

12-lipoxygenase promotes tumor progress by TGF- β 1-mediated epithelial to mesenchymal transition and predicts poor prognosis in esophageal squamous cell carcinoma

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Purpose: To clarify the effect of 12-lipoxygenase/12-hydroxyeicosatetraenoic acid (12-LOX/12-HETE) on progress of esophageal squamous cell carcinoma (ESCC) and the possible mechanism.

Patients and methods: We performed cell experiments including chemical treatment, transfection, Western blotting and transwell assay to investigate the function of 12-LOX/12-HETE. Slices of tumor tissues were obtained from ESCC patients treated in Qilu Hospital of Shandong University. Immunohistochemical (IHC) staining was done to find their correlation with prognosis and clinicopathological characteristics.

Results: In ESCC cells, inhibition of 12-LOX caused a decrease in transforming growth factor- β 1 (TGF- β 1)-mediated epithelial-mesenchymal transition (EMT) level, and abilities of migration and invasion were also inhibited. Nevertheless, the inhibition could be partly relieved when treated with 12-HETE or TGF- β 1. Analyses of IHC staining indicated a positive correlation between the expression of 12-LOX and EMT level, and an inverse correlation between 12-LOX and overall survival (OS). Univariate and multivariate analyses further suggested that 12-LOX was an independent prognostic factor for ESCC patients.

Conclusion: In conclusion, our study proved that 12-LOX/12-HETE-promoted tumor migration and invasion might partly be through TGF- β 1-mediated EMT in ESCC, and 12-LOX could be a promising biomarker for predicting prognosis in ESCC patients.

Keywords: 12-LOX, 12-HETE, Baicalein, tumor metastasis, overall survival

Introduction

Esophageal cancer (EC) is one of the most common virulent tumors with poor prognosis and high mortality worldwide.¹ Esophageal squamous cell carcinoma (ESCC) accounts for >90% of EC in China.² Despite the advances achieved in surgery, chemoradiotherapy (CRT) and other treatment methods, the 5-year overall survival rate (OS) remains poor due to the high recurrence rate.³ Migration and invasion make up the main reasons for poor prognosis and it is urgent to clarify the underlying mechanisms.

12-lipoxygenase (12-LOX) is one of the key enzymes involved in the metabolism of arachidonic acid (AA); it functions to accelerate the process of AA turning to the metabolite 12-hydroxyeicosatetraenoic acid (12-HETE).⁴ Recent studies revealed that 12-LOX/12-HETE were critical regulators in progression of ovarian

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cancer,⁵ prostate cancer,⁶ renal cell carcinoma⁷ and bladder carcinoma.⁸ In EC, the expression of 12-LOX was up-regulated compared to normal esophageal squamous epithelia, suggesting that AA metabolism pathway and 12-LOX may contribute to the progress of EC.⁹ While the specific mechanisms that how the 12-LOX pathway participates in the progress remain to be found, our present research was designed to identify the role of 12-LOX/12-HETE in ESCC.

Epithelial-mesenchymal transition (EMT) is a process that cells transit from epithelial phenotype to mesenchymal phenotype and gain more migratory and invasive abilities. There were several pathways reported to be associated with the regulation of EMT process, including transforming growth factor β 1 (TGF- β 1), Wnt, Notch, Hedgehog and other signaling pathways.^{10,11} TGF- β 1, first described as an inducer of EMT in normal mammary epithelial cells, was a cytokine with multiple biological functions, and several subsequent studies had presented important roles of TGF- β 1-induced EMT in tumor metastasis.¹² Our study revealed a possible mechanism of 12-LOX/12-HETE regulation in progress of ESCC through TGF- β 1-mediated EMT. Nevertheless, more detailed mechanism remained to be elucidated.

Baicalein, a common-used inhibitor of 12-LOX,¹³ was proved to be correlated to the increase of apoptosis and decrease of proliferation in several cancers, including hepatocellular carcinoma cells,¹⁴ epidermoid carcinoma cells,¹⁵ gastric cancer cells¹⁶ and lung non-small carcinoma cells.¹⁷ In this study, we used Baicalein and 12-LOX siRNA (si12-LOX) to inhibit the expression of 12-LOX, exploring the function of 12-LOX/12-HETE in ESCC cells, and attempted to explain the relationship of 12-LOX and progress of ESCC.

Materials and methods

Cell culture and treatment

Eca109 (Procell Life Science & Technology Co., Ltd) and Kyse150 (Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai) cells were chosen as the representative cell lines of Human ESCC. The cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., USA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc., USA) and 1% penicillin-streptomycin antibiotic solution. All cultures were maintained in a humidified incubator with 5% CO₂ atmosphere at 37°C. ESCC cells were

treated with Baicalein (Selleck, China) or DMSO (Solarbio, China) for 24 hrs with or without pretreated with 100 nM 12(S)-HETE (Cayman Chemical, USA) 4 hrs prior to the inhibitor. Or they were transfected with 12-LOX-specific siRNA (si12-LOX, Stealth RNAi siRNA Card for human Alox12, Catalog #1299003; three 12-LOX-specific 20–25 nt siRNA construct, Invitrogen, USA) and non-targeting siRNA (siNC) using EndoFectin™-MAX (GeneCopoeia, China) with or without treated with 100 nM 12(S)-HETE/5 ng/mL TGF- β 1 (Peprotech, USA) for an additional 24 hrs.

