

12-Lipoxygenase promotes invasion and metastasis of human gastric cancer cells via epithelial-mesenchymal transition

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Abstract. The role of 12-lipoxygenase (12-LOX) in tumorigenesis has been well established in several types of human cancer, including gastric cancer. It was reported that epithelial-mesenchymal transition (EMT) contributes to tumor invasion and metastasis. However, whether 12-LOX promotes the invasion and metastasis of human gastric cancer cells via EMT remains to be elucidated. In the present study, the expression of 12-LOX and EMT markers, N-cadherin and E-cadherin, was evaluated in gastric cancer and adjacent normal mucosa samples by immunohistochemical analysis. 12-LOX-overexpressing gastric cancer cells were established via lentiviral transfection of SCG-7901 cells. Wound-healing and Transwell assays were performed to examine the regulation of cell metastasis and invasion by 12-LOX. Furthermore, the regulation of N-cadherin expression by 12-LOX was evaluated using reverse transcription-quantitative polymerase chain reaction and western blotting. The results revealed that the expression of 12-LOX and N-cadherin was significantly higher in gastric cancer compared with that in adjacent normal mucosa tissues ($P < 0.05$). By contrast, the expression of E-cadherin was significantly decreased in gastric cancer compared with that in adjacent normal mucosa tissues ($P < 0.05$). Furthermore, the expression of 12-LOX was positively associated with N-cadherin expression in gastric cancer tissues. 12-LOX-overexpressing gastric cancer cells exhibited significantly increased invasion and migration abilities compared with the empty vector and control groups. The expression of N-cadherin in 12-LOX-overexpressing gastric cancer cells was increased compared with that in the empty vector and control groups. The present study suggests that EMT may be involved in the promotion of the invasion and metastasis of human gastric cancer cells by 12-LOX.

Introduction

Gastric cancer (GC) is a type of gastrointestinal cancer that is commonly diagnosed at an advanced stage. Gastric cancer remains the second leading cause of cancer mortality in China (1). Invasion and metastasis are hallmarks of cancer, and affect the mortality rates of GC (2).

12-LOX is an isozyme of the LOX superfamily, and previous studies have suggested that LOX isozymes, including 12-LOX, are implicated in tumor progression (3). 12-LOX, or its metabolic product, 12-HETE, promotes progression and metastasis in several types of solid tumors, including prostate cancer (4), breast cancer (5), colon cancer (6) and melanoma (7). The role of 12-LOX in the invasion and metastasis of human GC, and its underlying mechanism, remain to be elucidated.

Tumor cells acquire invasive/metastatic properties due to epithelial-mesenchymal transition (EMT), whereby the epithelial cell layers lose polarity and cell-cell adhesion due to remodeling of the cytoskeleton (8). EMT is involved in a variety of processes that characterize tumor progression, including cell invasion and metastasis (9,10). The hallmarks of EMT are the upregulation of N-cadherin and the downregulation of E-cadherin expression (11). N-cadherin is a member of the cadherin superfamily, which regulates cell-cell adhesion (12), and has been demonstrated to increase the motility and migration abilities of a number of types of cancer cells (13-15) and be a marker of EMT (16,17). E-cadherin is a Ca^{2+} -dependent cell-cell adhesion molecule that is important for maintaining the integrity and polarity of epithelial cells (18). Numerous studies have indicated that downregulation of E-cadherin results in tumor progression, metastasis and a poor prognosis for patients with GC (19-22), cervical carcinoma (23), colorectal cancer (24) and cholangiocarcinoma (25).

The aim of the present study was to investigate the role of 12-LOX in the invasion and metastasis of human GC, and to determine whether the effects of 12-LOX are mediated through EMT.

