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Research Article

LOXL2 promotes tumor proliferation and metastasis by FAK/Src signaling in esophageal squamous cell carcinoma ☆

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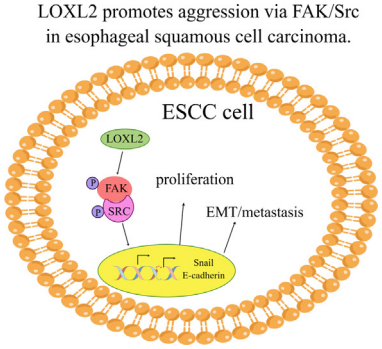
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Esophageal squamous cell carcinoma is the most common malignant tumor of the upper gastrointestinal tract, which is prone to metastasis and has a poor prognosis. Lysine oxidase-like protein 2 (LOXL2) is an important intracellular protein that is highly expressed in a variety of tumors, leading to reduced patient survival. Studies have shown that LOXL2 is closely associated with tumor metastasis. However, the effect of LOXL2 on the biological function of esophageal squamous cell carcinoma (ESCC) and its related mechanisms are not fully understood. This study aimed to evaluate the potential prognostic and treatment value of LOXL2 in patients with ESCC.

Results: The expression of LOXL2 was higher in ESCC tissues than in adjacent tissues. Positive LOXL2 expression was associated with poor tumor differentiation, lymph node metastases, and poor prognosis in ESCC patients. LOXL2 is an independent prognostic risk factor for ESCC. *In vitro* experiments showed that LOXL2 significantly promoted ESCC cell proliferation, migration, and invasion ability. LOXL2 altered the expression of the EMT marker, upregulating the mesenchymal marker (Snail) and downregulating the epithelial marker (E-cadherin). Mechanistically, LOXL2 induces EMT in esophageal squamous cell carcinoma by increasing FAK and SRC phosphorylation levels.

Conclusions: Taken together, LOXL2 regulates the FAK/Src signaling pathway to promote malignant biological behavior and EMT process in ESCC cells. LOXL2 is a marker of poor prognosis, and LOXL2 could be a promising therapeutic target for the treatment of esophageal squamous carcinoma.

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1. Introduction

Esophageal cancer is the most prevalent digestive system cancer worldwide, with an estimated 604,100 individuals diagnosed, and 544,076 patients deaths in 2020 [1]. More than half of the world's esophageal cancer incidence and mortality occur in China [2]. Esophageal cancer is divided into two main pathological types: squamous cell carcinoma and adenocarcinoma. In Asia, esophageal squamous cell carcinoma (ESCC) is the most common histologic type [3]. The early symptoms of ESCC patients are not obvious, the disease progresses easily to distant metastases, and the 5-year survival rate of patients is less than 30% [4]. Patients with ESCC who are unable to be treated surgically are primarily treated with chemo-radiotherapy and targeted therapies; nevertheless, the efficacy of treatment options remains controversial, and therapy lacks clearly defined and effective therapeutic targets [5]. Therefore, investigating the mechanisms involved in ESCC metastasis can provide a better understanding of the progression of ESCC and potentially lead to the discovery of novel targeted treatment drugs.

The lysine oxidase (LOX) family is a CU-dependent amino oxidase family composed of five enzymes: LOX, LOXL1, LOXL2, LOXL3, and LOXL4, which participate in biological processes such as cardiac fibrosis, osteogenesis, and cancer progression [6]. They have copper binding sites, lysine-tyrosine-quinone residues, and cytokine receptor-like domains in their highly conserved C-ends. However, the N-terminal sequences of the family members are diverse, resulting in different members having different functions [7]. The N-terminus of LOXL2 contains four contiguous scavenger receptor cysteine-rich (SRCR) structural domains. Through enzyme-dependent activity or independently of its catalytic activity, LOXL2 participates in the remodeling of the tumor microenvironment and promotes cell migration, invasion, angiogenesis, and metastasis [8]. Previous research has shown that LOXL2 is highly expressed in a variety of malignancies, such as hepatocellular carcinoma, gastric carcinoma, and colorectal carcinoma, and its high expression is usually indicative of poor patient prognosis [9,10,11]. In breast cancer, overexpression of enzymatically active LOXL2 promotes increased cross-linking and rearrangement of extracellular matrix fibers, enhancing breast cell invasiveness and leading to distant metastasis [12,13]. However, the function of LOXL2 in ESCC progression is not fully understood.

The metastasis of tumors depends on the migration and invasion ability of tumor cells and the remodeling of the extracellular matrix [14]. The focal adhesion kinase (FAK) belongs to the Src family of non-receptor tyrosine kinases, which is an important molecule that mediates cell-to-cell and cell-to-extracellular matrix signal transduction [15]. Src binds directly to the Tyr397 autophosphorylation site of FAK through its SH2 structural domain to form the FAK-Src complex [16]. FAK/Src regulates cytoskeletal dynamics and cell motility signaling pathways by affecting actin polymerization and focal flipping of adherent spots, thereby altering the tumor microenvironment and promoting the processes of tumorigenesis and metastasis [17]. Interestingly, LOXL2 promotes cytoskeletal reorganization and cell invasion by interacting with cytoplasmic actin-binding proteins [18]. Tanaka et al. [19] demonstrated that high expression of LOXL2 in pancreatic cancer induced activation of the FAK/Src signaling pathway, which silenced E-cadherin expression by regulating Snail transcription, ultimately leading to epithelial-mesenchymal transition (EMT) and promoting tumor cell migration and invasion. However, whether LOXL2 can synergize with the FAK/Src signaling pathway to promote metastasis in esophageal squamous cell carcinoma is unclear.

In the present study, we focused on the function of LOXL2 in ESCC and its potential mechanisms. We found upregulation of LOXL2 in ESCC tissues, and positive LOXL2 expression was associated with lymph node metastasis and poor prognosis. LOXL2 interacts with the FAK/Src signaling pathway to promote the proliferation, invasion, migration, and EMT processes of ESCC cells. This research explains the importance of LOXL2 in ESCC progression and suggests that it may be a new therapeutic target.

2. Materials and methods

2.1. Clinical samples

The tissues and information of 204 patients with esophageal squamous cell carcinoma who attended the Cancer Hospital of Xinjiang Medical University from 2015 to 2017 were collected. The ESCC tumor tissue and the corresponding adjacent normal tissues of 158 stage I-III patients from surgical resected specimens, and the tumor tissue of 46 stage IV patients were from esophageal endoscopic biopsy. All cases' diagnosis was based on the pathological examination. All specimens were preserved with 10% formalin solution and stored them in paraffin before use. Before surgery, no one of the patients had received any either local or systemic therapy. The seventh edition of the AJCC [20] (American Joint Committee on Cancer) TNM classification was used to define the stages of ESCC. All patients and their family who registered were informed and gave their consent. The study protocol was approved by the Ethics Committee of the Affiliated Tumor Hospital, Xinjiang Medical University (Urumqi, China), with the approval number K-2019053.

