


ORIGINAL ARTICLE

Lysyl oxidase: A colorectal cancer biomarker of lung and hepatic metastasis

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Introduction

Colorectal cancer (CRC) is the third most common malignant neoplasm worldwide and the third leading cause of cancer death.¹ Metastasis and recurrence are the most common causes of death in CRC patients. The overall metastasis rate of CRC is approximately 50%. Nearly 25% of CRC patients have developed synchronous liver metastasis at the time of initial diagnosis, and 60–70% of CRC patients experience recurrence in the liver after local primary tumor resection.² The lung is the second most frequent location of metastasis in CRC patients.³ At diagnosis, > 10% of CRC patients may have pulmonary metastasis.⁴

Abstract

Background: Colorectal cancer (CRC) is a common and lethal disease in which distant metastasis remains the primary cause of death. Paradoxical roles of LOX have been reported in CRC, and the intracellular function of LOX has also recently been determined. Correlations of LOX expression and its intracellular localization with clinicopathological features in CRC patients remain largely unknown. The aim of the present study was to explore the potential roles of LOX in CRC.

Methods: LOX messenger RNA expression was assayed by quantitative PCR in eight paired normal mucosa and tumor tissues. Immunohistochemistry was conducted using tissue arrays to investigate LOX expression in 201 CRC patients. Regulation of LOX by YAP and TEAD4 was explored by YAP or TEAD4 short hairpin RNA interference in a LoVo cell line.

Results: LOX messenger RNA expression was elevated in some CRC specimens, and LOX nuclear localization was detected in CRC tumor tissues. LOX nuclear localization was found to correlate with lung/hepatic metastasis, elevated serum carcinoembryonic antigen concentration, and mucinous tumor type ($P < 0.05$). Nuclear LOX expression was found to be associated with poor overall and disease-free survival ($P < 0.05$), and postoperative lung/hepatic metastasis ($P < 0.05$). Knockdown of YAP or TEAD4 induced downregulation of LOX expression.

Conclusions: LOX nuclear localization was significantly associated with poor survival in patients with CRC. Nuclear LOX expression was correlated with synchronous or postoperative lung/hepatic metastasis. LOX may prove to be a potential target gene of YAP and TEAD4.

Furthermore, lung metastasis occurs in 10–20% patients who undergo curative resection.⁵ Surgery remains the optimal option for the treatment of metastatic CRC. When surgical resection is possible, the five-year survival rate approaches 35%.⁶ However, only 20–35% patients with metastatic CRC are eligible for surgery,² and relapse is common, occurring in approximately 75% of such patients.⁷ To improve long-term survival, early and timely identification of patients at high risk of metastatic dissemination is therefore required.

Cancer progression involves constant and dynamic interaction between tumor cells and components of the

surrounding extracellular matrix (ECM),⁸ a substance formed by macromolecules that provides structural and biochemical support to cancer cells, vessels, and surrounding stromal cells. LOX is a member of the secreted copper-dependent amine oxidase family that plays a central role in ECM remodeling and maturation by mediating the cross-linking of collagen and elastin in ECM.⁹ The LOX multi-gene family comprises five members (LOX, LOXL1, LOXL2, LOXL3, and LOXL4), all of which share a highly conserved homologue sequence within their carboxy terminal region.¹⁰ LOX is an important ECM whose matrix remodeling function has been studied extensively.¹¹ Elevated LOX expression is reported to be associated with invasion and metastasis in various types of solid tumors, such as breast,^{12,13} melanoma,¹⁴ and head and neck tumors.¹⁵

The role of LOX in CRC has recently been comprehensively investigated, but the results reported are conflicting.^{11,16} Csiszar *et al.* reported that LOX messenger RNA (mRNA) expression is decreased in CRC patients with non-metastatic disease, suggesting that LOX functions as a tumor suppressor.¹⁷ However, a later study by Baker *et al.* showed that LOX expression is significantly elevated in tumor tissues, suggesting that it functions as a tumor promoter.¹⁶ Therefore, the role of LOX and its correlation with clinicopathological features in CRC remains inconclusive.¹⁸ The intracellular function of LOX in CRC also remains unclear. The aim of the present study was to determine whether LOX is involved in the regulation of cancer cell differentiation, migration, and adhesion, and to clarify the role of LOX nuclear localization in CRC.