Patients and methods

Patient selection and tissue preparation. The present study was approved by the Ethics Committee of the Fujian Medical

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University Union Hospital (Fuzhou, China; reference no. 2014KY031) and written informed consent was obtained from each patient. A total of 105 paraffin-embedded GC tissue samples and 43 adjacent normal gastric mucosa tissue samples were randomly selected from the Department of Pathology, Fujian Medical University Union Hospital (Fuzhou, China), for retrospective study. The GC cases consisted of 80 men and 25 women, aged 34–81 years (mean age, 57 years), between October 2011 and December 2014. Tumors were staged according to the 7th edition of the AJCC Cancer Staging Manual: Stomach (26). The clinicopathological characteristics of the patients are summarized as follows (some data missing): Tumor size (<5 cm, 53 cases; ≥5 cm, 52 cases); tumor invasion (T1–T2, 28 cases; T3–T4, 76 cases); differentiation (poor, 52 cases; middle-well, 42 cases); clinical stage (I+II, 44 cases; III+IV, 61 cases); lymph nodes metastasis (negative, 26 cases; positive, 78 cases). All patients were treated by radical surgical resection and had not received chemotherapy or radiotherapy prior to surgery.

Immunohistochemistry. Formalin-fixed and paraffin-embedded specimens from the Department of Pathology were sectioned at a thickness of 4- μ m, then deparaffinized twice in 100% dimethylbenzene for 30 min, then rehydrated in a graded ethanol series (100, 95, 70 and 50%). The slides were placed in antigen retrieval buffer (sodium citrate 10 mM, pH 6.0) and microwaved at high power for 15 min, followed by blocking endogenous peroxidase activity in 0.3% hydrogen peroxide for 20 min at room temperature. Protein expression was detected using the following primary antibodies: 12-LOX (dilution 1:100; cat. no. GTX80966), E-cadherin (dilution, 1:500; cat. no. GTX100443) and N-cadherin (dilution 1:150; cat. no. GTX12221; all GeneTex, Inc., Irvine, CA, USA), which were incubated with the sections overnight at 4°C. The slides were then incubated with secondary antibodies for 20 min in a humidified chamber at 37°C. All slides were stained with 10% 3'-diaminobenzidine (OriGene Technologies, Inc., Beijing, China) for 2 min at room temperature, washed with PBS and then stained with 0.1% hematoxylin for 3 min at room temperature. The sections were washed again with PBS and then washed with running tap water for 10 min. All the slides were observed under a light microscope (magnification, x400) in 10 randomly selected fields of view, and qualitatively scored by 2 pathologists. The final score was based on the percentage of positively stained cells as follows: 0, 1%; 1, 1–25%; 2, 25–50%; 3, 50–75%; and 4, >75%. This was multiplied by the intensity of the staining, which was scored as follows: 0, none; 1, weak; 2, moderate, and 3, strong. Scores of ≤3 were considered to indicate negative expression, whereas scores ≥4 were considered to indicate positive expression.

Cell culture. The human GC cell line, SGC-7901 (The Department of Gastroenterology, Fujian Medical University Union Hospital), was cultured in DMEM (HyClone; GE Healthcare, Chicago, IL, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare) at 37°C in 5% CO₂.

Lentivirus vector construction and transfection. The lentivirus vector for 12-LOX gene overexpression was constructed by

Shanghai GeneChem Co., Ltd. (Shanghai, China). An empty green fluorescent protein (GFP) vector was used as a negative control (Shanghai GeneChem Co., Ltd.). A total of 6x10⁴ SGC-7901 cells were seeded in each well of a 6-well plate. When confluency reached 30–40%, the 12-LOX overexpression vector (2x10⁷ TU/ml) or the empty GFP vector (2x10⁷ TU/ml) was transfected into the SGC-7901 cells. The status of cells was observed by fluorescence microscope (magnification, x200) for 8–12 h, according to the lentivirus vector manufacturer's instructions, and the medium was replaced. When confluency reached 90%, the cells was transferred into a culture flask. Following transfection, cells were used in subsequent experiments after 2 passages. Three groups of cells were used in the subsequent experiments: Untransfected cells (control group), cells transfected with the empty GFP vector (LV-vector group) and cells transfected with the 12-LOX expression vector (LV-12-LOX group).

