2 (Figure 3E). There was a positive correlation between LOX expression and MMP2 expression in resected ESCC tissues (Figure 3F). In addition, we could confirm the significant relationship between *MMP2* and *LOX* ($P < .001$) using GEPIA (Figure 3G). Although MMP9 is also a well-known molecule that is associated with cell invasion, the relationship between LOX and MMP9 was not observed (Figure S2). These data indicate that LOX enzymatic activity plays a crucial role in the invasion of esophageal cancer cells, by promoting MMP2 and thus promoting malignancy.

3.5 | Epigenetic regulation of *LOX* expression

To determine whether LOX expression is regulated epigenetically, we treated TE-1 cells with the demethylating agent 5-AZA and found that there was a significant increase in LOX expression in this cell line ($P < .001$) (Figure 4A). Moreover, we confirmed a decrease in LINE-1 methylation level (from 64% to 35%) after treatment with 5-AZA (Figure 4B). Importantly, we also observed an increase in *MMP2* expression (LOX downstream gene) following treatment with 5- AZA (Figure 4C). Thus, we suggest that LOX expression might be regulated epigenetically by LINE-1 hypomethylation (genome-wide hypomethylation).

3.6 | Lysyl oxidase expression and global hypomethylation in esophageal cancer

To confirm the relationship between LOX expression and genome-wide hypomethylation, we undertook mRNA expression array analyses to determined upregulated and downregulated genes in LINE-1 hypomethylated and hypermethylated ESCC specimens. Similar analyses were carried out using public array data of cell lines for comparison. In hypomethylated tumors we identified 328 upregulated genes in tissue biopsies, whereas data for cell lines revealed 667 genes. An integrated analysis of genes in tissues and cell lines revealed 4 mostly upregulated genes (more than 2-fold change) with LOX being most highly upregulated gene in LINE-1 hypomethylated cases (Figure 5A). Moreover, we examined the relationship between LOX expression and LINE-1 methylation in clinical samples using LOX protein reactivity (immunohistochemistry) and LINE-1 methylation levels (previously measured by pyrosequencing) in 284 ESCC cases and found that tumors with high LOX expression had significantly lower LINE-1 methylation levels ($P = .010$, paired *t*

