Secreted LOXL2 is a novel therapeutic target that promotes gastric cancer metastasis via the Src/FAK pathway

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The purpose of this study was to investigate invasion- and metastasis-related genes in gastric cancer. To this end, we used the transwell system to select a highly invasive subcell line from minimally invasive parent cells and compared gene expression in paired cell lines with high- and low-invasive potentials. Lysyl oxidase-like 2 (LOXL2) was overexpressed in the highly invasive subcell line. Immunohistochemical analysis revealed that LOXL2 expression was markedly increased in carcinoma relative to normal epithelia, and this overexpression in primary tumor was significantly associated with depth of tumor invasion, lymph node metastasis and poorer overall survival. Moreover, LOXL2 expression was further increased in lymph node metastases compared with primary cancer tissues. RNA interference-mediated knockdown and ectopic expression of LOXL2 showed that LOXL2 promoted tumor cell invasion in vitro and increased gastric carcinoma metastasis in vivo. Subsequent mechanistic studies showed that LOXL2 could activate both the Snail/E-cadherin and Src kinase/Focal adhesion kinase (Src/FAK) pathways. However, secreted LOXL2 induced gastric tumor cell invasion and metastasis exclusively via the Src/FAK pathway. Expression correlation analysis in gastric carcinoma tissues also revealed that LOXL2 promoted invasion via the Src/FAK pathway but not the Snail/E-cadherin pathway. We then evaluated secreted LOXL2 as a target for gastric carcinoma treatment and found that an antibody against LOXL2 significantly inhibited tumor growth and metastasis. Overall, our data revealed that LOXL2 overexpression, a frequent event in gastric carcinoma progression, contributes to tumor cell invasion and metastasis, and LOXL2 may be a therapeutic target for preventing and treating metastases.

Introduction

Gastric cancer is second only to lung cancer as the cause of the most cancer deaths worldwide (1). Whereas overall gastric cancer incidence has declined, the incidence remains high in Asian countries (1,2). Although gastric cancer is curable if detected early, most patients are diagnosed with late-stage disease, wherein current therapeutic strategies are far from optimal (3). Surgery and combination chemotherapies have been shown to confer only modest survival benefits in advanced gastric cancer, resulting in an overall 5 year survival rate of <24% (4,5). Thus, a molecular understanding of the genetic factors involved in gastric cancer progression may contribute toward identifying novel gastric biomarkers and highlight potential avenues for targeted therapies.

Lysyl oxidase-like 2 (LOXL2) is a member of the lysyl oxidase (LOX) protein family that consists of five members (6). All the members of the family show a highly conserved catalytic domain located at the C-terminus, whereas the N-terminus region of the LOX isoforms is more divergent and is thought to determine the individual role and tissue distribution of each isoenzyme (7). The two best-characterized members of the LOX family, LOX and LOXL2, have been previously known as extracellular enzymes responsible for cross-link formation in fibrillar collagen and elastin (8). More recently, however, members of the LOX family have been localized both intracellularly and extracellularly (9) and implicated in various other biological functions, including processes important to cancer progression, namely cell growth, adhesion, motility and invasion (10,11). In early LOXL2 studies, a nearly 60-fold decrease of LOXL2 messenger RNA expression was found in RAS-transformed rat fibroblasts (12) and the LOXL2 gene was identified as a downregulated transcript in some tumors including head and neck squamous cell carcinomas and ovarian tumors (13–15). However, some recent studies showed that LOXL2 overexpression promotes the invasiveness of breast cancer cells (16), and its upregulation has been reported in breast, colon and esophageal cancer (16,17). Furthermore, one recent article noted that intracellular LOXL2 was able to induce epithelial-to-mesenchymal transition by interacting with Snail, a transcription factor essential for epithelial-to-mesenchymal transition (18).

In this study, we demonstrate that LOXL2 is involved in gastric cancer invasion and is overexpressed in gastric cancer versus normal tissues. The clinical significance and biological involvement of LOXL2 in the progression of gastric cancer have not yet been elucidated. Therefore, immunohistochemical studies were performed on 274 primary gastric specimens and LOXL2 overexpression was associated with depth of tumor invasion, lymph node metastasis and poorer overall survival. Furthermore, we showed that secreted LOXL2 promotes gastric cancer metastasis via Src kinase/Focal adhesion kinase (Src/FAK) pathway, which is a good therapeutic target.