Materials

Patients

Two hundred and one CRC patients who were admitted and received treatment at Xinhua Hospital of Shanghai Jiaotong University School of Medicine (Shanghai, China) between January 2008 and December 2012 were enrolled in the study. The institutional review board of the hospital approved the research protocol, and informed consent was obtained from all patients. Eight CRC specimens and paired normal colorectal tissues were used for real-time PCR analysis. Normal colon mucosa and CRC specimens were used for tissue microarray construction and immunohistochemical analysis. All patients underwent surgery and were followed-up postoperatively on an outpatient basis every three months for the first two years. Follow-up examination included chest X-ray; abdominal ultrasound; computed tomography (CT) scans of the thorax, abdomen, and pelvis; and blood biochemistry, including carcinoembryonic antigen (CEA). In the three to five years following

surgery, follow-up examinations were performed every six months.

Cells culture, transfection, infection and 5-aza-dC treatment

CRC HCT116 and LoVo cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a 5% CO₂ atmosphere. Transfection was performed using Lipofectamine 2000 according to manufacturer guidelines (Invitrogen, Carlsbad, CA, USA). Stable cells were established by lentiviral or retroviral transduction and selected in 2 µg/mL puromycin or 200 µg/mL hygromycin, respectively, for two to three weeks to achieve stable knockdown or overexpression. Cells were seeded at 3 × 10⁵ cells per well in six-well plates 24 hours before 5-aza-dC treatment. Different concentrations of 5-aza-dC were added to the medium (1 µM and 5 µM), which were harvested in 48 hours. Total RNA was isolated and the LOX mRNA level was detected by quantitative real-time PCR (qRT-PCR).

RNA isolation and quantitative real-time-PCR

Total RNA was extracted using RNAiso Plus (TaKaRa, Dalian, China) and retro-transcribed into cDNA using a PrimeScript RT-PCR Kit (TaKaRa). qRT was performed using SYBR Premix Ex Taq (TaKaRa) on ABI 7500. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The sequences for qRT-PCR primers were as follows: GAPDH forward, 5'-GTCATC-CAACGGGAATGCA-3'; GAPDH reverse, 5'-TGATCGGT TA CCGTGATCAAAA-3'; LOX forward, 5'-TGGCAGTC-TATGTCTGCACC-3'; LOX reverse, 5'-CTATGGCTACC ACAGGCGAT-3'; TEAD4 forward, 5'-GTATGAGAGCC CCGAGAACAT-3'; TEAD4 reverse, 5'-GGATGCGGTAA GAGTAGTGTC-3'.

Western blotting

Cells were harvested and lysed with 1% NP-40 lysis buffer. An equal amount of protein was separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the nitrocellulose membrane. The membrane was blocked with 5% milk, followed by incubation with the following primary antibodies at 4°C overnight: LOX (1:1000; Proteintech, Chicago, IL, USA), YAP (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and β-ACTIN (1:1000; Sigma-Aldrich, St Louis, MO, USA). After washing, the membrane was incubated with horseradish peroxidase-labeled secondary antibodies. The

proteins were visualized using enhanced chemiluminescence reagents (Millipore, Billerica, MA, USA).

Immunohistochemistry

For immunohistochemical staining, sections were deparaffinized in xylene and then rehydrated in a series of ethanol solutions. Sections were pretreated by microwave for five minutes on high mode and then 10 minutes on medium mode in citrate buffer (pH 6.5). Peroxidase activity was blocked with 3% H₂O₂-methanol and sections were incubated with bovine serum albumin to eliminate non-specific staining. Anti-human LOX polyclonal antibodies (1:100; Proteintech) were applied and then sections were washed three times with phosphate buffered saline and incubated with secondary antibody (1:1000; Gene Co. Ltd., Chai Wan, Hong Kong) for another 30 minutes at room temperature. Following three five-minute rinses in phosphate buffered saline, staining was completed with 10-minute incubation with 3,3'-diaminobenzidine-tetrahydrochloride solution. The sections were then counterstained with 0.1% hematoxylin and topped with a coverslip, and immunoreactive scores were recorded, as previously described.¹⁹

Statistical analysis

Statistical analysis was performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Correlations were assessed with the Spearman correlation coefficient or Pearson chi-square test. Kaplan–Meier survival curves were generated and survival data were analyzed with the log-rank test and Cox proportional hazards regression. $P < 0.05$ was considered statistically significant.