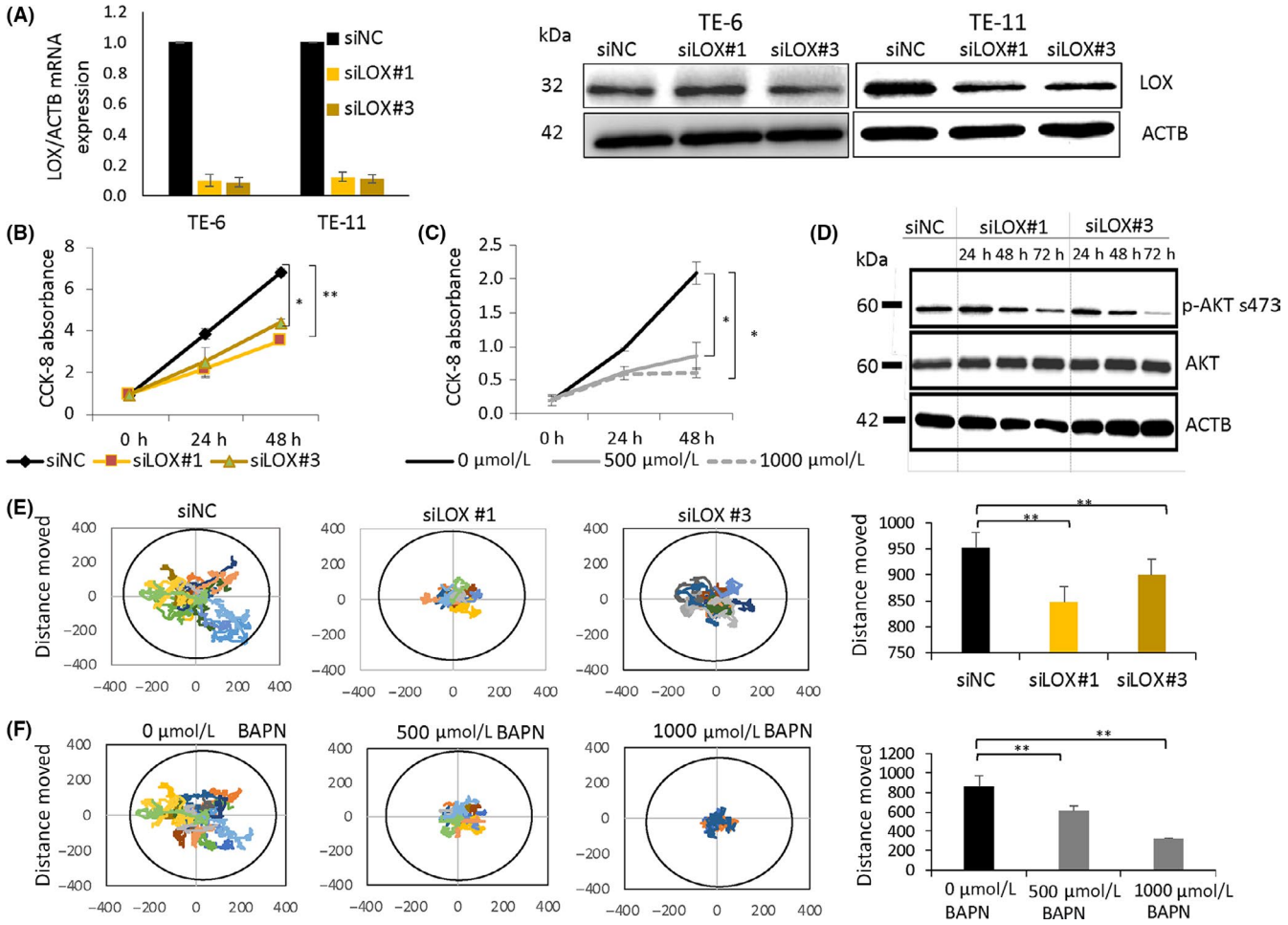

FIGURE 2 Effects of lysyl oxidase (LOX) on esophageal tumor progression in vitro. A, Suppression of LOX expression by transfection with 2 LOX siRNAs (siLOX#1 and siLOX#3) as determined by quantitative RT-PCR and western blot analyses in TE-6 and TE-11 cells. B, Growth curves for LOX knockdown in TE-11 cells. *P < .05, **P < 0. 01. C, Growth curves for LOX inhibition by β-aminopropionitrile (BAPN) in TE-11 cells. *P < .05. D, Downstream effect of LOX knockdown. Western blots showing reduction in the expression of phosphorylated AKT (p-AKT) following LOX knockdown by the 2 LOX siRNAs. There was no change in expression of AKT. E, Left panels: migration patterns of cancer cells in the ECM on LOX knockdown as determined by time-lapse imaging for 24 h. Right panel: calculated distances moved by the displayed cells. LOX knockdown reduced the migration ability of the cells (represented as means + SD). **P < .01. F, Left panels: migration patterns of cancer cells in the ECM on LOX knockdown as determined by time-lapse imaging for 24 h. Right panel: calculated distance moved by the displayed cells. LOX inhibition reduced the migration ability of the cells (represented as means + SD). **P < .01. ACTB, β-actin; siNC, normal control siRNA

test) (Figure 5B). Furthermore, a negative correlation was observed between LOX expression and LINE-1 methylation levels in frozen esophageal cancer specimens in vitro (Figure 5C). These findings certainly support that, in ESCC, LOX expression is related to LINE-1 hypomethylation (ie, global DNA hypomethylation).

3.7 | Lysyl oxidase promoter methylation

Utilizing pyrosequencing, we measured *LOX* promoter methylation level in resected specimens. First, we found that *LOX* promoter methylation levels in tumors were not different from those in normal tissues (P = .91) (Figure S3A). Second, *LOX* promoter methylation levels are not associated with *LOX* expression in tumors (P = .052) (Figure S3B). Third, LOX promoter methylation levels are inversely related with LINE-1 methylation level (P = .0049) (Figure S3C). We further studied *LOX* promoter methylation in cancer cell line TE-1 following 5-AZA treatment. The *LOX* promoter is hypomethylated in TE-1 cells and there was no significant difference in methylation levels following 5-AZA treatment (Figure S3D). Compared to the earlier findings, we showed that *LOX* is highly expressed in LINE-1 hypomethylated tumors and is inversely associated with tumor LINE-1 methylation. These findings have therefore clearly defined how DNA methylation influences LOX expression in esophageal cancer.