2.2. Cell lines and culture

ESCC cells including Eca-109 (CL-0077, Procell, Wuhan, China), KYSE150 (CL-0638, Procell, Wuhan, China), KYSE450 (YS3155C, YaJiBiological, Shanghai, China), KYSE30 (YS321C, YaJiBiological, Shanghai, China) and TE-1 (YS290C, YaJiBiological, Shanghai, China), were incubated in RPIM 1640 medium (11875-093, GIBCO, USA) with 10% fetal bovine serum (FBS) (10100147, ExCell Bio, Guangzhou, China) and 1% penicillin–streptomycin (25200-056, ExCell Bio, Guangzhou, China) supplements. ESCC cells were incubated at 37°C with 5% CO2.

2.3. Lentiviral infection

For the shRNA-mediated knockdown of LOXL2 targeting shRNAs were synthesized [21] (Genechem, Shanghai, China). The LOXL2-shRNA1 target sense sequences: 5′-cgATTACTCCAACAACAT CAT-3′. The LOXL2-shRNA1 target antisense sequences: 5′-ATGAT GTTGTTGGAGTAATCGTTTTTg-3′. The LOXL2-shRNA2 target sense sequences 5′-gaGAGGACATACAATACCAAA-3′. The LOXL2-shRNA2 target antisense sequences: 5′-TTTGGTATTGTATGTCCTCTCTTTTTg. The LOXL2-shRNA3 target antisense sequences: 5′-gaAGGAGA

CATCCAGAAGAAT-3′ and antisense sequences: 5′-ATTCTTCTG GATGTCTCCTTCTTTTTg-3′. The cell culture situation was modified to standard DMEM high glucose medium (11965092, GIBCO, USA) including FBS and penicillin–streptomycin after transfection in 293T cells (K1711, American Type Culture Collection, Manassas, VA, USA) for 8 h. After 48 h of transfection, viral particles were collected. The viral titer was evaluated by measuring the amount of green fluorescent protein-positive counts. The cells were treated with 500 ng/ml Puromycin [22] (PZ0234, Sigma, USA) for 48 h at 37°C to induce LOXL2 knockdown.

Lentiviral infection was conducted on ESCC cells by infecting them with the lentivirus at a multiplicity of infection of 20 in polybrene [23] at a concentration of 5 μg/mL (TR-1003, Sigma, USA). Lentiviral short hairpin RNAs (shRNA) targeting LOXL2 were acquired from Genechem (Shanghai, China). Puromycin at a concentration of 5 μg/ml was used to select the stable LOXL2 knockdown or overexpression cell lines, subsequently using the stable cell lines in the cellular studies that followed.

2.4. Cell proliferation assay

A total of 96-well microplates with 1000 ESCC cells in each well were planted with the cells (100 μL/ well). LOXL2 shRNA lentivirus and negative control shRNA were added according to the group. After the intervention, the medium was discarded and 100 μL of 10% CCK-8 solution were treated with Cell Counting Kit-8 [24] (Transgen, Beijing, China) for 1 h at 37°C after being cultured for 24 h. Then, using an enzyme-linked immunosorbent assay plate reader (BioTek Elx 800, USA), the optical density was measured at 450 nm.

2.5. Apoptosis and cell cycle assay

The cells were trypsinized, washed in PBS, and resuspended at a density of 10^6 cells per milliliter in 1X binding buffer. The 100 μl suspension and Annexin V-PE (5 μl) and 7-AAD (10 μl) [25] were poured into a 5 ml tube. The samples were mixed at 4°C for 10 min while they were kept in the dark. Red fluorescence and light scattering were detected by flow cytometry at excitation wavelength 488 nm. Flow cytometry (BD Biosciences, USA) was used to measure apoptosis rate, and FlowJo was used to analyze the data.

The cells were exposed to 80% ice-cold ethanol for an entire night in order to analyze the cell cycle. Propidium iodide (PI: 20 μg/ml, catalog number 550825) was applied to the cells for 30 min at 4°C while they were kept in the dark. Red fluorescence and light scattering were detected by flow cytometry at excitation wavelength 488 nm. Analysis software for cell DNA content was examined using a flow cytometer (BD Biosciences, San Jose, CA, USA) for cell cycle.

2.6. Migration and invasion assays

Transwell assays were used to evaluate cell migration and invasion [26,27]. First, the cells were cultured in DMEM without FBS for 24 h. The cells were suspended with serum-free medium, counted, and the concentration was adjusted to 5×10^5 cells /ml. The upper chambers received 5×10^4 cells that had been resuspended in 100 μL DMEM without FBS. The lower chambers were full with DMEM containing 10% FBS, which was allowed to develop for 48 h before being fixed to the insert membranes with 4% paraformaldehyde and stained with crystal violet solution. A total of 400 μl Giemsa dye solution B was added and continued staining for 1 min. To investigate cell migration, Transwell Chambers without a matrix coating were used. The lower chambers were filled with DMEM containing 10% FBS, while the upper chambers were filled with 2×10^4 cells. Following a 24 h growth period, fixed with 4% paraformaldehyde and stained using a crystal violet staining solution, 800 μl Giemsa dye solution B was added and continued staining for 6 min.

2.7. qRT-PCR analysis

TRIZOL reagent (Ambion, USA) was applied to retrieve the total RNA from patient samples and ESCC cells. The 5X All-In-One RT MasterMix (abm, Canada) was applied to reverse-transcribe the same amount of total RNA into cDNA. The q-PCR tests were carried out using a Real-Time PCR apparatus (ABI, USA) and the EvaGreen Express 2qPCR MasterMix-Low Rox (abm, Canada). To normalize gene expression levels, the housekeeping gene GAPDH was used. We used the following primer pairs to identify genes, as shown in Table 1. Relative gene expression levels were calculated using the comparative cycle threshold (Ct) ($2^{-\Delta\Delta}$Ct) method [28,29] and then converted to fold changes.

2.8. Western blotting analysis

Western blotting analyses were carried out utilizing conventional techniques. The cells were washed with PBS before being collected using a RIPA lysis buffer, which contained protease inhibitors (Boster Biological Technology, Wuhan, China). We added the same quality protein to each sample and then loaded each sample onto 10% SDS–polyacrylamide gels for electrophoresis. The proteins were transferred to PVDF membranes (Millipore, MA, USA) and being blocked with QuickBlock Blocking Buffer (Sangon Biotechnology, Shanghai, China). Primary antibody was incubated overnight at 4°C and then secondary antibody for 2 h at 37°C, followed by electrochemiluminescence detection (Thermo Fisher Scientific, Shanghai, USA). To account for variations in protein loading, the same membrane was stripped and incubated with the anti-β-actin [30] antibody.