Wound-healing assay. A total of 4x10⁵ cells were seeded into each well of a 6-well plate and allowed to grow in DMEM supplemented with 10% fetal bovine serum (both HyClone; GE Healthcare, Chicago, IL, USA) until ~100% confluent. A 200- μ l pipette tip was used to scratch across the cell monolayer. Cellular debris was removed by washing with PBS three times, and the plate was cultured for another 24 h. Images were captured at 0 and 24 h after wounding using an inverted light microscope. The extent of wound-healing was quantified using the following formula: $(W_{0h} - W_{24h}) / W_{0h}$, where W_{0h} and W_{24h} represent the width of the wound at 0 and 24 h, respectively.

Cell invasion and migration assay. A 24-well plate containing Transwell inserts with a pore size of 8 μ m (Merck KGaA, Darmstadt, Germany) was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) for the invasion assay and left uncoated for the migration assay. The following steps were then performed for the two assays. A total of 1.5x10⁴ cells suspended in serum-free DMEM were placed in the upper chamber. DMEM containing 10% fetal bovine serum was placed in the lower chamber as a chemoattractant. The cells were incubated for 24 h at 37°C prior to removal of cells remaining on the upper side of the chamber with a cotton swab. Cells on the lower side of the membrane were fixed in 4% paraformaldehyde for 10 min at room temperature and dyed with 0.1% crystal violet for 15 min at room temperature. The number of cells was then counted under an inverted light microscope (magnification, x400) by a technician blinded to the experimental settings in 5 randomly selected fields of view in each plate. The experiments were repeated 3 times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RT-qPCR was used to detect the mRNA expression of 12-LOX and N-cadherin in the transfected and untransfected cells. Total RNA was extracted using TRIzol (Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized using 2 μ g total RNA using the PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd. Dalian, China). RT-qPCR analysis was performed using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus; Takara) with 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific,

Table I. Primer sequences for 12-LOX, N-cadherin and GAPDH.

Gene	Primer (5'-3')
12-LOX	Forward: ATGGCCCTCAAACGTGTTTAC Reverse: GCACTGGCGAACCTTCTCA
N-cadherin	Forward: GCGTCTGTAGAGGCTTCTGG Reverse: AAATCTGCAGGCTCACTGCT
GAPDH	Forward: CATCAGCAATGCCTCCTGCAC Reverse: TGAGTCCTTCCACGATACCAA AGTT
12-LOX, 12-lipoxygenase.	

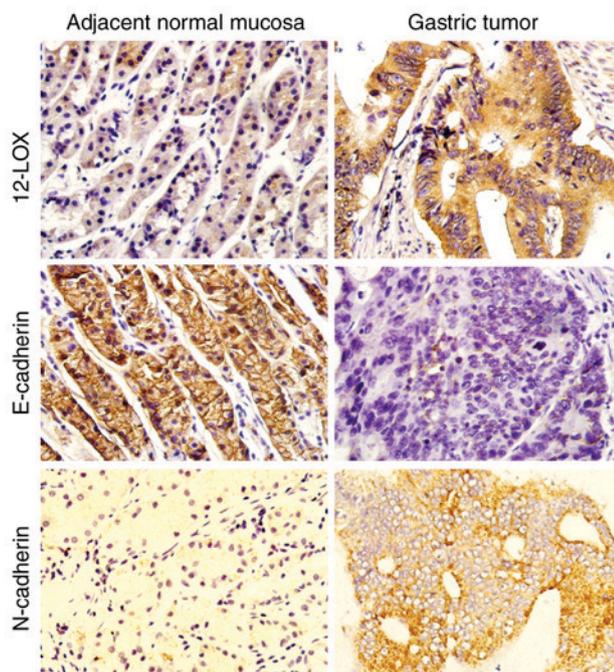


Figure 1. Representative expression levels of 12-LOX, E-cadherin and N-cadherin in GC and adjacent normal gastric mucosa tissues, as analyzed by immunohistochemistry (magnification, x400). The protein expression level of 12-LOX and N-cadherin was increased, and that of E-cadherin was decreased, in GC tissue compared with adjacent normal gastric mucosa tissue. 12-LOX, 12-lipoxygenase; GC, gastric cancer.