4 | DISCUSSION

Lysyl oxidase is an amine oxidase that functions to ensure tissue integrity in the ECM through the cross-linking of collagen and elastin.[20] In this study, using esophageal cancer specimens and cell lines we revealed

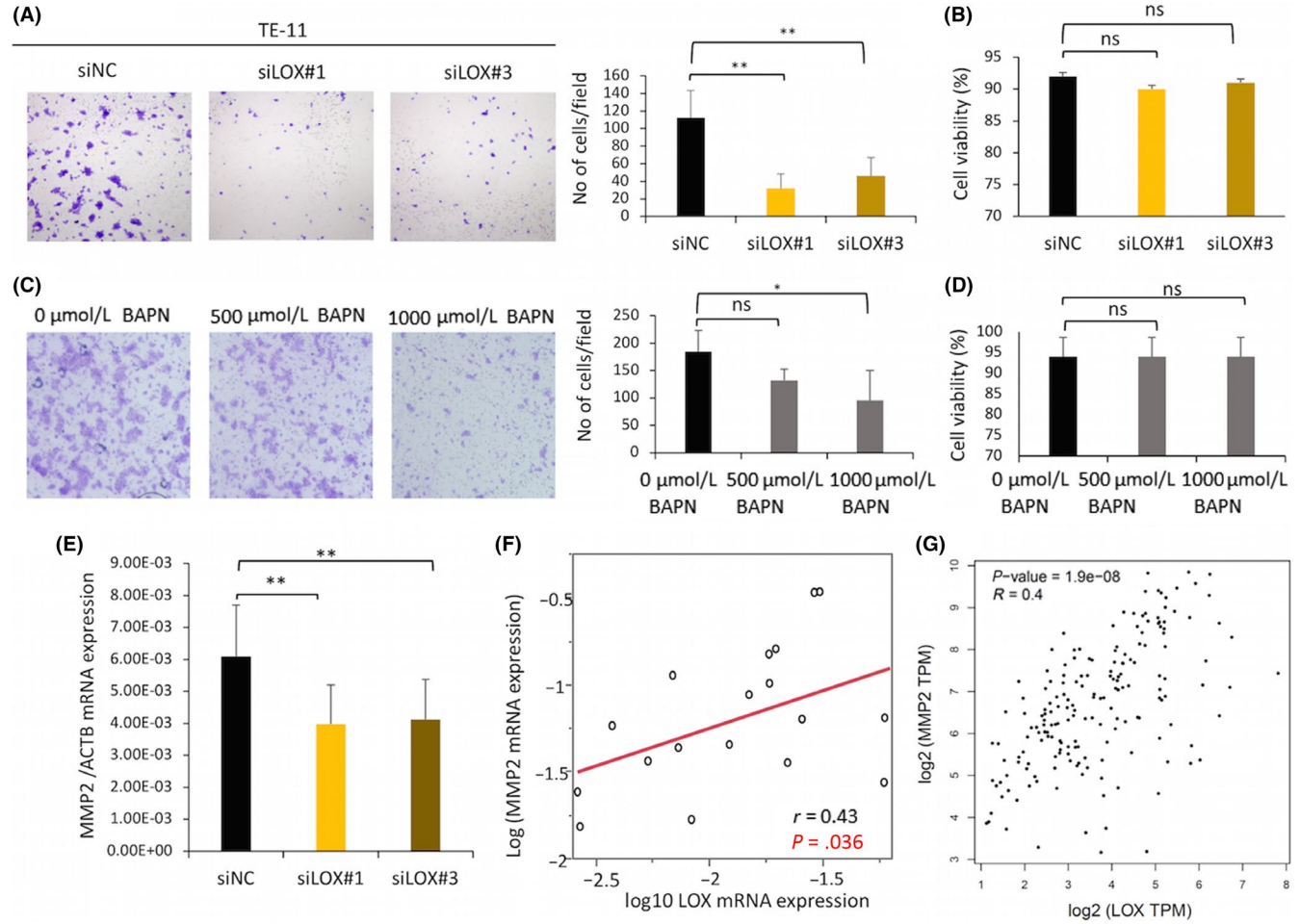

FIGURE 3 Effects of lysyl oxidase (LOX) on esophageal tumor invasion. A, Transwell invasion assay for siRNA-transfected cells. Right panel: number of invaded cells per field (represented as means + SD). $^{**}P < .01$. B, Plots show cell viability after transfection with LOX siRNAs and relative to their controls. LOX knockdown reduced cell invasiveness (represented as means + SD). C, Transwell invasion assay for β-aminopropionitrile (BAPN)-treated cells. Right panel: number of invaded cells per field (represented as means + SD). $^{*}P < .05$. D, Plots show cell viability after transfection with LOX siRNAs and relative to their controls. LOX knockdown reduced cell invasiveness (represented as means + SD). E, MMP2 expression upon LOX suppression by 2 siRNA for LOX in TE-11 cells (represented as means + SD). $^{**}P < .01$. F, Relationship between LOX and MMP2 expression in frozen esophageal cancer specimens. G, Relationship between *LOX* and *MMP2* expression in silico. ns, not significant; siNC, normal control siRNA

that LOX was highly expressed in esophageal cancer, suggesting that LOX is involved in ESCC pathogenesis. Overexpression of LOX has been reported in various cancers including nasopharyngeal carcinoma, head and neck tumors, breast cancer, hepatocellular carcinoma, and colorectal cancer and has been associated with poor clinical outcome.