Cell migration and invasion assay

ESCC cells were lysed and centrifuged, then mixed with 100 μ L 1640 medium at the density of 5.0×10^5 /mL and seeded in the upper chamber (8- μ m pore size, 6.5-mm diameter, Corning, USA). 600 μ L medium supplemented with 15% FBS was added in the lower chamber and acted as a chemotactic agent. After incubated for 24 hrs, the migrated cells on the upper chamber were fixed using 100% methanol for 30 mins and stained with crystal violet (Solarbio, China) for another 30 mins. Cells were observed and calculated in five filed under a microscope randomly. The procedures of invasion assay were similar to the migration assay except that the membrane of the upper chamber was coated with matrigel (Corning, USA) and the time of incubation was prolonged to 36 hrs.

Western blotting analyses

The ESCC cells were lysed and the overall protein was extracted. A BCA kit (Beyotime Biotechnology, China) was used to determine the concentration of the protein. Equal amount of protein was separated using 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Then, the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline Tween (TBST, pH 7.4) for 1 hr, and then incubated with primary antibodies against 12-LOX (Novus Biologicals, USA, diluted 1:700), TGF- β 1 (Affinity Biosciences, China, diluted 1:1000), vimentin (CST, USA, diluted 1:1000), N-cadherin (Proteintech, USA, diluted 1:1000), E-cadherin (CST, USA, diluted 1:1000) and GAPDH (Santa Cruz Biotechnology, USA, diluted 1:2000) for 4°C overnight. Subsequently, after washed with TBST, the membranes were incubated with secondary antibody (Affinity Biosciences, China, diluted 1:5000) for 1 hr at room

temperature. The membranes were washed and detected using ECL solution (Beyotime Biotechnology, China).

Patients and samples

We collected 86 tumor tissue samples of patients who received esophagectomy in Qilu Hospital of Shandong University (Jinan, China) during 2008. All cases were confirmed as ESCC pathologically. Samples in the following condition were excluded from the study: 1) if the patient was diagnosed with more than one primary tumor; 2) if the patient received neoadjuvant therapy before surgery; 3) if the patient was lost to follow-up. The clinical basic data, including age, gender, history of smoking and alcohol consumption, differentiation degree, invasion depth (T stage), lymph node metastasis (N stage), pathological TNM (pTNM) and adjuvant treatment after surgery were acquired from the medical records. Our study was approved by the Ethics Committee of Qilu Hospital. All patients were anonymous and wrote informed consents.

Follow-up

All the patients were followed-up after surgery to enquire about their recovery and recurrence, and give them more detailed instructions according to tumor progression. In the first 2 years, patients were followed-up by telephone calls every 3 months. Subsequently, the information of patients was obtained every 6 months until November 2013, unless they were either lost to follow-up or dead.

Immunohistochemistry (IHC) staining

The paraffin-embedded ESCC tissues were sliced into 5- μ m sections and mounted on the microslides. IHC staining was performed according to the manufacturer's protocol. Before de-waxing in xylene and rehydrating in alcohol gradient, tissue sections were baked at 60°C in the oven for 1 hr. Antigen retrieval was performed at 93°C in the microwave oven for 20 mins using citrate buffer (pH =6.0). Then, 3% H₂O₂ was used to block the non-specific protein-binding sites and inactivate the endogenous peroxidase at room temperature for 10 mins. After blocked with 5% BSA for 30 mins at room temperature, the sections were incubated overnight at 4°C with primary antibody against 12-LOX (Novus Biologicals, USA, diluted 1:50), TGF- β 1 (Affinity Biosciences, China, diluted Affinity Biosciences, China, diluted 1:50), vimentin (CST, USA, diluted 1:70), N-cadherin (Proteintech, USA, diluted 1:70) and E-cadherin (CST, USA, diluted 1:70). Then the sections were washed with PBS and incubated with biotinylated secondary

antibodies and Strept Avidin-Biotin Complex (SABC) (Boster, California, USA) successively at 37°C for 30 mins. Finally, sections were incubated with DAB. Finally, the slides were counterstained with hematoxylin, dehydrated in alcohol gradient and xylene, covered with coverslips using neutral balsam to observe.

The immunoreactivities of IHC staining were assessed by scanning whole tumor sections under Olympus IX81 microscope (Olympus, Japan) using a well-established score system in which the score was generated by incorporating both the percentage of positive tumor cells and the intensity of staining (three view fields per section).¹⁸ A score <100 was considered low expression (-), and score >100 was considered overexpression (+).

Statistical analysis

All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., USA). For cell experiments, the data were presented as mean \pm SD, and 3 individual experiments were performed in triplicates. One-way analysis of variance followed by Tukey's test was used to compare the data from different groups. χ^2 test was used, respectively, to evaluate the associations between the expression of 12-LOX and clinicopathological factors of patients, and examine the relationships between the expression of TGF- β 1, E-cadherin, N-cadherin, vimentin and 12-LOX. Kaplan-Meier analysis method and log-rank test were used to assess the difference of survival rate between two subgroups. Cox regression model was adopted in univariate and multivariate analyses to identify independent prognostic factors. $P < 0.05$ was considered statistically significant in all cases.