Inc.). The PCR amplification was performed under the following conditions: 95°C for 2 min, then 45 cycles of denaturation at 95°C for 15 sec, annealing at 63°C for 15 sec and elongation at 72°C for 20 sec. The primer sequences for 12-LOX, N-cadherin and GAPDH are listed in Table I (Shanghai Yaolin Bio-Technology Co., Ltd., Shanghai, China). The gene expression levels were normalized to GAPDH and are presented as relative fold change compared to the control (27). The data were analyzed using the $2^{-\Delta\Delta C_t}$ method. A total of 3 independent repeat experiments were performed.

Western blotting analysis. Total protein was extracted using cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing 1 mmol/l phenylmethylsulfonyl fluoride.

Table II. Expression of 12-LOX, N-cadherin and E-cadherin in 105 cases of gastric cancer and 43 gastric normal mucosa tissues.

Protein expression	Gastric cancer tissue	Gastric normal mucosa tissue	P-value
12-LOX			
Positive	65	14	0.001
Negative	40	29	
N-cadherin			
Positive	63	11	<0.001
Negative	42	32	
E-cadherin			
Positive	41	24	0.046
Negative	64	19	

χ^2 test. 12-LOX, 12-lipoxygenase.

Protein concentration was determined using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). A total of 40 μ g protein per lane was subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare, IL, Chicago). The membrane was blocked in 5% skimmed milk dissolved by PBS for 2 h at room temperature and subsequently incubated with the following rabbit polyclonal primary antibodies overnight at 4°C: 12-LOX (dilution 1:80; cat. no. GTX80966), N-cadherin (dilution 1:80; cat. no. GTX12221; both GeneTex, Inc.) or GAPDH (dilution 1:1,500; cat. no. TA309157; OriGene Technologies, Inc.). The membrane was then incubated with the appropriate HRP-conjugated Goat anti-Rabbit IgG (H+L) (dilution 1:2,000; cat. no. ZB2301; OriGene Technologies, Inc.) for 1 h at room temperature. The proteins were visualized using an ECL Advance Detection system (Origene Technologies, Inc.).

Statistical analysis. Data are expressed as the mean \pm standard deviation. The differentiation between expression levels and clinicopathological parameters were analyzed using the χ^2 test. The associations between groups were compared using analysis of variance followed by the Least-Significant-Difference test. Statistical analyses were performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of 12-LOX, N-cadherin and E-cadherin in GC tissue. Immunohistochemical analysis was used to evaluate the protein expression levels of 12-LOX, N-cadherin and E-cadherin in GC tissue and adjacent normal mucosa tissue. It was demonstrated that 12-LOX and N-cadherin were mainly expressed in the cytoplasm of tumor cells, whereas E-cadherin was localized in the cell membrane (Fig. 1). The protein expression level of 12-LOX and N-cadherin was significantly increased in GC tissue compared with that in

Table III. Association between 12-LOX, E-cadherin and N-cadherin protein expression level and clinicopathological features in 105 cases of gastric cancer (some patient data is missing).

Characteristic	12-LOX expression			E-cadherin expression			N-cadherin expression		
	Low, n	High, n	P-value	Low, n	High, n	P-value	Low, n	High, n	P-value
Age, years									
≤60	18	29	0.751	34	13	0.031 ^a	21	26	0.291
>60	24	34		30	28		20	38	
Sex									
Male	35	45	0.164	46	34	0.198	31	49	0.912
Female	7	18		18	7		10	15	
Tumor size, cm									
<5	24	29	0.296	32	21	0.902	27	26	0.011 ^a
≥5	18	34		32	20		14	38	
Tumor invasion									
T1-T2	11	17	0.986	18	10	0.645	14	14	0.145
T3-T4	30	46		45	31		26	50	
Differentiation									
Poor	32	20	0.670	14	38	0.021 ^a	29	23	0.121
Middle-well	24	18		21	21		30	12	
Clinical stage									
I+II	17	27	0.811	29	15	0.381	19	25	0.466
III+IV	25	36		35	26		22	39	
Lymph node metastasis									
Negative	9	17	0.562	16	10	0.908	11	15	0.645
Positive	32	46		47	31		29	49	