[30-33] Similarly, we found that esophageal cancer patients highly expressing LOX had a poorer prognosis in terms of shorter OS and cancer-specific survival. From the results, it is clear that LOX expression is clinically implicated in poor prognosis in esophageal cancer. In addition, high LOX expression had a significant correlation with the presence of lymph node metastasis. This is consistent with a previous study in esophageal cancer,[34] but our findings are slightly superior because we used a larger cohort of patients and we further determined the function of LOX in esophageal cancer progression using in vitro experiments.

Lysyl oxidase has been reported to function intracellularly and is involved in the regulation of cell differentiation, migration, and motility. Recent evidence indicates that LOX enzyme specifically plays a role in tumor progression,[4] but reports in esophageal cancer are limited. In this study, LOX functional analysis was evaluated in vitro by knockdown of LOX with siRNA and inhibition of LOX enzymatic activity with BAPN. We showed that LOX knockdown and inhibition by BAPN led to significant reductions in cell proliferation. Moreover, we observed a reduction in the expression of p-AKT in LOX knockdown cells, which implies that LOX regulates cell proliferation through the PI3K-AKT signaling pathway; a similar conclusion was reached by Pez et al in colorectal cancer.[35] Of course, we acknowledge that the other molecules, such as vascular endothelial growth factor and hypoxia inducible factor-1 could play a crucial role downstream of LOX. Future studies are needed to examine other potential mechanism(s) by which LOX affects tumor behavior. We also focused on the role of LOX in cell migration and interestingly we showed that the migration ability of cells was reduced following LOX knockdown and enzymatic inhibition.

"Esophageal cancer is notoriously aggressive and invasive in nature."[36] In this study, we addressed the role of LOX in esophageal