Materials and methods

Cell lines and cell culture

Human gastric cancer cell lines PAMC82 (19), BGC823 (20) and others were maintained in RPMI 1640 (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). To select a highly invasive subpopulation, PAMC82 cells were seeded on the Matrigel coating, 8 µm pore transwell (Costar, Cambridge, MA). The cells that invaded through the membrane and attached to the lower well were harvested and expanded for second-round selection. Serial selection of cells for increased invasiveness was continued for three generations, and the sublines from the different generations were designated as PAMC82-P1, PAMC82-P2 and PAMC82-P3.

Anti-LOXL2 polyclonal antibody preparation and immunoprecipitation

The affinity-purified rabbit anti-human LOXL2 polyclonal antibody was designed against the C-terminal end (EHSGILLNNQLSPQ), which was produced by GL Biochem Ltd (Shanghai, China). The sequence is unique among the LOX family (17). Antibody specificity was tested by immunoblots and immunoprecipitation. For immunoprecipitation, BGC823 cells were grown on plates and lysated as described previously (21). Protein extracts were produced by GL Biochem Ltd (Shanghai, China). The sequence is unique among the LOX family (17). Antibody specificity was tested by immunoblots and immunoprecipitation. For immunoprecipitation, BGC823 cells were grown on plates and lysated as described previously (21). Protein extracts were precleared with protein A/G, then incubated with normal rabbit IgG or anti-LOXL2 antibody (own production) overnight at 4°C and pulled down with protein A/G for 60 min. Pulled-down proteins were analyzed with anti-LOXL2 antibody (R&D Systems, Minneapolis, MN) by western blot. Furthermore, inverse immunoprecipitation analysis was carried out. Protein extracts were incubated with normal mouse IgG or anti-LOXL2 antibody (R&D Systems) and pulled down with protein A/G. Pulled-down proteins were analyzed with anti-LOXL2 antibody (own production).

Abbreviations: H2O2, hydrogen peroxide; LOX, lysyl oxidase; LOXL2, lysyl oxidase-like-2; PBS, phosphate-buffered saline; rLOXL2, recombinant human lysyl oxidase-like-2; RNAI, RNA interference; shEGFP, enhanced green fluorescent protein RNA interference; Src/FAK, Src kinase/Focal adhesion kinase.

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Secreted LOXL2 is a novel therapeutic target in gastric carcinoma

Female mice aged 4 weeks were used in these experiments. For the spontaneous metastasis assay, stable RNA interference (RNAi)-mediated LOXL2 knockdown BGC823 cells (shLOXL2-1# and shLOXL2-2#) and enhanced green fluorescent protein RNA interference (shEGFP) cells were subcutaneously injected into mice. The mice were killed 7 weeks after injection and examined for subcutaneous tumor growth or development of metastases.

Anti-LOXL2 antibody treatment of tumor-bearing mice

Tumor-bearing mice were size matched and divided into groups. The weights of mice were similar within each treatment cohort. Treatment began 3 days after injection of the tumor cells. Three groups were treated with a 200 μg per mouse dose (~10 mg/kg, high dose), a 100 μg per mouse dose (~5 mg/kg, moderate dose) or a 50 μg per mouse dose (~2.5 mg/kg, low dose) of anti-LOXL2 antibody (own production), whereas three groups of control received corresponding doses of normal rabbit antibody or PBS alone. Therapeutic or control doses were systemically administered celiac in 200 μl PBS once daily for 5 days, followed by twice weekly until treatment was completed. The mice were killed 27 days after injection of the tumor cells and examined for subcutaneous tumor growth. Another PBS, high-dose IgG or anti-LOXL2 anti-body treatment groups were maintained 60 days and examined lung metastasis.

Statistical analysis

Statistical analyses for Table 1 were carried out with Fisher’s exact test. For survival analysis, Kaplan–Meier survival curves were constructed and differences between them were tested by the log-rank test. P < 0.05 was considered statistically significant.