The primarily used antibodies included anti-LOXL2 (#PB0759, 1:500, Boster Biological Technology, China), anti-FAK (#bs-1340R, 1:400, Bioss, China), anti-p-FAK (#bs-3159R, 1:300, Bioss, China), anti-Src (#bs-1135R, 1:400, Bioss, China), anti-p-Src (#bs-3426R, 1:300, Bioss, China), anti-E-cadherin (#bs-1519R, 1:500, Bioss, China), anti-Snail (#bs-1371R, 1:400, Bioss, China), and anti-β-actin (#100166-MM10, 1:1000, sinobiological, China).

2.9. Immunohistochemistry analysis

The ESCC and corresponding adjacent normal paraffin-embedded tissues were divided into slices of 4 mm thickness, and LOXL2 immunohistochemical staining [31] procedures were carried out on each section. The standard protocol was followed while deparaffinizing, rehydrating, and treating the paraffin sec-

Table 1
Primer sequences for qRT-PCR.

Gene	Primer sequence (5′ to 3′)	Product size
LOXL2	Forward: GTGTGCAGCGACAAAAGGATT Reverse: CACGTAGCCCTCCATCACTG	150 bp
FAK	Forward: ACACTTGGAGAGCTGAGGTC Reverse: GACACCAGAACATTCCGAGC	158 bp
Src	Forward: TCAATGCAGAGAACCCGAGA Reverse: CATCGGCGTGTTTGGAGTAG	219 bp
E-cadherin	Forward: GCTGTGTCATCCAACGGGAA Reverse: CACCTTCCATGACAGACCCC	124 bp
Snail	Forward: CCCCAATCGGAAGCCTAACT Reverse: GACAGAGTCCCAGATGAGCA	157 bp 197 bp
GAPDH	Forward: TGTTGCCATCAATGACCCCTT Reverse: CTCCACGACGTACTCAGCG	202 bp

tions. The sections were treated with a monoclonal anti-human LOXL2 antibody (#TA807443, 1:200, ORIGENE, USA) or phosphate-buffered saline, and then incubated with a secondary antibody that was horseradish peroxidase-labeled for 30 min. After washed with PBS, the sections were stained with 3′,3-diaminobenzidine solution, labeled with DAB, and then counterstained with 0.1% hematoxylin. LOXL2 staining intensity and rate were graded. There are four levels of staining intensity: 0, no staining, 1, mild staining, 2, moderate staining, and 3, strongly positive. Staining rate criteria are as follows: 0, 0–10% positive cells; 1, >10% positive cells. The semi-quantitative score was calculated by multiplying these two values for each section (from 0 to 3). Two histopathologists blinded to clinical data reviewed and scored the slides.

2.10. Bioinformatics analysis

UALCAN [32] (http://ualcan.path.uab.edu/) was used to analyze LOXL2 expression data from samples (contains tumor and normal) of TCGA. There are the following conditions: step 1, datasets: esophagus cancer; step 2, gene symbol: LOXL2; step 3, expression: based on histology type.

GEPIA [33] (http://gepia.cancer-pku.cn/) was used to perform the correlation analysis. There are the following screening conditions: step 1, input gene: Gene A: LOXL2, Gene B: FAK; step 2, Correlation coefficient: Pearson; step 3, selecting datasets: ESCA tumor.

2.11. Statistical analysis

The SPSS software (version 22.0, IBM Corp, Armonk, NY, USA) and GraphPad Prism software (version 8.01, GraphPad Software Inc, San Diego, CA) were used for all statistical analyses. Values were expressed as the mean ± SD. Student's t-tests and Mann-Whitney U tests were used for comparing quantitative variables. Chi-squared test or Fisher's exact tests were used to compare qualitative variables. The Kaplan-Meier was used to draw survival curves, and the log-rank test was used to compare them. Molecular schematic representation was plotted by Figdraw. $^{*}P < 0.05$ was regarded as statistically significant.

3. Results

3.1. LOXL2 is upregulated in ESCC patients and is positively associated with aggressive clinicopathological characteristics

To clarify the expression of LOXL2 in esophageal squamous carcinoma, we first analyzed data from UCLCAN. The expression level of LOXL2 mRNA was significantly higher in esophageal cancer tissues than in normal tissues, especially the expression was increased in esophageal squamous cell carcinoma (Fig. 1a). Then, the expression of LOXL2 was detected by qRT-PCR in 15 pairs of esophageal squamous carcinoma patients, and the results showed that the relative expression of LOXL2 in esophageal squamous carcinoma tissues was 1.719 ± 0.521, which was significantly higher than that of 1.078 ± 0.305 in adjacent normal tissues (Fig. 1b). Finally, the expression of LOXL2 was further verified by immunohistochemical analysis, which showed that the rate of high expression of LOXL2 in esophageal squamous carcinoma tissues was 75.95% (120/158) and the rate of high expression of LOXL2 in adjacent normal tissues was 53.16% (84/158) (Fig. 1c).

Table 2 summarizes the relationship between clinicopathological features and LOXL2 expression in patients with ESCC. We divided 204 patients into a positive-LOXL2 group (n = 164) and negative-LOXL2 group (n = 40) based on IHC analysis. Patients with positive LOXL2 expression were more likely to exhibit clinical features such as poor tumor differentiation ($\chi^2 = 6.78$, $P < 0.05$), deep tumor infiltration ($\chi^2 = 9.88$, $P < 0.05$), regional lymph node metastasis ($\chi^2 = 4.61$, $P < 0.05$) and late TNM stage ($\chi^2 = 12.12$, $P < 0.05$) compared to those with negative LOXL2 expression. There were no statistically significant differences in LOXL2 expression with patient age, gender, and CEA expression. The above data suggest that LOXL2 expression is associated with the aggressive clinicopathological features of ESCC.

3.2. LOXL2 as a potential prognosis biomarker in ESCC patients

According to Kaplan-Meier survival analysis, patients with positive LOXL2 expression had a median survival of 20 months, which was considerably less than the 40 months for patients with negative LOXL2 expression (Fig. 1d). Patients with positive expression of LOXL2 had a lower survival rate than patients with negative expression of LOXL2. Univariate Cox proportional hazards models showed that significant variables for poorer OS included poor tumor differentiation, higher T stage, higher lymph node metastasis, later disease stage and positive LOXL2 expression (Table 3). Multivariate analysis shows that positive LOXL2 expression and late TNM staging are associated with shorter overall survival in ESCC patients (Table 3). These data reveal that LOXL2 is an independent prognostic factor for ESCC, and its high expression indicates a poor prognosis for patients.

3.3. LOXL2 promotes cell proliferation and inhibits apoptosis in ESCC cells

We examined LOXL2 expression in different ESCC cell lines by qRT-PCR. We select TE-1 cells as the LOXL2 high-expressing cell line and KYSE30 cells as the LOXL2 low-expressing cell line for the following experiments (Fig. 2a). TE-1 cells were transduced with three lentiviral LOXL2 shRNAs. The transfection efficiency of each group of ESCC cells was verified using qRT-PCR, and the shLOXL2-2 with the highest transfection efficiency was chosen to establish a sub-cell line with low LOXL2 expression (Fig. 2b). In contrast, the cell that successfully expressed LOXL2 ectopically in KYSE30 cells was chosen for functional analysis (Fig. 2c).