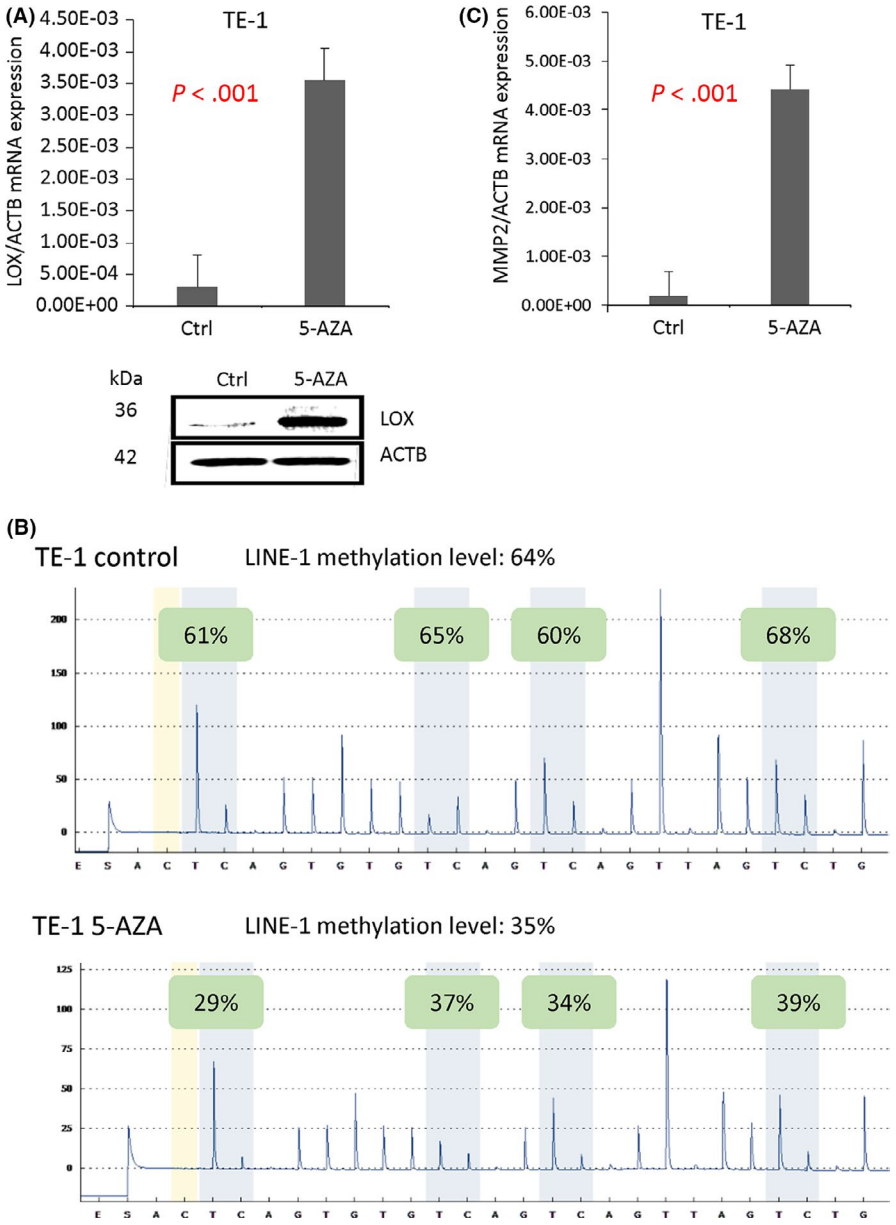

FIGURE 4 Epigenetic regulation of lysyl oxidase (LOX) expression in vitro. A, DNA demethylation with 5-aza-2′-deoxycytidine (5-AZA) causes upregulation of *LOX* mRNA expression and protein expression in the TE-1 esophageal cancer cell line ($P < .001$). B, Pyrogram showing reduction of long interspersed nucleotide element-1 (LINE-1) methylation levels after demethylation with 5-AZA. C, DNA demethylation with 5-AZA causes upregulation of MMP2 mRNA expression in the TE-1 cell line ($P < .001$). ACTB, β-actin; Ctrl, control

cancer invasion and revealed that knockdown of LOX with siRNA and inhibition of LOX enzyme with BAPN led to reduced invasiveness of TE-11 cells. These results go beyond previous reports,[12,15] showing that LOX regulates cell invasion through MMP2 as knockdown of LOX expression led to a decrease in MMP2 expression. Matrix metalloproteinase 2 has been reported to promote tumor invasion in various cancers including colorectal[37] and prostate cancer.[38] Our results cast a new light on the role of LOX in potentiating esophageal cancer progression. Moreover, using in vitro experiments, we have showed that the use of BAPN, an irreversible LOX inhibitor, could contribute significantly to reversing the effects caused by this enzyme. Therefore, as BAPN was reported to be useful in the treatment of cancers that overexpress LOX, such as breast cancer,[39,40] melanoma,[41,42] and head and neck carcinoma,[43,44] our findings suggest that targeting LOX therapeutically would improve the clinical outcome of esophageal cancer patients.