Results

LOXL2 is overexpressed in highly invasive subline PAMC82-P3

We used the parental cell line PAMC82 to select the highly invasive cancer cell line PAMC82-P3. The ability of PAMC82-P3 to invade Matrigel was ~8-fold greater than the parental PAMC82, but equal to the second-generation line PAMC82-P2 (Figure 1A), suggesting that the invasive potential of the sublines had reached a plateau after three rounds of selection. The different expression profiles of PAMC82 and PAMC82-P3 were analyzed by microarray. Based on a threshold of ≥2-fold, 289 genes were differentially expressed, of which 213 were upregulated and 76 were downregulated in PAMC82-P3 relative to PAMC82 (data not shown). Most of the dysregulated genes have been previously shown to be involved in tumor invasion and metastasis, such as matrix metallopeptidase 1 (23), cadherin 3 (24), lectin galactoside-binding soluble 1 (25), thymidine phosphorylase (26) and LY6/PLAUR domain containing 3 (27). One of the dysregulated genes, LOXL2, was of particular interest: western blot analysis revealed that LOXL2 was gradually upregulated from PAMC82 to PAMC82-P3 (Figure 1A). In order to confirm this finding, the association of LOXL2 expression and invasive potential was examined in additional human gastric cancer cell lines. It was found that LOXL2 expression correlated well with the invasive ability of the cells (Figure 1B). To perform the following experiments, we developed an anti-LOXL2 polyclonal antibody. Western blot and immunoprecipitation assay showed that our purified anti-LOXL2 antibody specifically recognized human LOXL2 protein (Figure 1C–F).

LOXL2 was overexpressed in gastric carcinomas and correlated with poor survival

We evaluated the expression of LOXL2 in a gastric carcinoma tissue microarray (from 100 cases of gastric carcinoma). Normal epithelia showed negative or weak immunoreactions, whereas 47 of 100 primary tumors exhibited positive or strongly positive LOXL2 staining. The intensity of LOXL2 staining was statistically higher in carcinomas than in normal epithelia (Figure 2A). In order to characterize LOXL2 expression in human metastasis loci, we examined LOXL2 expression in 140 gastric carcinoma patients having primary tumors and corresponding lymph nodes with metastasis. Among these 140 paired samples, LOXL2 was frequently overexpressed in 43.57% of primary tumors (61/140 ≥ level 2) and overexpressed in 69.29% of corresponding lymph node metastases (97/140 ≥ level 2) (Figure 2B). In addition, 44.29% (62/140) cases of lymph node metastases displayed a higher expression level of LOXL2 compared with the matched primary tumors (P < 0.01).

Gastric carcinoma tissue microarray and immunohistochemical staining

The expression of LOXL2 in gastric cancer was determined by assessing its staining using tissue microarrays from 514 clinical cases, of which 274 of the gastric cancer specimens had clinical follow-up records. In addition, 100 of these specimens had paired normal epithelia and 140 of these specimens had paired lymph node metastasis. For immunoassaying of LOXL2, a DAKO CSA kit (DAKO, Glostrup, Denmark) was used. Anti-LOXL2 antibody (own production, 5 μg/ml) was incubated for 1 h in citrate buffer. After staining, slides were evaluated by two pathologists. Expression score was determined by staining intensity and percentage. Tissues with no staining were rated as 0, those with faint staining or moderate to strong staining in ≤25% of cells as 1, with moderate staining or strong staining in 25–50% of cells as 2 and with strong staining in >50% of cells as 3.

LOXL2 knockdown cells

Two short hairpin RNAs were designed, based on the LOXL2 sequence: shRNA1064 5'-GAATACGAGTGT-3' or shRNA1340 5'-CCAGAAGATTACGAGTGT-3'. The oligoduplexes were cloned into psiTarget™ 2.0-CMV-RFP-Neo siRNA vector (Genechem, Shanghai, China) and transfected into cells using Lipofectamine 2000 (Invitrogen). Transfected cells were selected for 14 days with 500 μg/ml G418. Cells stably expressing shRNA1064 or shRNA1340 were designated as shLOXL2-1# or shLOXL2-2#, respectively. Pooled populations of knockdown cells were injected into nude mice for in vivo experiments. Negative control cell lines were generated by infecting cells with a psiTarget™ 2.0-CMV-RFP-Neo construct targeting enhanced green fluorescent protein complementary DNA (5'-GAAGACGACGACTCTCT-3').

LOXL2 overexpressing cells

The human LOXL2 complementary DNA was cloned into the pcDNA3.1 expression vector (Genechem) containing a His epitope at the C-terminus to give a pcDNA3.1-LOXL2-His vector and then transfected into the gastric cancer cell line N87. Stable cell lines were selected by incubation with 500 μg/ml G418 for 2 weeks.

Western blot analysis

The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were blocked and then probed with antibodies against LOXL2 (3 μg/ml, R&D Systems), LOXL2 (3 μg/ml; own production), β-actin (1:5000, Sigma, St. Louis, MO), FAK (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA), phospho-FAK (Ser192, 1:400, Santa Cruz Biotechnology), Src (1:500, Santa Cruz Biotechnology), phospho-Src (Yyr106, 1:400, Santa Cruz Biotechnology), Snail (1:500, Santa Cruz Biotechnology) and E-cadherin (1:300, Santa Cruz Biotechnology). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence kit (Pierce, Rockford, IL).