Results

High LOX expression in colorectal cancer (CRC) specimens is associated with poor prognosis

To identify LOX mRNA expression in CRC, qRT-PCR analysis was performed in eight pairs of newly collected normal mucosa and matched tumor tissue specimens. Compared with the normal tissue specimens, LOX mRNA expression was upregulated in four CRC specimens and downregulated in the other four CRC specimens (Fig 1a). Because LOX has been reported to be both a tumor suppressor and a tumor promoter in CRC, and hypermethylation of the LOX promoter has been identified in some CRC cases, changes in LOX mRNA expression before and after 5-aza-dC treatment were detected in an HCT116 cell line. We found that 5-aza-dC treatment effectively

increased LOX mRNA expression (Fig 1b). When the cohort was stratified by median relative LOX level, patients with higher LOX mRNA expression showed poor OS.

Nuclear localization of LOX is associated with poor prognosis

To evaluate LOX protein expression in CRC and analyze the relationship between LOX protein expression and CRC development or progression, immunohistochemical analysis was performed using CRC tissue arrays. As shown in Figure 2a,b, normal tissues showed weak or mild positive staining (panels i–ii), while most CRC tissue specimens showed a low, medium, or high level of LOX protein expression (panels iii–vi). A significant portion of CRC specimens (84/201) were stained with nuclear LOX (panel iii–v). LOX protein intracellular nuclear localization was associated with prognosis in CRC patients. The 35-month overall survival rates were 49.3% and 81.4% for patients with or without positive LOX nuclear staining, respectively (Fig 2c).

Nuclear localization of LOX is correlated with lung/hepatic metastasis in CRC patients

The associations between LOX nuclear localization and clinicopathological characteristics in CRC patients are shown in Table 1. Nuclear LOX expression was correlated with distant metastasis ($P < 0.05$). Nuclear localization of LOX was significantly positively correlated with lung/hepatic metastasis, and the level of clinical metastasis markers, such as CEA, in CRC patients ($P < 0.05$). Interestingly, nuclear LOX expression was also associated with the mucinous tumor type ($P < 0.05$).

Nuclear localization of LOX is associated with postoperative lung/hepatic metastasis and poor disease-free survival

Of the 201 patients in this series, 142 CRC patients underwent radical resection and were followed-up routinely, particularly for postoperative local or distant relapse. Nuclear LOX expression was correlated with postoperative lung/hepatic metastasis (Fig 2e) and low disease-free survival (Fig 2d).

YAP and TEAD4 mediates LOX expression in CRC

The Hippo signaling pathway is known to play a role in the expansion of undifferentiated progenitor cells and the development of CRC. YAP protein is an important