Our study also evaluated the epigenetic regulation of LOX expression in esophageal cancer. Previous studies that reported on the epigenetic regulation of LOX identified *LOX* as a tumor suppressor gene that was suppressed by promoter hypermethylation.[45,46] In contrast, in our study, we found that LOX enzyme functions as a tumor promoter in ESCC and LOX expression was increased by DNA demethylation of TE-1 cells (a low LOX expression cell line) in vitro. These findings were further validated by our expression array analysis, in which *LOX* was shown to be one of the most upregulated genes in hypomethylated tumors. In addition, in clinical specimens, tumors with high LOX expression had significantly lower LINE-1 methylation levels. In contrast, we found that LOX expression was not related to the methylation status of the LOX promoter. Previously, using a CGH array, we suggested that LINE-1 hypomethylation contributes to the acquisition of aggressive tumor behavior in ESCC through genomic gain of oncogenes such as *CDK6*.[26] This

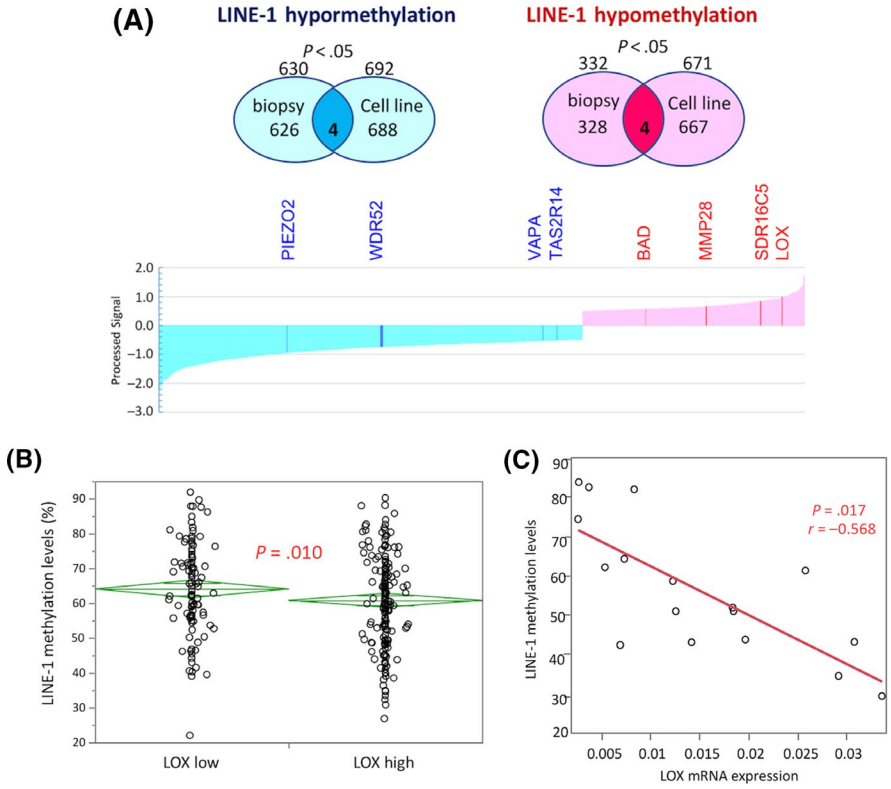

FIGURE 5 Relationship between lysyl oxidase (LOX) expression and long interspersed nucleotide element-1 (LINE-1) methylation in vitro. A, mRNA expression array analysis using 10 esophageal tumor biopsy specimens and data from 10 cell lines analyzed from a public database (GSE63941) for comparison, showing upregulated and downregulated genes in LINE-1-hypomethylated and LINE-1-hypermethylated tumors, respectively. LOX was the most highly upregulated gene with the highest fold change of the 4 identified genes in LINE-1-hypomethylated tumors ($P < .05$) in an integrated analysis. B, Association between LOX expression and LINE-1 methylation levels. Tumors with high LOX expression showed significantly lower LINE-1 methylation levels than tumors with low LOX expression ($P = .010$, paired *t* test). C, Relationship between LOX expression and LINE-1 methylation

study provides preliminary evidence that LOX expression is regulated epigenetically by DNA hypomethylation, and this confounding relationship might explain the prognostic impact of LINE-1 hypomethylation in esophageal cancer patients. Further research is needed to explore the specific mechanism behind this relationship.

In conclusion, the results of our study show that high LOX expression is associated with poor prognosis and aggressive tumor behavior in patients with ESCC. Moreover, our findings suggest that LOX expression is regulated epigenetically by DNA hypomethylation. Therefore, detecting LOX expression in patients, and therapeutically targeting LOX, could contribute to the prevention and treatment of esophageal cancer; thus, our study has clinical significance.

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CONFLICT OF INTEREST

No conflict of interests declared.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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