χ^2 test. 12-LOX, 12-lipoxygenase. ^aP<0.05.

adjacent normal gastric mucosa tissue (P<0.05; Table II). By contrast, the expression of E-cadherin was significantly decreased in GC tissues compared with that in adjacent normal gastric mucosa tissue (P<0.05). The associations between patient clinicopathological characteristics and the protein expression levels of 12-LOX, N-cadherin and E-cadherin were examined. As demonstrated in Table III, there was a close association between the levels of E-cadherin protein expression and two factors, patient age and tumor differentiation (P<0.05), whereas the level of N-cadherin protein expression was associated with tumor size (P<0.05). 12-LOX expression was higher in tumors ≥5 cm, with a depth of invasion of T3-T4 and clinical stage of III+IV, however, these differences were not statistically significant (P>0.05). The expression of 12-LOX was positively associated with that of N-cadherin in GC tissues (r=0.263; P=0.007; Table IV).

12-LOX promotes the migration and invasion of GC cells. A stably transfected 12-LOX-overexpressing GC SGC-7901 cell line was established, and the green fluorescent cells were >95% confluent prior to fluorescence microscopy (magnification, x200). The results of RT-qPCR and western blotting revealed that the expression of 12-LOX in the LV-12-LOX group was significantly increased compared with that in the LV-vector and control groups (Fig. 2A).

Table IV. Association between the expression levels of 12-LOX and N-cadherin protein expression in 105 gastric cancer tissues.

N-cadherin expression	12-LOX expression			R	P-value
	Low	High			
Low	23	18		0.263	0.007
High	19	45			

χ^2 test. 12-LOX, 12-lipoxygenase.

The results of the scratch wound-healing (Fig. 2B) and Transwell (Fig. 2C) assays demonstrated that 12-LOX promotes the migration and invasion of GC cells *in vitro*. Significantly more cells migrated in 24 h in the LV-12-LOX group compared with those in the LV-vector and control groups (P<0.05). The number of cancer cells that invaded through the Matrigel was significantly higher in the LV-12-LOX group compared with that in the LV-vector and control groups (P<0.05).

The results of RT-qPCR revealed that the mRNA level of N-cadherin was significantly increased in the LV-12-LOX