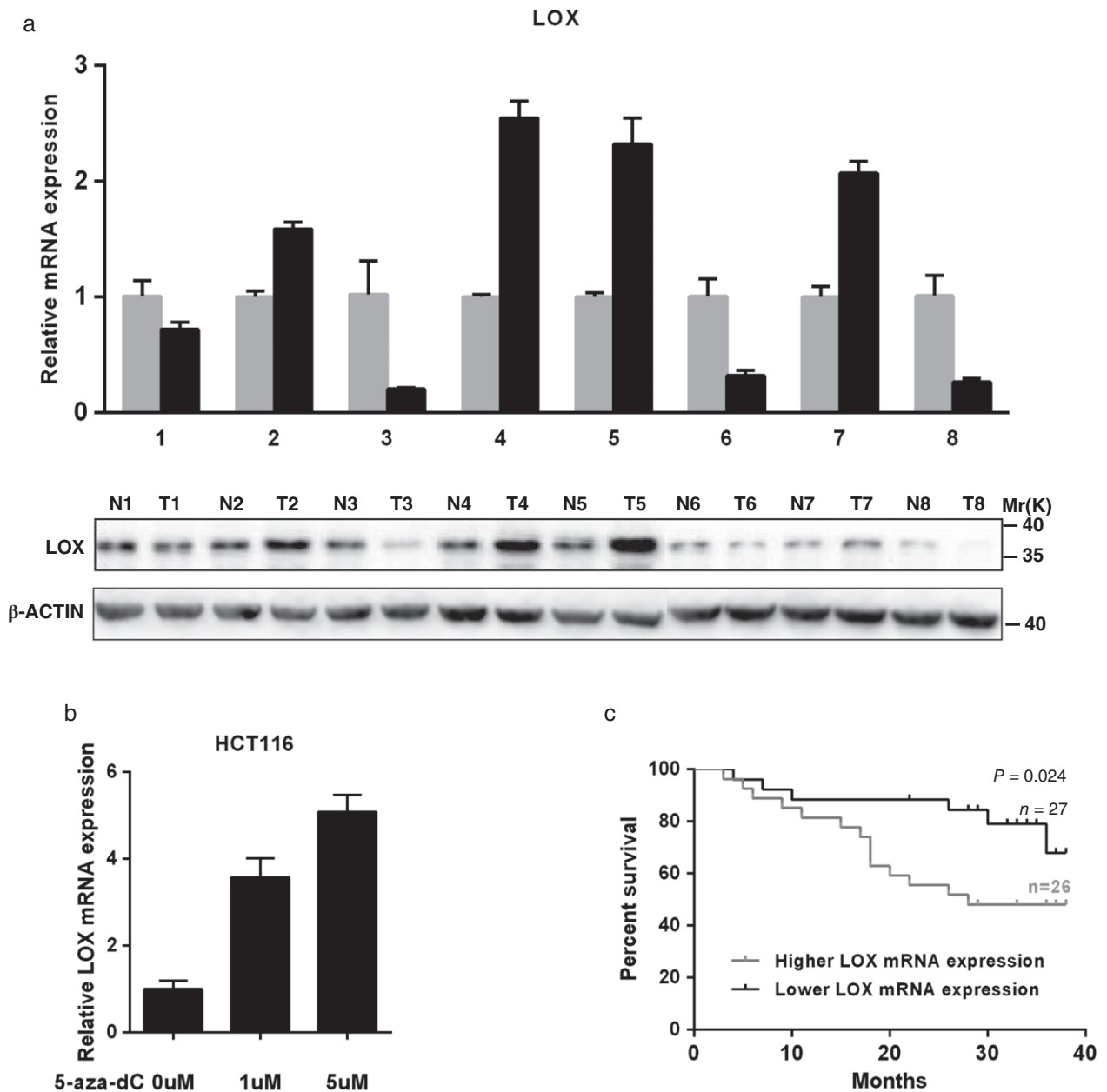


Figure 1 Upregulation of LOX messenger RNA (mRNA) expression in colorectal cancer (CRC) is correlated with poor prognosis. (a) Quantitative real-time PCR analysis of LOX mRNA level in eight fresh CRC and matched normal mucosa tissue specimens. (b) Relative expression of LOX in HCT116 cell line after 5-aza-dC treatment. (c) Kaplan–Meier plots of overall survival of CRC patients stratified by LOX mRNA expression. Log-rank test was performed to assess statistical significance. (■) normal tissue; (■) tumor tissue.

component of the Hippo pathway, and transcriptional factor proteins (TEADs) mediate most functions of YAP.²⁰ As *CTGF* is a classic YAP target gene and has been reported to play a role in regulating LOX expression,²¹ possible correlations between YAP and LOX expression were analyzed in 45 primary human CRC specimens using qRT-PCR (Fig 3a). The LOX mRNA expression level was related to the YAP mRNA level. In addition, knockdown of either

YAP or TEAD4 induced downregulation of LOX protein and mRNA expression (Fig 3b–d).

Discussion

LOX was initially identified as an ECM enzyme that regulated the tensile strength of tissues.¹¹ Some in vitro studies reported that the LOX gene was a tumor suppressor and