Results

Expression of 12-LOX was inhibited by Baicalein in ESCC cells

Baicalein of different concentrations (0 μ M, 20 μ M, 40 μ M) was added to the medium of Eca109 and Kyse150 cells, respectively. Figure 1 shows that expression of 12-LOX was down-regulated in direct proportion to the concentration of Baicalein, and we chose 40 μ M to perform the transwell assay since the expression of 12-LOX was inhibited to a large extent.

Effects of Baicalein and 12(S)-HETE on the migration and invasion ability in ESCC cells

Eca109 and Kyse150 cells were treated by 40 μ M Baicalein with or without pretreated with exogenous 12(S)-HETE. The transwell assay showed that both the migration and invasion

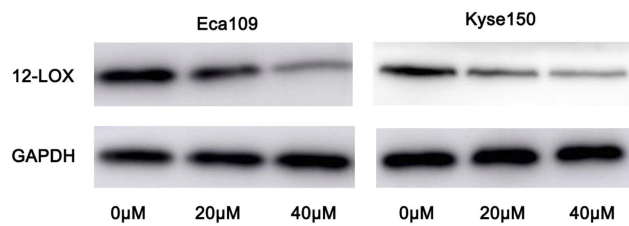


Figure 1 Expression of 12-LOX was inhibited by Baicalein in a concentration-dependent way in ESCC cells. The left panel showed that the expression levels of 12-LOX in Eca109 cells were gradually inhibited after treated with 20 μ M and 40 μ M Baicalein, and right panel indicated similar results observed in Kyse150 cells. **Abbreviation:** ESCC, esophageal squamous cell carcinoma.

ability were inhibited when treated by Baicalein. While the inhibition was partly relieved if the cells were treated with 12(S)-HETE in advance (Figure 2).

Effects of Baicalein and 12(S)-HETE on the expression of EMT markers in ESCC cells

EMT is one of the crucial mechanisms that promote metastasis of epithelium-originated cancers. Since the transwell assay had revealed the function of 12-LOX/12(S)-HETE in metastasis of ESCC cells, three representative EMT markers were chosen to evaluate the EMT level after treatment. Expression of E-cadherin represented the epithelial phenotype, and vimentin and N-cadherin were the markers of mesenchymal phenotype. Results showed that the EMT was inhibited (expression of E-cadherin was up-regulated, while that of vimentin and N-cadherin down-regulated) after treatment of Baicalein. The inhibition was partly relieved when exogenous 12(S)-HETE was added in advance (Figure 3).

Effects of Baicalein and 12(S)-HETE on the expression of TGF- β 1 in ESCC cells

TGF- β 1 was already known as one of the key pathways that participated in the acceleration of EMT process. Hence, we detected the expression of TGF- β 1 after treated by Baicalein and 12(S)-HETE. Results indicated that the expression of TGF- β 1 was reduced by Baicalein, while partly relieved by pretreated 12(S)-HETE as expected, suggesting that Baicalein and 12(S)-HETE regulated the process of EMT in ESCC cells partly through TGF- β 1 pathway (Figure 3).

Down-regulation of 12-LOX using siRNA and its regulation in EMT-related proteins in ESCC cells

Previous experiments had presented that Baicalein suppressed the process of TGF- β 1-mediated EMT process in

ESCC cells through inhibition of 12-LOX pathway. To further investigate the function of 12-LOX, we used si12-LOX to knockdown the expression of 12-LOX in ESCC cells and rescued with 12(S)-HETE. As shown in Figure 4, the expression of 12-LOX was successfully down-regulated by si12-LOX at the protein level compared with siNC. The left panel indicated that when the expression of 12-LOX was deregulated, the levels of TGF- β 1 and EMT were also inhibited, and the inhibition was relieved when 12(S)-HETE was added. To further investigate the effect of 12-LOX on TGF- β 1 reduced EMT, TGF- β 1 was added to perform the rescue experiments. The results showed that the expression of the epithelial marker was also increased when added TGF- β 1 (Figure 5). The results further supported our finding that 12-LOX promoted the progress of ESCC partly through TGF- β 1-mediated EMT.

The associations of 12-LOX and EMT markers in ESCC patients

To further confirm the relationship of 12-LOX and EMT in ESCC, we evaluated the expression of 12-LOX, EMT markers (E-cadherin, vimentin, N-cadherin), and also TGF- β 1 by IHC staining in 40 specimens of ESCC patients with surgery, and representative pictures and parallel correlation analyses are shown in Figure 6. Among the specimens, 55% of the patients expressed high level of 12-LOX. Analyses showed that there was a significant positive correlation among the expression of 12-LOX and TGF- β 1, vimentin, N-cadherin, and an inverse correlation between 12-LOX and E-cadherin. The results further indicated the possible association between 12-LOX and TGF- β 1-mediated EMT.