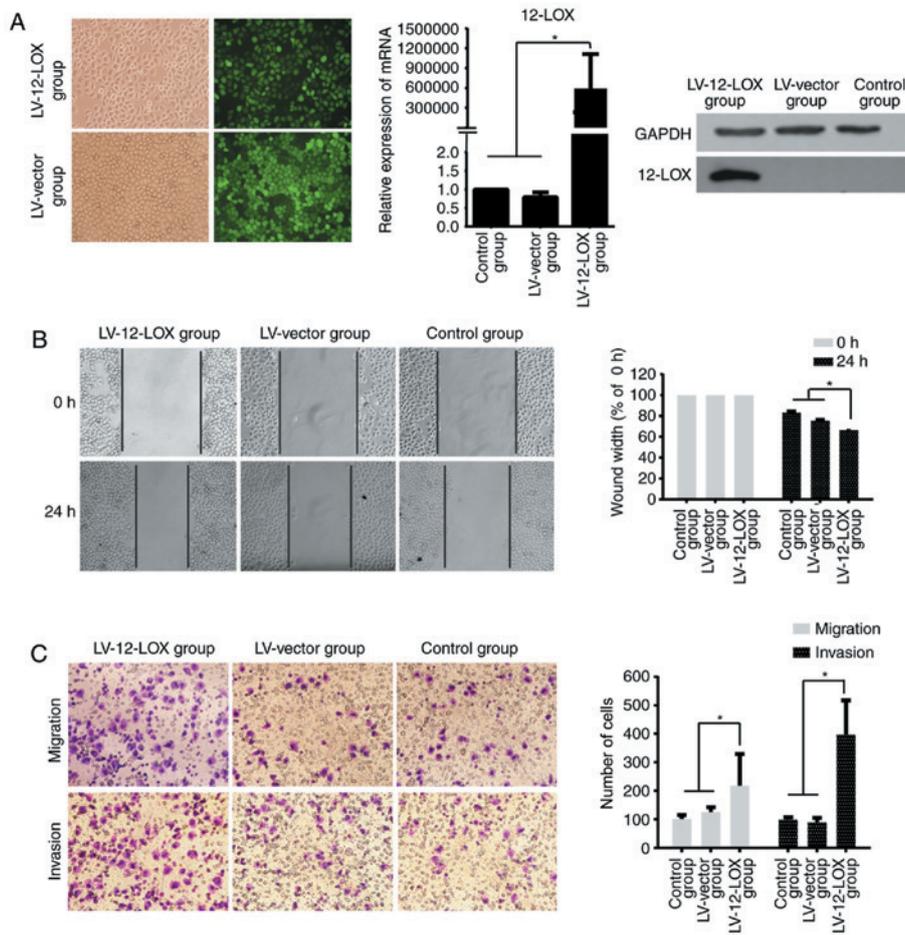


Figure 2. SGC-7901 GC cells were transfected with the 12-LOX overexpression vector or empty vector. (A) Successfully transfected cells fluoresced green. The mRNA expression of 12-LOX was significantly increased in the LV-12-LOX group compared with that in the LV-vector and control groups ($P<0.05$), which was reflected at the protein level. (B) The wound-healing assay demonstrated the migration of the 3 groups at 0 and 24 h. The migratory ability of LV-12-LOX GC cells was significantly increased compared with the LV-vector and control group cells ($P<0.05$). (C) Migration and invasion assays revealed that the number of cancer cells that migrated or invaded through the Transwell insert was significantly increased in the LV-12-LOX group compared with that in the LV-vector and control groups ($P<0.05$). 12-LOX, 12-lipoxygenase; LV, lentivirus; GC, gastric cancer.

group compared with that in the LV-vector and control groups (Fig. 3A; $P<0.05$). Western blotting revealed that the protein expression level of N-cadherin was markedly increased in the LV-12-LOX group compared with that in the LV-vector and control groups (Fig. 3B).

Discussion

12-LOX serves an important role in various inflammatory diseases, including diabetes, atherosclerosis (28) and nervous system diseases (29). Previous research has reported significant functional roles of 12-LOX and its product, 12-HETE, in the formation, development, invasion and metastasis of several types of cancer (30-32). Klampfl *et al* (6) reported that upregulation of 12-LOX induced a migratory phenotype in colorectal cancer cells. The available literature regarding the expression and involvement of 12-LOX in GC is limited; however, it has been reported that 12-LOX may be associated with GC cell apoptosis (33,34).

The present study demonstrated that 12-LOX was highly expressed in GC tissue compared with that in adjacent normal gastric mucosa tissue, indicating that 12-LOX overexpression may contribute to GC progression. LV-12-LOX