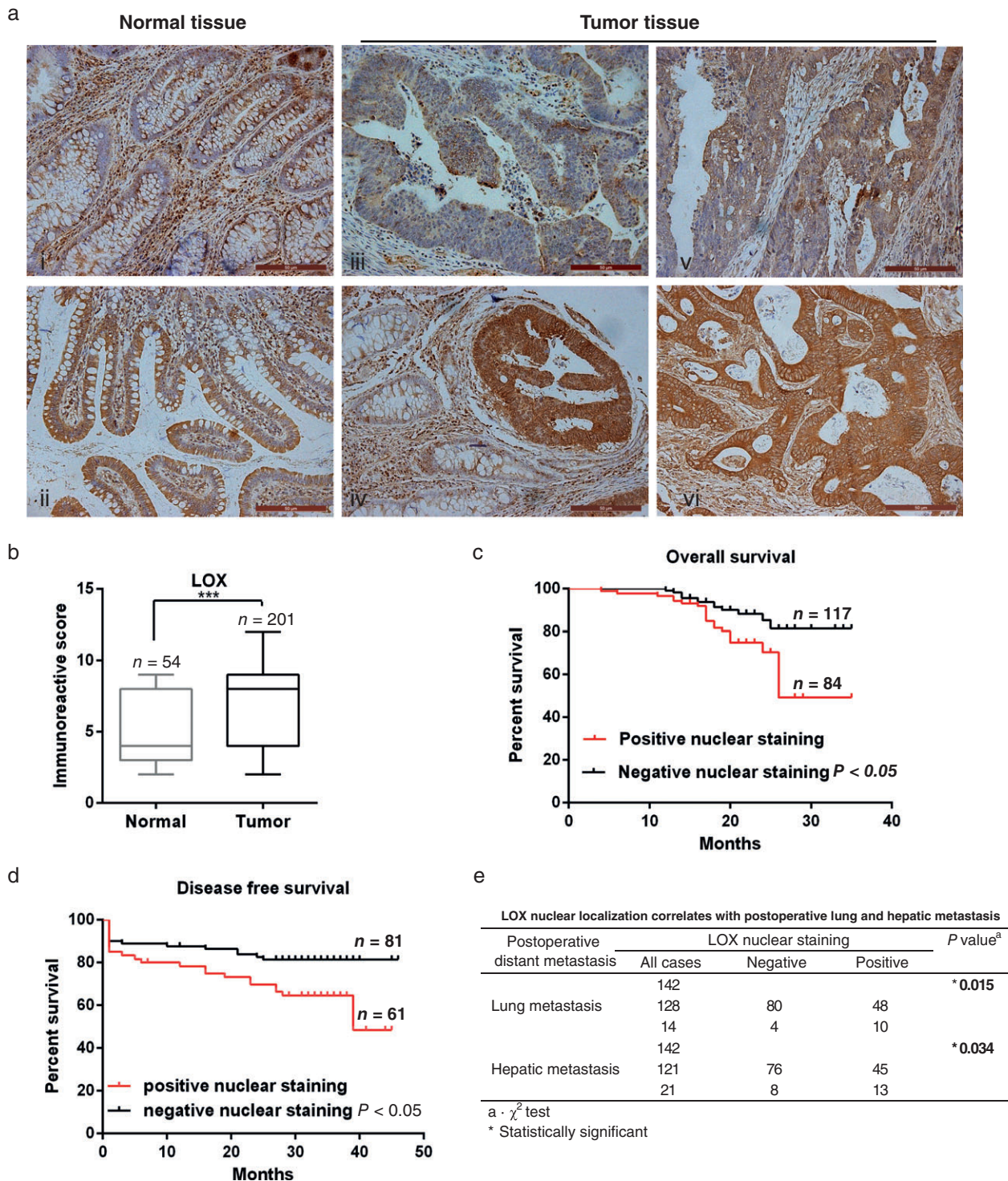


Figure 2 Overexpression and intracellular localization of LOX in colorectal cancer (CRC) and their clinical significance. **(a)** Immunohistochemical analysis of LOX in CRC and normal mucosa tissue specimens. Representative images of **(i)** weak and **(ii)** mild LOX expression in normal tissue, **(iii)** low level expression of LOX in CRC, **(iv)** medium level LOX expression, **(v)** high level/nuclear expression and **(vi)** high level/cytoplasm expression LOX in CRC. **(b)** Box-and-whisker plots of LOX expression levels in normal mucosa and CRC tissues. A Kruskal–Wallis H test was performed to assess statistical significance. *** $P < 0.001$. **(c,d)** Kaplan–Meier plots of overall and disease-free survival of CRC patients stratified by LOX intracellular localization. **(e)** Correlation of LOX nuclear localization and postoperative lung/hepatic metastasis in CRC. A Spearman rank coefficient test was performed to assess statistical significance.