Cell proliferation assays show that LOXL2 knockdown significantly reduces TE-1 cell viability compared to vector control (Fig. 2d). The opposite effect was obtained in KYSE30 cells overexpressing LOXL2 (Fig. 2e). These results indicate that LOXL2 can promote the growth of ESCC cells. LOXL2′s impact on the ESCC cell cycle was evaluated using flow cytometry. The results showed that the knockdown of LOXL2 in TE-1 cells decreased the proportion of S-phase cells and increased the proportion of G0/G1-phase cells compared with the control group (Fig. 3a). In contrast, KYSE30 cells overexpressing LOXL2 significantly increased the proportion of S-phase cells and decreased the proportion of G0/G1-phase cells (Fig. 3b). These findings suggest that LOXL2 promotes ESCC cell proliferation by participating in cell cycle differentiation. In addition, we analyzed the role of LOXL2 in apoptosis. In TE-1 cells, knockdown of LOXL2 significantly increased the percentage of apoptotic cells from 7.690% to 18.163%. Overexpression of LOXL2 reduced the percentage of apoptotic cells from 5.767% to 5.187% in KYSE30 cells (Fig. 3c, d). The above study showed that LOXL2 could promote ESCC cell proliferation and inhibit apoptosis.

3.4. LOXL2 enhances migration and invasion and promotes EMT of ESCC cells

Transwell assay results showed that knockdown of LOXL2 decreased the migratory and invasive abilities of TE-1 cells

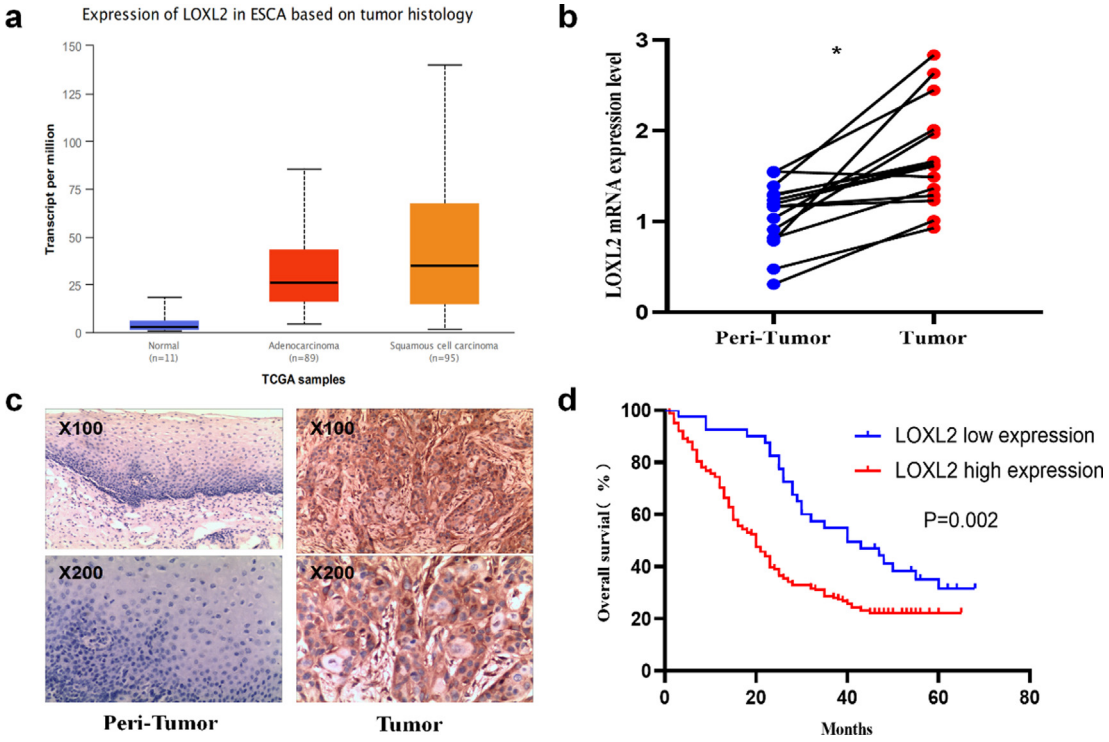

Fig. 1. LOXL2 expression is upregulated and associated with poor prognosis of ESCC patients. (a) Representative LOXL2 mRNA expression in esophageal carcinoma tissues versus esophageal cancer samples from the TCGA. (b) LOXL2 gene expression levels in 15 paired ESCC and tumor-adjacent tissues. *$P < 0.05$. (c) Representative images of IHC analysis of LOXL2 staining in ESCC and tumor-adjacent normal tissue. (d) Kaplan-Meier curve analysis revealed that ESCC patients in positive LOXL2 expression had shorter survival time.

Table 2
Association between the expression of LOXL2 and main clinical characteristics in ESCC patients.

Clinicopathologic feature	All cases	LOXL2 expression		Chi-square	*P* value
		Negative (n = 40)	Positive (n = 164)		
Gender					
Female	80	16	64	0.013	1.000
Male	124	24	100		
Age (years)					
<60	88	20	68	0.955	0.375
≥60	116	20	96		
CEA (μg/L)					
≤5	187	38	149	0.724	0.534
>5	17	2	15		
Tumor differentiation					
Well	18	7	11	**6.778**	**0.034***
Moderate	132	27	105		
Poor	54	6	48		
T stage					
T1	10	4	6	**9.879**	**0.020***
T2	28	10	18		
T3	134	23	111		
T4	32	3	29		
Lymph node metastasis					
Negative	77	21	56	**4.610**	**0.045***
Positive	127	19	108		
TNM stage					
I	4	2	2	**10.057**	**0.018***
II	76	14	62		
III	78	21	57		
IV	46	3	43		

Two-way ANOVA was used to evaluate significant differences,*$P < 0.05$. Statistical significance ($P < 0.05$) is shown in bold.

(Fig. 4a). In contrast, overexpression of LOXL2 increased the migratory and invasive abilities of KYSE30 cells (Fig. 4b). Accordingly, we hypothesized that LOXL2 promotes the migration and invasive abilities of esophageal squamous cell carcinoma. Owing to the fact that EMT usually occurs in the early stages of malignant metastasis, it allows tumor cells to invade and migrate by altering the

Table 3
Univariate and multivariate Cox hazards analyses of different clinical characteristics in ESCC patients.