Relationships between the expression of 12-LOX and clinicopathological characteristics in ESCC patients

Representative IHC staining of 12-LOX with positive and negative expression is shown in Figure 5. We systematically analyzed expression of 12-LOX in 86 cases of ESCC patients, among which 47 were positive and 39 negative. The basement characteristics of 86 ESCC patients are listed in Table 1. And the relationships between expression level of 12-LOX and clinicopathological characteristics are summarized in Table 2. The results showed a positive correlation of expression level of 12-LOX with N stage ($P=0.001$) and pTNM ($P=0.022$), while no significant

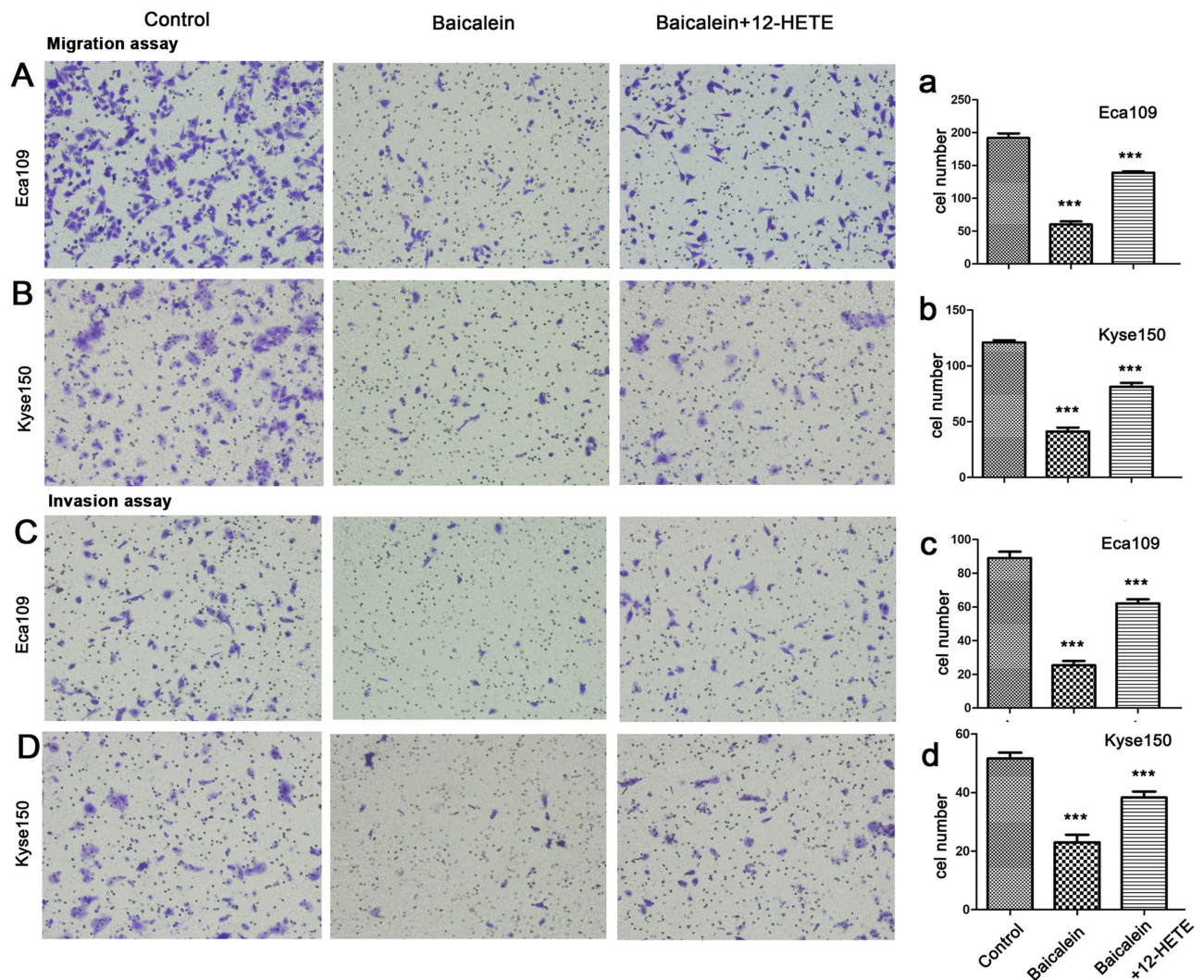


Figure 2 Effects of Baicalein and 12(S)-HETE on the migration and invasion ability in ESCC cells. Representative images of migrated cells (A, B) and invaded cells (C, D) stained by crystal violet. Left, control cells; second, cells treated by 40 μ M Baicalein; third, cells pretreated with 12(S)-HETE before treated by Baicalein (Eca109: A and C; Kyse150: B and D). Analyzed results of the cell migration and invasion assays are shown in a, b, c, d. The number of migrated cells and invaded cells in the Baicalein group was significantly less than those of the control ($P<0.001$), and the inhibition was partly relieved when pretreated with 12(S)-HETE. *** $P<0.001$ vs Control group. **Abbreviation:** ESCC, esophageal squamous cell carcinoma.

differences in gender, age, smoking, drinking, T stage and differentiation between the 2 groups. The results may be restricted by the small size of samples.