Cell adhesion assay

Cells were trypsinized, counted and then plated at a density of 5 × 10⁴ cells per well in 96-well plates coated with 100 ng/ml fibronectin or 100 ng/ml collagen I. The cells were incubated at 37°C for 30 min in a CO₂ incubator. Non-attached cells were removed by three washings with phosphate-buffered saline (PBS). Attached cells were analyzed by Cell Counting Kit-8 (Dojindo, Japan) according to the manufacturer’s instructions, and the optical density was measured at 450 nm. These experiments were performed in triplicate and repeated twice.

Matrigel chemoinvasion assay

Matrigel chemoinvasion assay was performed as described previously, with some modification (22). Briefly, for Matrigel chemoinvasion assays, cells (4 × 10⁵) were counted and seeded onto 6.5 mm Corning Costar transwells coated with 150 g/cm² Matrigel. After incubation for 24 h at 37°C, the cells that remained inside the insert were removed with a cotton swab, and cells that had penetrated the Matrigel to invade to the lower surface of the membrane were fixed in methanol:acetone (50:50) and stained with 4',6-diamidino-2-phenylindole. After air drying the membrane, the cells were counted at a magnification of ×100 in three random fields of view under a fluorescence microscope. These experiments were performed in triplicate and repeated twice. Therefore, the values represent the mean number of invasive cells per ×100 fields from 18 random ×100 fields. Differences in value distribution were statistically validated using the two-tailed t-test for unpaired data. Differences were considered to be significant at values lower than P < 0.05.

Xenograft assays in nude mice

Female nu/nu mice obtained from the Jackson Laboratory (Vitalriver, Beijing, China) were kept in a specific pathogen-free facility at the Experimental Center of the Chinese Academy of Medical Science that is accredited for animal care by the Chinese Association for Accreditation of Laboratory Animal Care.
In vivo behavior key question was whether LOXL2 RNAi could suppress metastatic a.

tary Figure S3 is available at BGC823 cells, shLOXL2-1# and shLOXL2-2#, were selected and metastatic potentials of gastric carcinoma shRNA-mediated LOXL2 knockdown attenuated invasive, adherent and metastatic potentials of gastric carcinoma.

The two shRNA vectors, shLOXL2-1# and shLOXL2-2#, both markedly suppressed the expression of LOXL2 in PAMC82-P3 and BGC823 (Figure 3A). And LOXL2 knockdown did not affect the expression of other members of LOX family (supplementary Figure S2 is available at Carcinogenesis Online). Downregulation of LOXL2 had no effect on cell proliferation and apoptosis in vitro (supplementary Figure S3 is available at Carcinogenesis Online) but did result in a >50% decrease in invasion and cell–matrix adhesion (Figure 3A).

A key question was whether LOXL2 RNAi could suppress metastatic behavior in vivo. To address this possibility, two LOXL2 knockdown BGC823 cells, shLOXL2-1# and shLOXL2-2#, were selected and tested in animal experiments. Tumor growth was suppressed by LOXL2 knockdown (Figure 3B). The tumor burden was reduced by ~50% after 49 days of subcutaneous injection of tumor cells (Figure 3B). As previous speculation, hematoxylin and eosin staining of tumor margins confirm the invasive growth patterns into the surrounding muscles in the parent BGC823 and shEGFP BGC823 groups, whereas the LOXL2 knockdown in BGC823 cells leads to a partial encapsulation of the primary tumor (Figure 3C); therefore, LOXL2 downregulation significantly decreased tumor cell invasion into surrounding tissues. Furthermore, LOXL2 knockdown dramatically inhibited spontaneous lung metastasis of BGC823 (Figure 3D).

We have proved that LOXL2 had no effect on cell proliferation and apoptosis in vitro. However, in the xenograft assay, LOXL2 knockdown can suppress tumor growth. To explore the discrepancy between these two results, we performed immunohistochemistry with the Ki-67 proliferation marker and the CD31 endothelial cell marker. We want to know whether LOXL2 RNAi in the primary tumors would affect cell proliferation and tumor angiogenesis. And we found that Ki-67+ cells density and the CD31 positivity (blood vessel density) in the center area of tumors was similar between shEGFP BGC823 tumors and shLOXL2 BGC823 tumors. This observation is in concordance with what was observed in vitro. Whereas in the shEGFP group, the CD31 positivity and Ki-67+ cells density were higher in the edge area than that of shLOXL2 groups (supplementary Figure S4 is available at Carcinogenesis Online). Thus, we speculate that vascularization at tumor edge might be a rate-limiting determinant of overall tumor growth and that inhibited invasiveness might afford the shLOXL2 cells worse access to the vasculature.