Table 1 Correlations between LOX nuclear localization and clinico-pathological features of CRC patients

Variables	LOX nuclear staining			P†
	All cases	Negative	Positive	
Age (year)‡	201			0.489
≤ 63	117	70 (59.8%)	47 (40.2%)	
> 63	84	47 (56.0%)	37 (44.0%)	
Gender	201			0.396
Male	114	69 (60.5%)	45 (39.5%)	
Female	87	48 (55.2%)	39 (44.8%)	
Tumor size (cm)	201			0.261
≤ 5 cm	137	83 (60.6%)	54 (39.4%)	
> 5 cm	64	34 (53.1%)	30 (46.9%)	
TNM staging	201			0.536
I	19	11 (57.9%)	8 (42.1%)	
II	76	50 (65.8%)	26 (34.2%)	
III	84	48 (57.1%)	36 (42.9%)	
IV	22	8 (36.4%)	14 (63.6%)	
Invasion	201			0.525
T ₁	4	1 (25.0%)	3 (75.0%)	
T ₂	20	11 (55.0%)	9 (45.0%)	
T ₃	78	46 (59.0%)	32 (41.0%)	
T ₄	99	59 (59.6%)	40 (40.4%)	
No. of positive nodes	201			0.58
0	99	61 (61.6%)	38 (38.4%)	
1–3	74	38 (51.4%)	36 (48.6%)	
> 3	28	18 (64.3%)	10 (35.7%)	
Distal metastasis	201			0.03*
M ₀	179	109 (54.2%)	70 (45.8%)	
M ₁	22	8 (36.4%)	14 (63.6%)	
CEA level	201			0.045*
0–10 ng/mL	140	88 (62.9%)	52 (37.1%)	
> 10 ng/mL	61	29 (47.5%)	32 (52.5%)	
Tumor type	201			0.038*
Non-mucinous	186	112 (60.2%)	74 (39.8%)	
Mucinous§	15	5 (33.3%)	10 (66.7%)	
Lung metastasis	201			0.025*
No	189	113 (60.9%)	76 (39.1%)	
Yes	11	3 (27.3%)	8 (72.7%)	
Hepatic metastasis	201			0.034*
No	184	112 (58.7%)	72 (41.3%)	
Yes	17	6 (35.3%)	11 (64.7%)	
Tumor site¶	201			0.219
Proximal colon	23	11 (47.8%)	12 (52.2%)	
Distal colon	91	51 (56.0%)	40 (44.0%)	
Rectum	87	55 (63.2%)	32 (36.8%)	
Differentiation	201			0.789
Well	23	14 (60.9%)	9 (39.1%)	
Moderate	160	92 (57.5%)	68 (42.5%)	
Poor	18	11 (61.1%)	7 (38.9%)	
Ki67	153			0.617
Negative	112	66 (58.9%)	46 (41.1%)	
Positive	41	26 (63.4%)	15 (36.6%)	
Pelvic cavity metastasis	201			0.199
No	191	113 (58.6%)	78 (41.4%)	
Yes	10	4 (40.0%)	6 (60.0%)	

Table 1 Continued

Variables	LOX nuclear staining			P†
	All cases	Negative	Positive	
CA242 level	201			0.718
0–20 u/mL	149	85 (57.0%)	64 (43.0%)	
> 20 u/mL	52	32(61.5%)	20 (38.5%)	
CA199 level	201			0.226
0–39 U/mL	168	94 (56.0%)	74 (44.0%)	
> 39 U/mL	33	23 (69.7%)	10 (30.3%)	

*Statistically significant. †χ² test. ‡Median age at operation. §The mucinous type includes mucinous adenocarcinoma and signet ring cell carcinoma. ¶Proximal colon tumors develop in the cecum, ascending colon, hepatic flexure, or transverse colon; distal colon tumors develop in the splenic flexure, descending colon, or sigmoid colon; and rectal tumors develop in the rectosigmoid or rectum. CA, carbohydrate antigen; CEA, carcinoembryonic antigen; CRC, colorectal cancer; TNM, tumor node metastasis.

its re-expression could revert H-Ras-mediated transformation of NIH 3T3 fibroblasts.²² However, LOX mRNA expression is either upregulated or downregulated in some cancer types, including CRC. Our results showed that LOX expression is both increased and decreased in tumor tissues compared to the matched normal colon tissues. Loss of chromosome 5q14–5q31 and hypermethylation may be the cause of LOX downregulation in CRC.^{17,23} To investigate whether promoter hypermethylation could decrease LOX expression, HCT116 cells were treated with 5-aza-dC, an inhibitor of DNA methylation. Hypermethylation occurred in the LOX promoter region as a result. LOX mRNA expression was significantly increased after 5-aza-dC treatment, suggesting that hypermethylation plays a role in downregulating LOX expression.