Relationships between the expression of 12-LOX and OS in ESCC patients

Kaplan-Meier analysis showed that, compared to low-expression of 12-LOX, its overexpression was significantly associated with decreased OS ($P=0.002$) (Figure 7). Univariate analyses showed that N stage, pTNM, CRT and 12-LOX were significantly associated with OS (for N stage and pTNM, $P<0.001$, for CRT and 12-LOX, $P=0.024$ and 0.003, respectively). We included the significant factors

($P<0.05$) into the multivariate analyses, and the result indicated that 12-LOX overexpression was an independent prognostic factor for OS [HR (95% CI): 2.371 (1.250, 4.496), $P=0.008$] (Table 3).

Discussion

The AA-metabolizing enzyme 12-LOX and its metabolite 12-HETE have been reported to be key regulators of cell proliferation, motility, apoptosis and angiogenesis in several cancers.⁵⁻⁸ Up-regulation expression of 12-LOX was found in ESCC compared to normal esophageal squamous epithelia, suggesting that AA metabolism pathway and the expression of 12-LOX may play important roles in ESCC,⁹

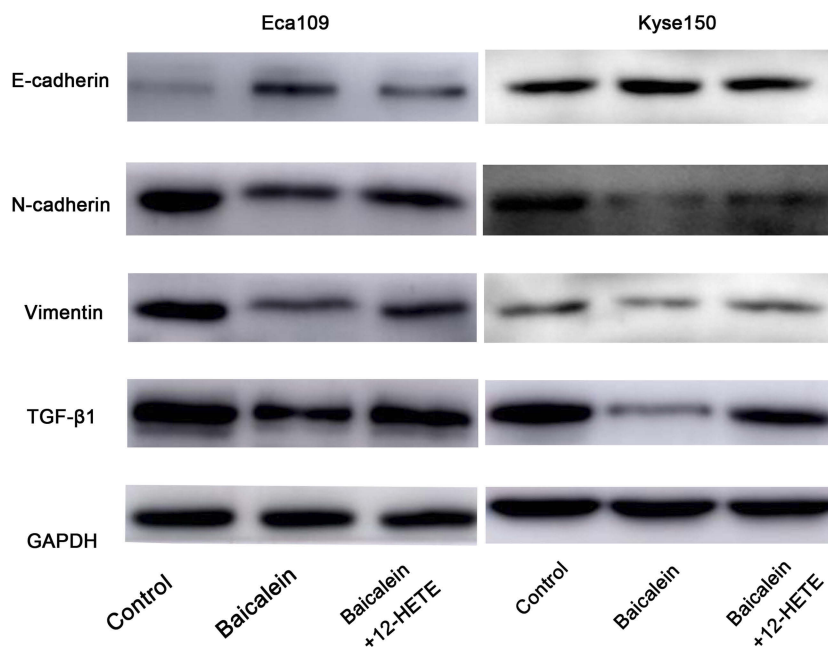


Figure 3 Expression of 12-LOX/12(S)-HETE was associated with TGF-β1 and EMT markers in ESCC cells. The left panel showed that the expression levels of TGF-β1, vimentin and N-cadherin in Eca109 cells were inhibited after treated by Baicalein and that of E-cadherin was increased, and the down-regulated level of EMT was partly relieved when pretreated with 12(S)-HETE; right panel indicates similar results observed in Kyse150 cells.

Abbreviations: ESCC, esophageal squamous cell carcinoma; EMT, epithelial-mesenchymal transition.