Clinicopathologic Feature	Univariate analysis		P	Multivariate analysis		P
	HR	95% CI		HR	95% CI	
Gender (Male vs. Female)	0.924	0.669–1.276	0.629			
Age (years) (≥60 vs. < 60)	0.973	0.707–1.339	0.866			
CEA (μg/L) (>5 vs. ≤ 5)	1.548	0.907–2.641	0.109			
Tumor differentiation (Poor, Moderate vs. Well)	1.503	1.144–1.975	0.003*	1.144	0.868–1.507	0.339
T stage (T2-4 vs. T1)	1.884	1.450–2.448	<0.001*	1.126	0.837–1.517	0.433
Lymph node metastasis (Positive vs. Negative)	3.125	2.153–4.537	<0.001*	0.953	0.566–1.605	0.856
TNM stage (II–IV vs. I)	3.593	2.787–4.633	< 0.001*	3.366	2.371–4.778	< 0.001*
LOXL2 expression (Positive vs. Negative)	1.937	1.265–2.965	0.002*	1.737	1.112–2.713	0.015*

CI: confidence interval; HR: hazard ratio. ANOVA was used to evaluate significant differences, *P < 0.05. Statistical significance (P < 0.05) is shown in bold.

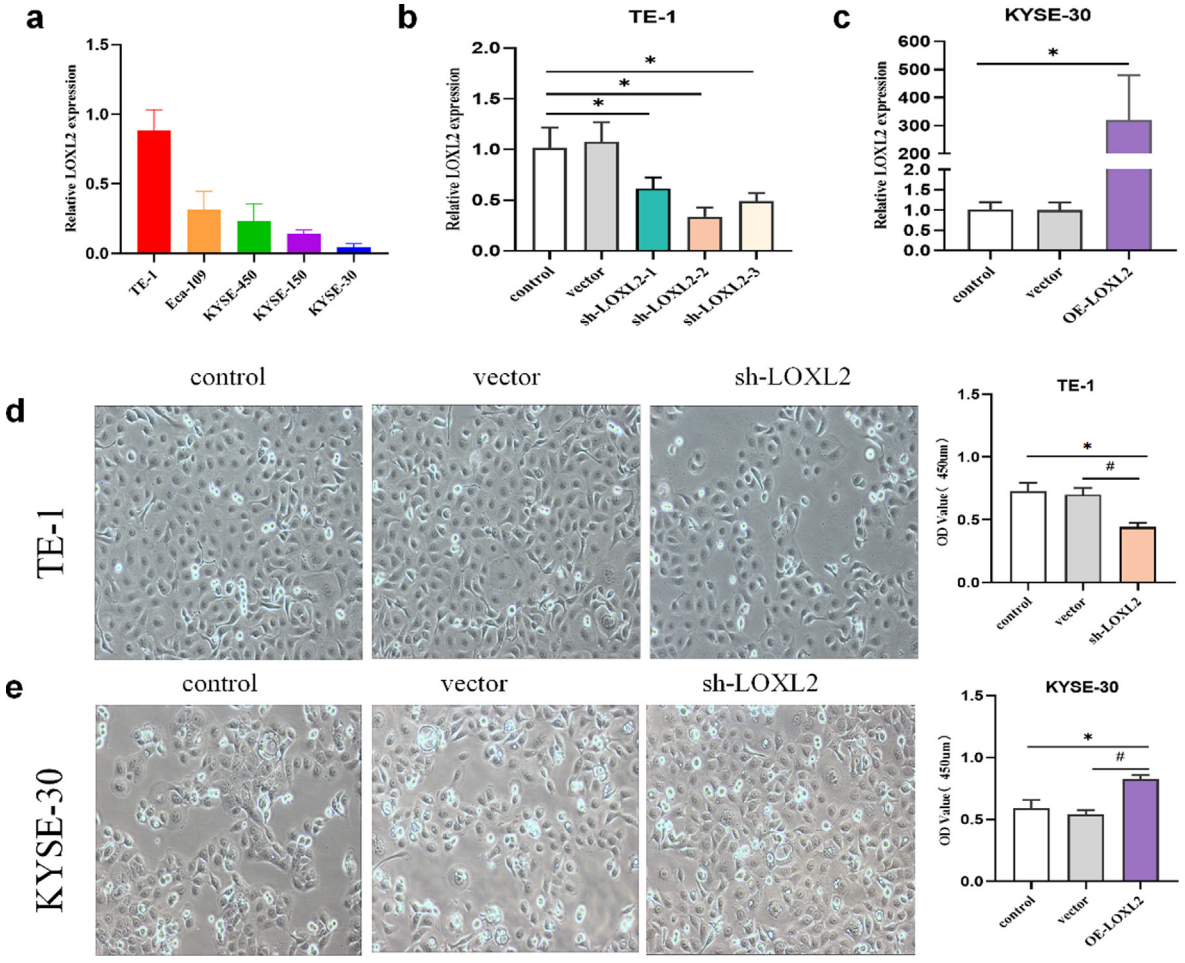

Fig. 2. LOXL2 promotes ESCC cell proliferation. (a) qRT-PCR identified LOXL2mRNA expression in ESCC cells. (b) qRT-PCR identified that the expression of LOXL2 was lower in sh-LOXL2 1/2/3-transfected cells than in corresponding negative control group cells. (c) qRT-PCR identified the expression of LOXL2 was overexpression in OE-LOXL2-transfected cells than corresponding negative control group cells. (d,e) The viability of ESCC cell lines after LOXL2 knockdown or overexpression was determined using the CCK-8 assay. All cellular experiments were performed in triplicate or more, data are expressed as mean ± SEM, *P < 0.05, #P < 0.05.

tumor microenvironment [34,35]. Therefore, we used qRT-PCR and WB to detect EMT markers in the aforementioned cells. The results showed that the expression of the epithelial marker E-cadherin was upregulated while the expression of the mesenchymal marker Snail was significantly downregulated in TE-1 cells with LOXL2 knockdown (Fig. 4c). Overexpression of LOXL2 led to a decrease