Previous studies have reported that the LOX protein is a metastasis promoter in breast, neck, and oral and oropharyngeal squamous cancers.^{15,24} However, few studies have reported the relationship between LOX expression and CRC metastasis. Recently, Erler *et al.* showed that LOX expression was significantly elevated in tumor tissue compared to normal colon tissues, with the greatest increase observed in the metastatic tissue.¹⁶ Our results indicate that the tumor tissue immunoreactive score is significantly higher than in normal tissue. In addition, nuclear and cytoplasmic localization of LOX protein was detected in CRC cells. Recent studies have demonstrated that LOX plays novel intracellular roles, including its ability to regulate gene transcription, migration, and cell adhesion. These diverse functions have led researchers to hypothesize that LOX may play multiple roles in affecting both extracellular and intracellular cell function(s).²⁵ In a previous study, LOX protein was processed extracellularly to produce a ~30kDa active molecular form. The sequence of the

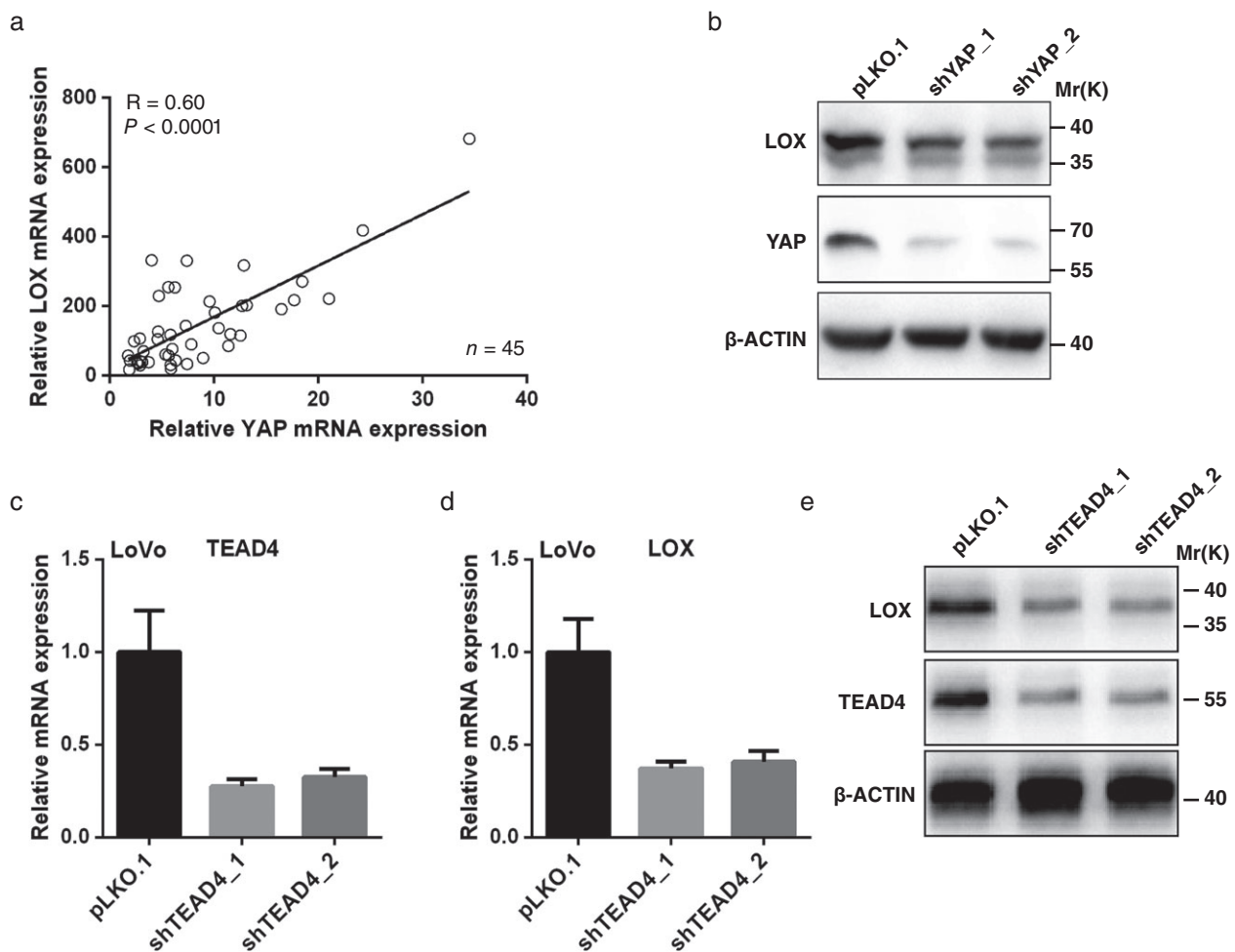


Figure 3 Regulation of LOX by YAP and TEAD4. (a) Correlation between YAP and LOX relative messenger RNA (mRNA) levels in 45 primary human colorectal cancer (CRC) specimens. The Spearman rank coefficient was used as a statistical measure of correlation. (b) Representative Western blot result of LOX protein level in the control LoVo cells and YAP knockdown cells. (c–e) Quantitative real-time-PCR and Western blot analysis of mRNA level of TEAD4 and LOX in LoVo cells with control pLKO.1 or TEAD4 short hairpin RNA virus.

proteolytic processing site in pro-LOX resembled that of the fibrillary procollagen C-terminal pro-peptide processing site cleaved by procollagen C-proteinase.²⁶ Indeed, the propeptide contains a putative nuclear localization sequence,²⁷ suggesting that it may be actively directed to the nucleus once released from the proenzyme, and has been shown to be capable of both repressing the oncogenic *bcl-2* gene in breast cancer²⁸ and inhibiting FGF-2 signaling in prostate cancer.²⁷