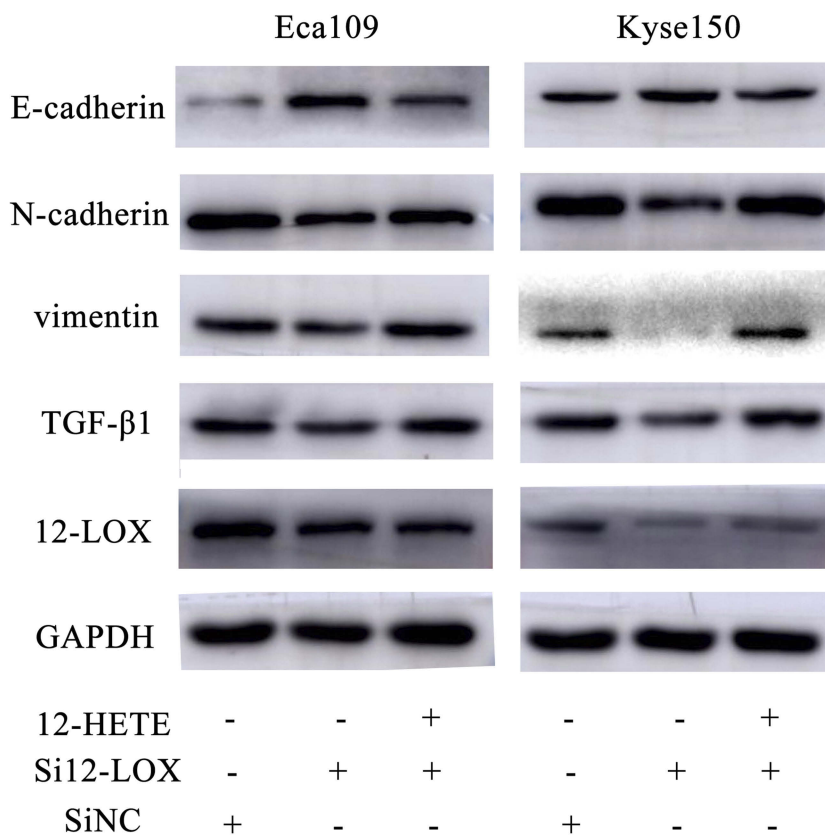


Figure 4 Regulation of si12-LOX and 12(S)-HETE in EMT-related proteins in ESCC cells. The left panel showed that the expression of 12-LOX, TGF-β1, vimentin and N-cadherin in Eca109 cells were reduced after treated by si12-LOX and that of E-cadherin was increased, and the changes were partly relieved after addition of 12(S)-HETE; right panel shows the similar results in Kyse150 cells.

Abbreviations: ESCC, esophageal squamous cell carcinoma; EMT, epithelial-mesenchymal transition.

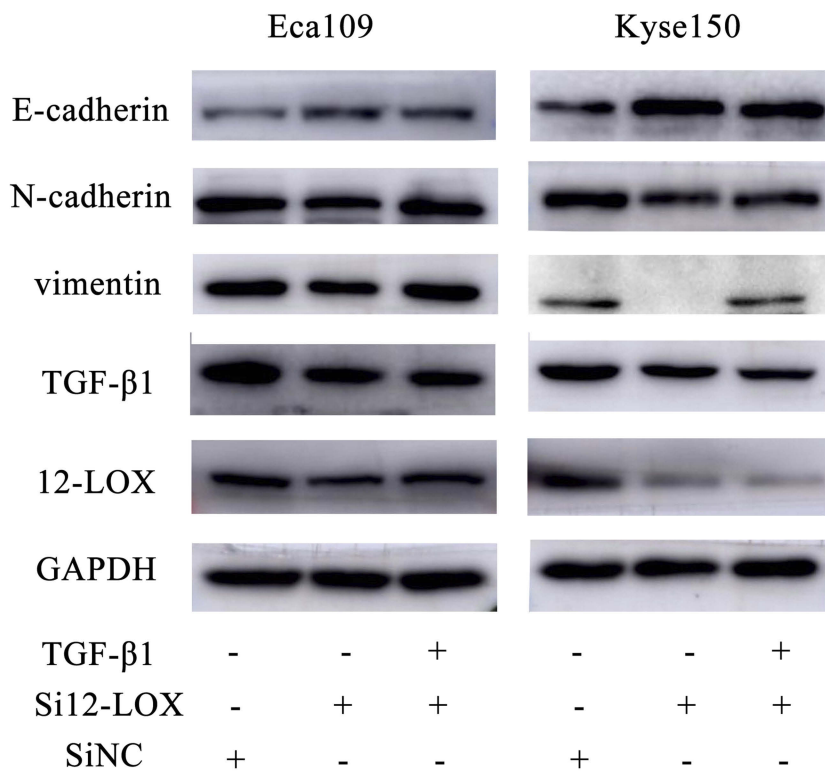


Figure 5 Regulation of si12-LOX and 12(S)-HETE in EMT-related proteins in ESCC cells. The left panel showed that the expression of 12-LOX, TGF-β1, vimentin and N-cadherin in Eca109 cells were reduced after treated by si12-LOX and that of E-cadherin was increased, and the changes were relieved after addition of TGF-β1 to some extent; right panel indicates similar results observed in Kyse150 cells.

Abbreviations: ESCC, esophageal squamous cell carcinoma; EMT, epithelial-mesenchymal transition.