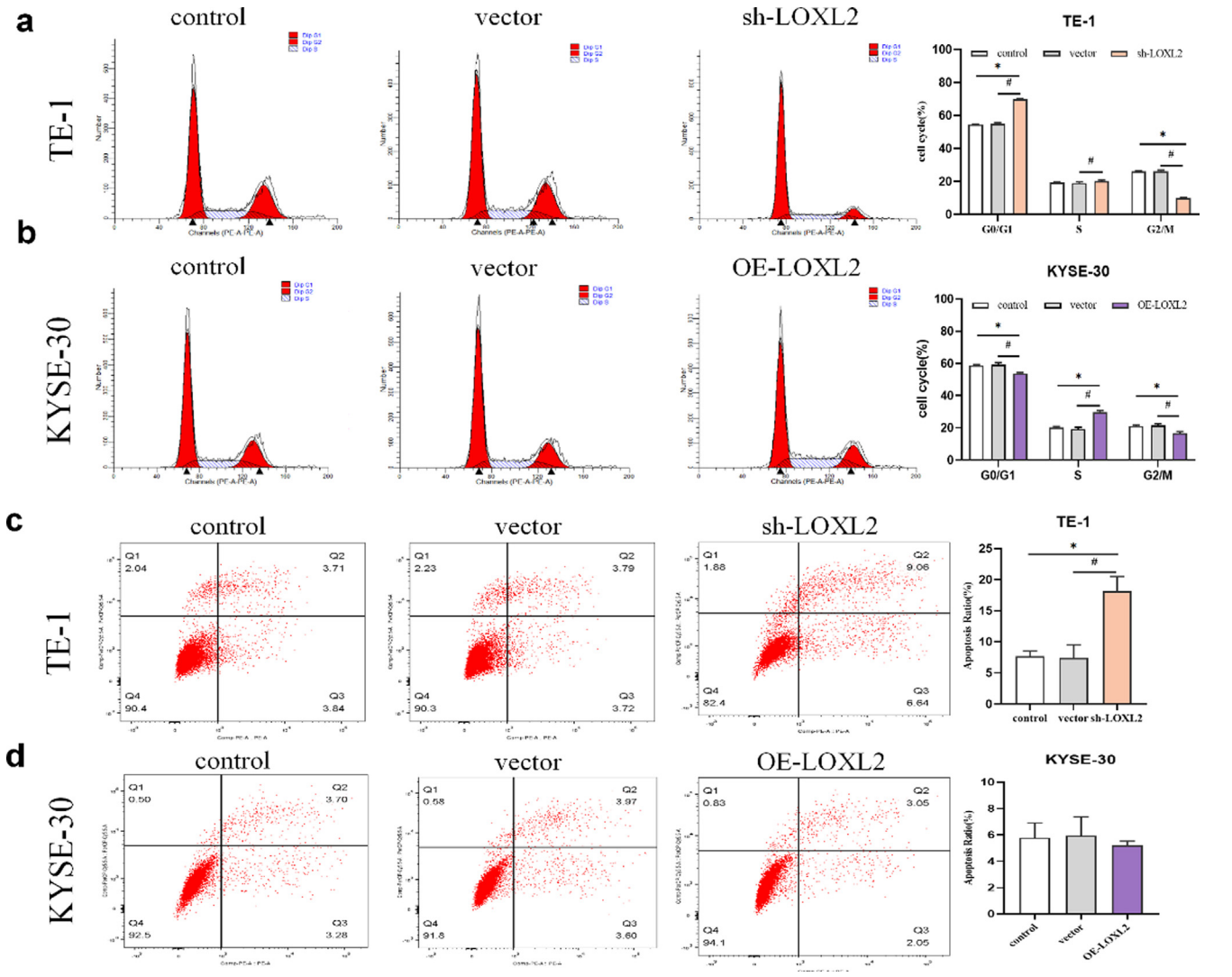

Fig. 3. LOXL2 regulates ESCC cell cycle. (a,b) The cell cycle of ESCC cell lines after LOXL2 knockdown or overexpression was determined using the flow cytometry. (c,d) The cell apoptosis of ESCC cell lines after LOXL2 knockdown or overexpression was determined using the flow cytometry. All cellular experiments were performed in triplicate or more data are expressed as mean ± SEM; $^{*}P < 0.05$, $^{\#}P < 0.05$.

in E-cadherin expression and an increase in Snail expression in KYSE30 cells (Fig. 4d). Taken together, the above data suggest that LOXL2 may enhance the migration and invasion ability of ESCC cells by inducing the EMT process.

3.5. LOXL2 induces the activation of FAK/Src signaling of ESCC cells

Previous studies show that LOX promotes tumor growth and metastasis through the activation of FAK/SRC signaling [36]. We discovered that the expression levels of LOXL2 and FAK were positively linked by GEPIA analysis (Fig. 5a). Therefore, we hypothesized that LOXL2 regulates ESCC cell biological functions may through the FAK/Src signaling pathway. The expression of FAK, pFAK, Src and pSrc in ESCC cell lines was examined by qRT-PCR and WB. In TE-1 cells with stable knockdown of LOXL2, the protein and mRNA levels of FAK and SRC did not change much, while the levels of pFAK and pSrc levels significantly decreased (Fig. 5b,d, e). However, pFAK and pSrc levels were significantly increased in KYSE30 cells when LOXL2 was overexpressed (Fig. 5c,d,e). In summary, LOXL2 promotes ESCC cell migration, invasion and EMT processes by phosphorylating pFAK and pSrc, and activating the FAK/Src signaling pathway.

4. Discussion

LOXL2 is an 87 kDa enzyme protein with 774 amino acids that is located on chromosome 8p21-22. Its function is to promote extracellular matrix (ECM) remodeling by catalyzing the covalent cross-linking of lysine residue molecules in collagen and elastin, as well as to participate in a variety of pathological processes related to connective tissue [37]. LOXL2 promotes malignant tumor progression and metastasis by regulating the synergistic effect of multiple signaling pathways. In osteosarcoma, Wnt9a and Wnt7 induce LOXl2 expression in an autocrine and paracrine manner, thereby regulating collagen accumulation and affecting osteoblast differentiation and tumor aggressiveness [38]. Fan et al. [39] demonstrate that intracellularly active LOXL2 upregulates the HIF-1α/VEGF signaling pathway through the Snail-FBP1 axis and thus plays an important role in the progression of hepatocellular carcinoma. Meanwhile, LOXL2 overexpression may promote vasculogenic mimicry formation and tumor metastasis by collaborating with SNAIL and VE-cadherin in HCC [40]. Reynaud et al. [41] study found that colorectal cancer cells with overexpressed LOXL2 can produce more IL-6 and activate the STAT3 signaling pathway, which promoting the production and differentiation of RANKL-