However, our data fail to support the hypothesis that LOX nuclear localization might act as a tumor suppressor in CRCs. We found that LOX nuclear localization is associated with lung/hepatic metastasis and poor OS. Nuclear LOX expression is correlated with elevated CEA concentration. A recent study showed that LOX mRNA expression is associated with diffuse cytoplasmic expression of CEA, which is consistent with our finding.²⁹ LOX nuclear

localization is correlated with postoperative lung/hepatic metastasis and poor DFS in patients who undergo radical resection. We conclude that LOX may act as a tumor promoter in CRC, partially by translocation into the nucleus, although the mechanism needs to be further explored.

LOX expression induced by TGF- β ,³⁰ TNF- α ,³¹ and IFN- γ ^{24,32} has been implicated in earlier studies. LOX expression is induced under hypoxic conditions through hypoxia-inducible factor-1 transcription factor binding to a functional hypoxia-responsive element in the promoter region.²⁴ Crosstalk of the signaling pathway is common in tumor genesis and development. The Hippo pathway plays a role in the development of CRC.³³ YAP overexpression is frequently detected in CRC and is correlated with poor prognosis.³⁴ Earlier studies have reported that knocking down *CTGF*, a direct target gene of YAP and TEADs, plays a role in LOX expression.²¹ Bioinformatics studies have

shown potential TEAD protein binding sites on the promoter region of LOX. We found that knockdown of both YAP and TEAD4 induces LOX downregulation, suggesting that LOX is a potential target gene of YAP and TEAD4.

In summary, although recent studies have reported the intracellular functions of LOX, no studies of the possible association between LOX nuclear localization and the clinicopathological features of CRC have been published. The results obtained in this study have for the first time revealed a significant correlation between the LOX nuclear localization and synchronous or postoperative lung/hepatic metastasis. In addition, LOX intracellular localization was found to be a prognostic marker of a worse outcome in CRC patients. The Hippo pathway plays a role in regulating LOX expression in CRC. However, whether LOX is a direct target gene of TEAD4/YAP needs to be further clarified.

Acknowledgments

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Disclosure

No authors report any conflict of interest.

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