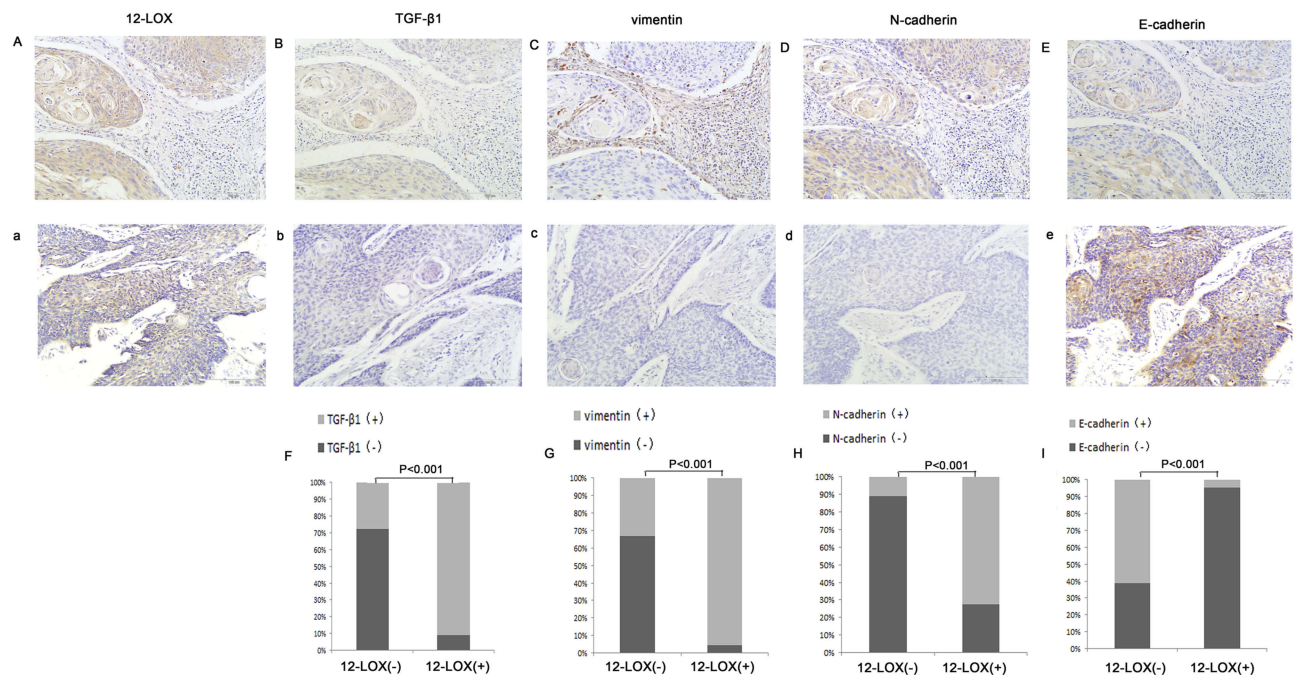


Figure 6 Immunohistochemical staining of 12-LOX in ESCC tissues. The expression of 12-LOX was localized mostly to the cytoplasm of the cells. Figure A and a represented overexpression and low expression of 12-LOX (200 x), respectively. Figure B, b, C, c, D, d, E, e represent the expression of TGF-β1, vimentin, N-cadherin and E-cadherin (200 x). Compared with inferior panel, upper panel represented overexpression of 12-LOX, TGF-β1, vimentin, N-cadherin, and low expression of E-cadherin, which was observed from the tissue from the same patient. F, G, H, I showed the significant correlation between expression of 12-LOX and TGF-β1, vimentin, N-cadherin, E-cadherin ($P < 0.001$).

Table 1 Basemnt characteristics of 86 ESCC patients

Characteristics		Patients, n%
Gender	Female/Male	19 (22.1%)/67 (77.9%)
Age	Mean ± SD Median (range)	60.63±9.072 61.000 (32–84)
Smoking	Yes/No	49 (57.0%)/37 (43.0%)
Drinking	Yes/No	56 (65.1%)/30 (34.9%)
Differentiation degree	Well Middle Poor	17 (19.8%) 40 (46.5%) 29 (33.7%)
T stage	T1~2 T3~4	37 (43%) 49 (57%)
N stage	N0 N1~3	45 (52.3%) 41 (47.7%)
TNM stage	I~II III	48 (55.8%) 38 (44.2%)
Adjuvant treatment	None Radiotherapy Chemotherapy CRT	44 (51.2%) 17 (19.8%) 3 (3.5%) 22 (25.6%)
I2-LOX	Low/Overexpression	39 (45.3%)/47 (54.7%)

Abbreviation: CRT, radiochemotherapy; ESCC, esophageal squamous cell carcinoma.

Table 2 The correlation of clinicopathologic features and the expression of I2-LOX in ESCC tissues

Clinicopathological features		I2-LOX		P-value
		Low (n=39)	Over (n=47)	
Age	<60	8	11	0.748
	≥60	31	36	
Gender	Male	18	15	0.176
	Female	21	32	
Smoking	No	21	28	0.593
	Yes	18	19	
Drinking	No	27	29	0.466
	Yes	12	18	
Differentiation	Well	8	9	0.882
	Moderate	17	23	
	Poor	14	15	
T stage	T1~2	19	18	0.331
	T3~4	20	29	
N stage	N0	28	17	0.001*

(Continued)

Table 2 (Continued).

Clinicopathological features		I2-LOX		P-value
		Low (n=39)	Over (n=47)	
pTNM	N1~3	11	30	0.022*
	I~II	12	11	
	III	6	11	

Notes: P-value: Chi-square test. *P<0.05.

Abbreviation: ESCC, esophageal squamous cell carcinoma.

while the molecular mechanisms of how I2-LOX/I2-HETE regulates progress of ESCC were imperfectly understood until now.

Our results suggested that the migration and invasion ability of ESCC cells were inhibited when the expression of I2-LOX was inhibited by Baicalein, while the inhibition was partly relieved when pretreated with I2(S)-HETE. Meanwhile, since EMT was a critical process associated with migratory and invasive properties of cells, we detected the expression levels of TGF-β1 and EMT markers by Western blotting. Results demonstrated