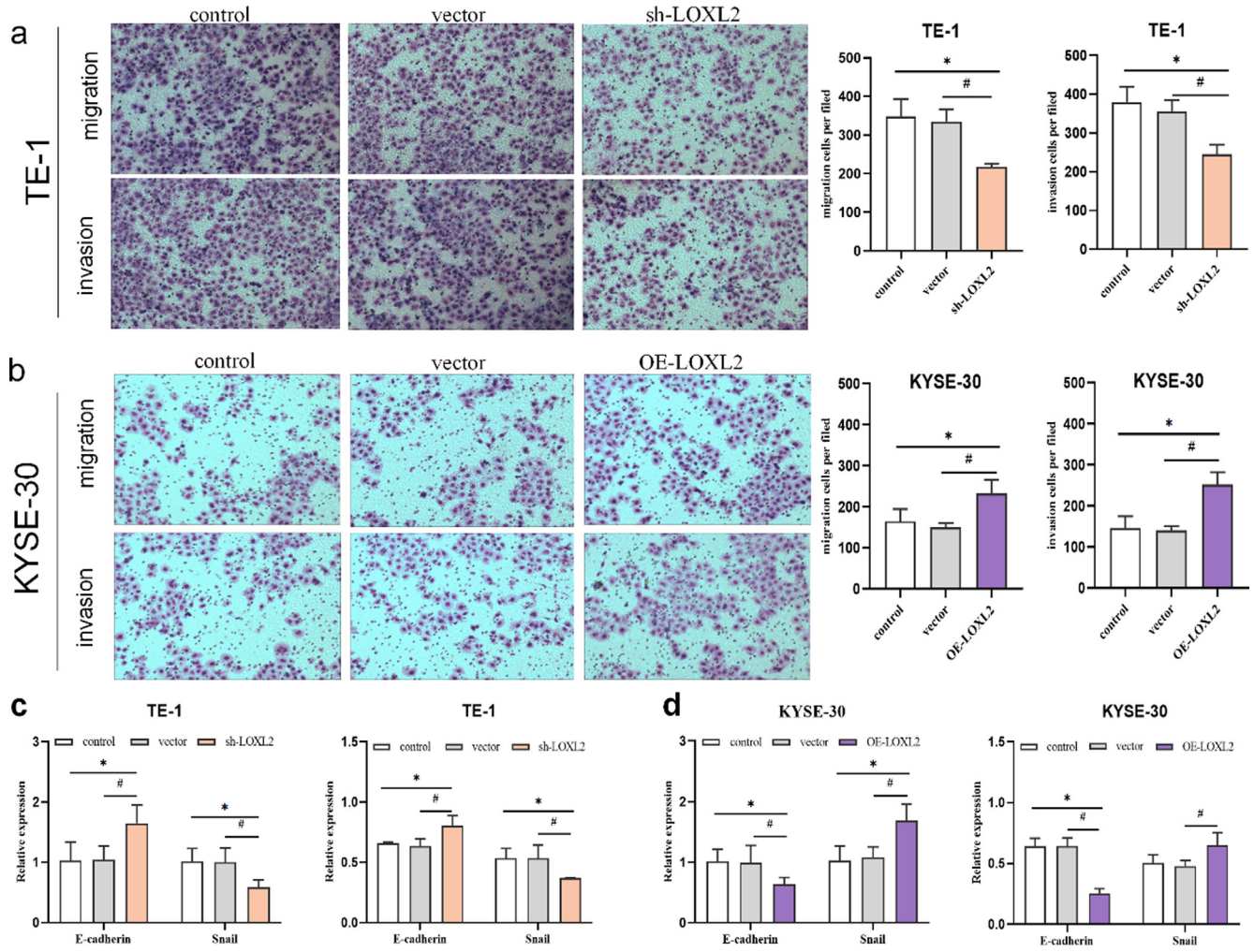

Fig. 4. LOXL2 promotes ESCC migration, invasion and EMT. (a) Cells migration and invasion abilities in TE-1 cells with LOXL2 knockdown were determined by transwell assay. (b) Cells migration and invasion abilities in KYSE30 cells with LOXL2 overexpression were determined by transwell assay. (c,d) Expression of EMT markers, E-cadherin and Snail upon LOXL2 knockdown or overexpression was analyzed by qRT-PCR and western blotting. All cellular experiments were performed in triplicate or more data are expressed as mean ± SEM, $^{*}P < 0.05$,$^{\#}P < 0.05$.

dependent osteoclasts and leading to colorectal cancer bone metastasis. The PI3K/Akt signaling is another pathway that LOXL2 can activate to promote the development of tumors and distant metastases [42]. While LOXL2 is involved in tumor development via multiple signaling pathways, the specific mechanism of LOXL2 is still unknown in ESCC.

In this study, we for the first time showed the expression of LOXL2 in advanced ESCC tissues. According to the findings, the rate of LOXL2 positive expression increased in ESCC tissues, and the proportion of positive expression increased with TNM stage, and stage IV patients had up to 93.48% positive LOXL2 expression. Meanwhile, LOXL2 protein levels were strongly correlated with the tumor differentiation, T stage, and lymph node metastasis, which is consistent with the findings of Zhang et al. [43] in a meta-study that concluded that patients with positive LOXL2 expression had poorer OS and poorer clinicopathological parameters. As a result, we believe that LOXL2 may be a potent biomarker to evaluate the prognosis of ESCC. Furthermore, in vitro experiments were also conducted to determine LOXL2′s role in ESCC progression. Our data demonstrate that LOXL2 knockdown significantly inhibits cell proliferation and induces apoptosis in ESCC cells. However, ESCC cell growth ability significant improvement was observed when LOXL2 was overexpressed. These findings indicate that LOXL2 positively regulates ESCC cell growth, and they are consistent with a previous report that LOXL2 promotes breast cancer cell malignant behavior [44]. Peng et al. [45] found that LOXL2 may act on the key EMT-transcription factor snail in cervical cancer, attenuating snail degradation mediated by GSK3beta, thus reducing E-cadherin expression. Meanwhile, this study found that Small molecule inhibitors of LOXL2 ((2-Chloropyridin-4-yl) methanamine hydrochloride) significantly reduce the invasive capacity of cervical cancer by reversing the LOXL2-induced EMT process. In our investigation, we found findings that were similar. Our results show that overexpression of LOXL2 leads to decreased E-cadherin expression and increased expression of the mesenchymal marker Snail, suggesting that LOXL2 may be a key regulator of the EMT process in ESCC cells. More importantly, we found that overexpression or knockdown of LOXL2 can regulate the activity of the FAK/Src signaling in ESCC cells, and we hypothesized that the activity of this pathway may be important for the functional realization of LOXL2 in ESCC. FAK is a crucial regulator of integrin-mediated cell adhesion and migration, and FAK upregulation and hyperphosphorylation have been shown to increase invasiveness in a variety of human cancer types, including gastric cancer and breast cancer [46,47,48]. Phosphorylation of FAK on Try397 is the major phosphorylation site, and p-FAK leads