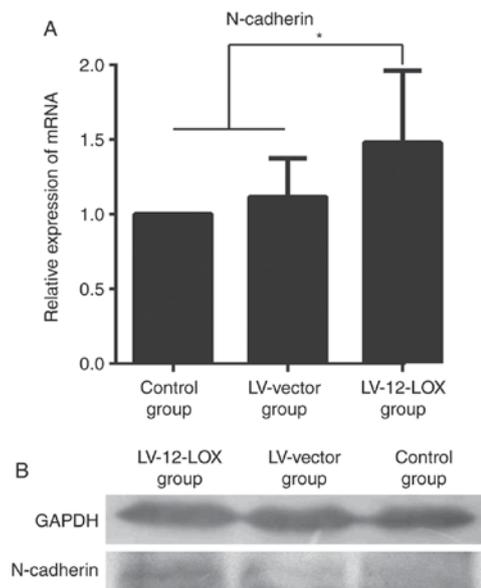


Figure 3. 12-LOX promotes N-cadherin expression in gastric cancer cells. (A) The mRNA expression level of N-cadherin was determined by reverse transcription-quantitative polymerase chain reaction ($P<0.05$). (B) The protein expression level of N-cadherin was determined by western blotting. 12-LOX, 12-lipoxygenase; LV, lentivirus.

GC cells exhibited significantly enhanced migratory and invasive abilities compared with the LV-vector and control groups. These results indicate that 12-LOX facilitates GC cell migration and invasion and are consistent with previous studies that reported that 12-LOX promotes invasion and metastasis in numerous types of tumor cells (35-37). However, the specific underlying mechanism remains to be elucidated.

Immunohistochemical analysis revealed that GC tissue exhibited increased E-cadherin and decreased N-cadherin protein expression levels compared with those of adjacent normal gastric mucosa tissues. Abnormal expression of E-cadherin was demonstrated to be significantly associated with tumor grade and patient age, which was also reported by Torabizadeh *et al* (38). Anbiaee *et al* (39) and Lazăr *et al* (40) reported a significant correlation between the abnormal expression of E-cadherin and tumor grade, but no correlation with patient age. The present study also revealed a significant association between the abnormal expression of N-cadherin and tumor size, suggesting that N-cadherin may promote the growth of GC.

The present study also demonstrated that the expression level of 12-LOX was positively associated with that of N-cadherin in GC tissue. To the best of our knowledge, the regulation of N-cadherin expression by 12-LOX has not been previously reported. N-cadherin is a member of the calcium-dependent cell adhesion molecule family, and a marker of interstitial cells (41). N-cadherin is also a marker of EMT (42,43), and serves a key role in tumor cell migration, invasion and metastasis (44,45). It has been reported that EMT is involved in the invasion and metastasis of GC (46), which indicates that 12-LOX may affect GC progression via EMT.

The present study suggests that EMT may be involved in the progression of GC, which is in accordance with previous study results (9,10,46). Therefore, we hypothesize that 12-LOX promotes the invasion and metastasis of GC cells via EMT. Han and Xu (47) reported that epithelial membrane protein 3 is induced by twist family BHLH transcription factor 1/2 and regulates the EMT of GC cells. Song *et al* (48) demonstrated that the Wnt/ β -catenin and phosphoinositide 3-kinase/protein kinase B signaling pathways regulate EMT in GC. Chen *et al* (49) reported that the tumor necrosis factor- α -inducing protein of *Helicobacter pylori* induces EMT in GC cells through activation of the interleukin-6/signal transducer and activator of transcription 3 signaling pathway. Numerous other pathways are reportedly involved in EMT, including transforming growth factor β (50), notch (51), nuclear factor- κ B (52) and mitogen-activated protein kinases/extracellular signal-regulated kinase signaling (53). The underlying mechanisms of the promotion of EMT by 12-LOX require further investigation.

In conclusion, it was demonstrated that EMT may be involved in the promotion of the invasion and metastasis of human gastric cancer cells via 12-LOX; therefore, it may be a novel target for the treatment of gastric cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CZ performed the experiment, analyzed the experiment data and wrote the manuscript. MZ was responsible for the collection of the patient selection and tissue preparation. XW and JL were responsible for the design of experiment. ZC and YH were responsible for guiding the experimental technique, analysis of data and revising the manuscript. FC was accountable for designing the experiment, performing data interpretation and revising the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Fujian Medical University Union Hospital (Fuzhou, China; reference no. 2014KY031).

Consent for publication

Written informed consent was obtained from each patient for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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