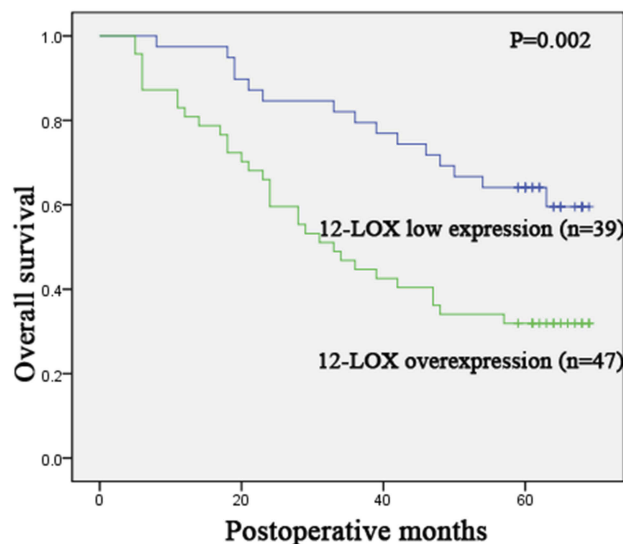


Figure 7 Kaplan-Meier analysis and log-rank test of the expression of 12-LOX for OS. Overexpression of 12-LOX significantly predicted decreased OS, while 12-LOX low-expression mostly indicated a better OS ($P=0.002$).

that the expression of 12-LOX was positively associated with TGF- β 1 and EMT level in ESCC cells. We further detected the change of TGF- β 1 during the process because it was one of the vital pathways that participated in the regulation of EMT,^{10,11} and its role in ESCC had already been elucidated in ESCC.¹⁹ The results were consistent with the change of EMT as respected. Furthermore, we transfected the ESCC cells with siRNA of 12-LOX and either 12(S)-HETE or TGF- β 1 was added to perform the rescue experiments to further verify the results. IHC staining performed in ESCC tissues suggested the positive correlation between expression level of 12-LOX, TGF- β 1 and EMT level. In addition, Kaplan-Meier analysis proved

that overexpression of 12-LOX predicted poor OS, univariate and multivariate analyses indicated that 12-LOX could be served as an independent factor predicting prognosis in ESCC patients.

The mechanisms underlying 12-LOX/12(S)-HETE-regulated progress of ESCC had not been verified. It was proven that 12-HETE inhibits cell apoptosis and facilitates cell survival by up-regulating and activating the integrin-linked kinase/NF- κ B pathway in ovarian cancer.⁵ In prostate cancer, inhibition of 12-LOX was found to lead to a specific G1-arrest-related growth inhibition and induction of apoptosis via caspase and Bcl-mediated mechanisms,⁶ and interactions of 12-(S)-HETE and its receptor 12-HETER were proven to result in activation of ERK1/2, MEK and NF κ B, thus promoting cell invasion.²⁰ In addition, 12-LOX-mediated MMP9 secretion via activation of PI3K/Akt/NF- κ B signaling may account for the invasive and angiogenic potential in prostate cancer.²¹ Role of 12-LOX/12(S)-HETE in EMT-mediated tumor metastasis was only found in gastric cancer²² and breast cancer,²³ while its involvement in other tumors including ESCC remained unclear. In our study, the results demonstrated that 12-LOX/12(S)-HETE promoted the metastasis of ESCC through EMT and presented TGF- β 1 as one of the corresponding pathway involved.

Several limitations remain in our research. First, as a retrospective cohort study, the sample number is small, which might have influenced the reliability of the results. A study with larger amount of samples is required to verify our results. Second, we are unsure whether there could have been more suitable cutoff values for the overexpression and the reduced expression of 12-LOX, TGF- β 1 and EMT

Table 3 Univariate and multivariate analysis of prognostic variables in ESCC with respect to overall survival

Variable	Univariate analysis	Multivariate analysis	
	P-value	HR (95% CI)	P-value
Gender (Male vs Female)	0.684		
Age (<60 vs \geq 60)	0.127		
Smoking (Yes vs No)	0.184		
Drinking (Yes vs No)	0.495		
T stage (T1~2 vs T3~4)	0.064		
N stage (N0 vs N1~3)	<0.001*	2.387 (1.245, 4.573)	0.009*
Differentiation (Well vs Moderate vs poor)	0.130		
pTNM (I-II vs III)	<0.001*	2.429 (1.299, 4.542)	0.005*
CRT (None vs RT vs CT)	0.024*	0.715 (0.552, 0.926)	0.011*
12-LOX (Low vs.Over)	0.003*	2.371 (1.250, 4.496)	0.008*

Note: * $P<0.05$.

Abbreviations: CI, confidence interval; RT, radiotherapy; CT, chemotherapy; CRT, radiochemotherapy; ESCC, esophageal squamous cell carcinoma.

markers that could predict the correlation and prognosis in a better way. Third, more detailed elucidation of the mechanism of 12-LOX/12(S)-HETE promoting the progress of ESCC remains to be discovered. Further studies are expected to put forward some interesting methods targeting the elevated expression of 12-LOX and thereby help to improve the prognosis.

Conclusion

In conclusion, our research presented that 12-LOX/12(S)-HETE functioned to promote tumor migration and invasion in ESCC, and raised the possible mechanism that they acted might partly be through TGF- β 1-mediated EMT, and the expression of 12-LOX could be a promising marker for predicting prognosis in ESCC patients.

Consent for publication

Written informed consent had been obtained to publish the details from the participants.

Ethics approval and informed consent

Our study was approved by the Ethics Committee of Qilu Hospital. All patients in the study were anonymous and their written informed consents were obtained.

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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