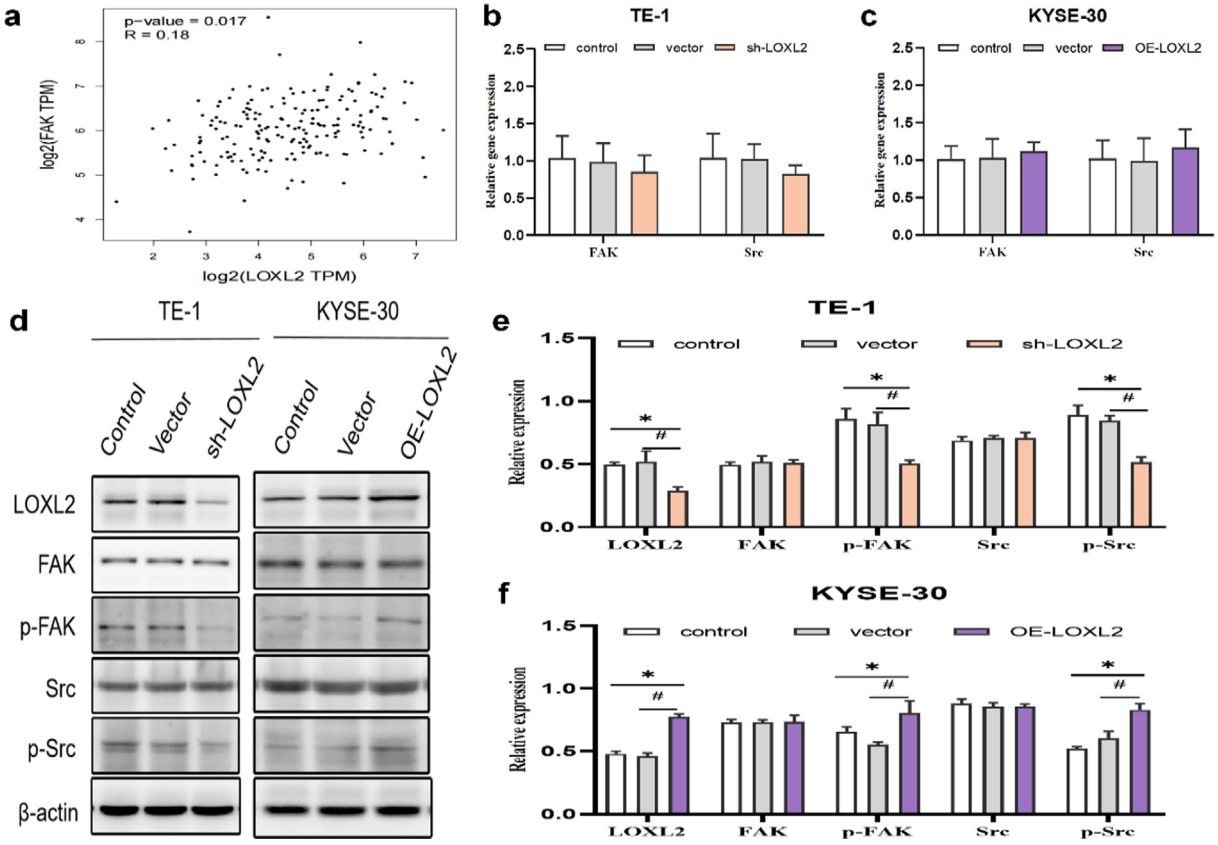

Fig. 5. LOXL2 promotes activation of FAK/Src signaling pathway in ESCC. (a) FAK was to predicted to have a positive correlation with LOXL2 in ESCC using GEPIA (http://gepia.cancer-pku.cn/) (P = 0.017). (b,c) Expression of FAK signaling upstream regulators upon LOXL2 knockdown or overexpression was analyzed by qRT-PCR. (d,e,f) Expression of FAK signaling upstream regulators upon LOXL2 knockdown or overexpression was analyzed by western blotting. All cellular experiments were performed in triplicate or more data are expressed as mean ± SEM, $^{*}P < 0.05$, $^{\#}P < 0.05$.

to tumor cell progression by promoting tumor cell migration and invasion [49]. Hong et al. [50] discovered that silencing LOXL2 inhibited the migration, invasion and epithelial-mesenchymal transition of renal cell carcinoma cells through the FAK/Src signaling pathway. Our findings reveal that LOXL2 regulates FAK phosphorylation at Tyr397 site in ESCC cells and the knockdown of LOXL2 decreased p-FAK and p-Src. However, p-FAK and p-Src were significantly upregulated in ESCC cells overexpressing LOXL2. In this research, we found that LOXL2 activates the FAK/Src signaling pathway and induces EMT, thereby contributing to ESCC progression.

In summary, our findings suggest that LOXL2 may induce EMT in esophageal squamous carcinoma through FAK/Src signaling. LOXL2 may be an important oncogene and potential therapeutic target in esophageal squamous cell carcinoma. However, we did not evaluate the effect of LOXL2 blockers on esophageal squamous carcinoma, which needs to be confirmed by further studies.

5. Conclusions

Taken together, our study is the first to examine LOXL2 expression in patients with stage I-IV ESCC. Our findings demonstrated that LOXL2 was significantly upregulated in ESCC and served as a potential prognosis biomarker in ESCC patients. LOXL2 overexpression promotes proliferation, invasion, and migration while suppressing apoptosis and possibly promoting EMT through FAK phosphorylation, which activates the FAK/Src signaling pathway in ESCC (Fig. 6). Therefore, it is important to further investigate LOXL2 as a possible target for ESCC treatment.

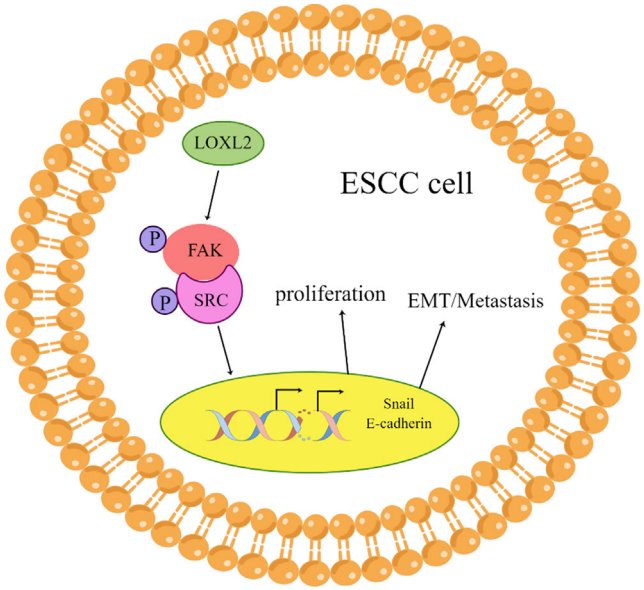

Fig. 6. LOXL2 promotes aggression of ESCC by activating FAK/Src signaling pathway.

Ethical approval

All subjects provided written informed consent. The study protocol was approved by the Ethics Committee of the Affiliated

Tumor Hospital, Xinjiang Medical University (Urumqi, China), with the approval number K-2019053.

Author contribution statement

- Study conception and design: ZQ Fan; XY Liu.
- Data collection: W Nian; XT Huang; SY Hou.
- Analysis and interpretation of results: YM Liu; F Chen; QQ Yang.
- Draft manuscript preparation: YM Liu; Xinya Liu; F Chen.
- Revision of the results and approval of the final version of the manuscript: ZQ Fan; XY Liu.

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Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

We would like to thank the study participants for their contributions.

Data availability

We used online software for our bioinformatics analysis. Here are the links to access these data.

1.-F1a: https://ualcan.path.uab.edu/cgi-bin/TCGAExResult-New2.pl?genenam=LOXL2&ctype=ESCA. There are the following conditions: step 1, datasets: esophageal cancer; step 2, gene symbol: LOXL2; step 3, expression: based on histology type.

2.-F5a: http://gepia2.cancer-pku.cn/#correlation. There are the following screening conditions: step 1, input gene: Gene A: LOXL2, Gene B: FAK; step 2, correlation coefficient: Pearson; step 3, selecting datasets: ESCA tumor.

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