Overexpression of LOXL2 in gastric cancer cells induces invasion and adhesion

To examine the functional consequence of elevated LOXL2 expression in gastric cancer cells, we overexpressed His-tagged LOXL2 in N87 and MKN45 gastric cancer cells (which exhibit a low endogenous level of LOXL2) (Figure 3E). We examined whether LOXL2 overexpression promoted cell invasion and cell–matrix adhesion. In invasion assay, LOXL2 overexpression significantly increased invasion of N87 and MKN45 cells relative to control cells (Figure 3E). In cell–matrix adhesion assays, overexpression of LOXL2 led to a significant enhancement of cell adhesion to fibronectin and collagen I matrix (data not shown). In addition, overexpression of LOXL2 in BGC823 cells restored the invasive ability, which was attenuated by shLOXL2 transfection (supplementary Figure S5 is available at Carcinogenesis Online).

Anti-LOXL2 antibody efficiently reduces the invasive, adherent and metastatic potentials of gastric carcinoma

We produced an anti-LOXL2 antibody and assessed its impact on PAMC82-P3 and BGC823 cells. Treatment with the anti-LOXL2 antibody led to a significant and dose-dependent decrease in cell invasion and cell–matrix adhesion (Figure 4A). In addition, we also observed that the effect on cell invasion of anti-LOXL2 antibody was not due to inhibition of cell proliferation (supplementary Figure S6 is available at Carcinogenesis Online). We then examined whether the anti-LOXL2 antibody suppressed BGC823 growth and spontaneous metastasis in vivo. In the human xenograft model described above, in which BGC823 cells were injected subcutaneously into nude mice, tumor growth was markedly inhibited in a dose-dependent manner by the anti-LOXL2 antibody (Figure 4B). Notably, high dose of the anti-LOXL2 antibody significantly decreased tumor cell invasion into surrounding tissues and spontaneous lung metastasis of BGC823 (Figure 4C and D). We also determined the effects of anti-LOXL2 antibody on PAMC82-P3 growth and spontaneous metastasis in vivo. Similarly, anti-LOXL2 antibody inhibited PAMC82-P3 growth and spontaneous metastasis as well (supplementary Figure S7 is available at Carcinogenesis Online). In addition, we performed the in vivo tail vein metastasis assay. A high dose of the anti-LOXL2 antibody significantly inhibited experimental lung metastasis of BGC823 (supplementary Figure S8 is available at Carcinogenesis Online).

<p>| Table I. LOXL2 protein expression in 274 primary gastric tumors determined by immunohistochemistry |</p>
<table>
<thead>
<tr>
<th>Variables</th>
<th>Score of LOXL2 expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, year</td>
<td>0.584</td>
<td>0.0001</td>
</tr>
<tr>
<td>SD</td>
<td>12.8</td>
<td>11.9</td>
</tr>
<tr>
<td>Gender</td>
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<tr>
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<td>67</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>PT (primary tumor)</td>
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<td></td>
</tr>
<tr>
<td>PT1</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>PT2</td>
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<td>PT3</td>
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<tr>
<td>PT4</td>
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<td>8</td>
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<tr>
<td>PN (lymph node metastasis)</td>
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<tr>
<td>PN1</td>
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<td>19</td>
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<tr>
<td>PN2</td>
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<td>PN3</td>
<td>9</td>
<td>27</td>
</tr>
<tr>
<td>PN4</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>PM (distant metastasis)</td>
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<td>87</td>
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<tr>
<td>PM1</td>
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<tr>
<td>Differentiation</td>
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<tr>
<td>High</td>
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<td>5</td>
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<tr>
<td>Moderate</td>
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<tr>
<td>Low</td>
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(A) LOXL2 staining and clinicopathologic characteristics in 274 gastric carcinoma patients

(B) Cox multivariate analysis

*Statistical analysis was performed by a χ² test, P values <0.05 were considered to be statistically significant.

The prognostic significance of LOXL2 was determined by assessing its staining using tissue microarrays containing 274 cases with known clinical follow-up records. Statistical analysis indicated that LOXL2 expression in the gastric carcinoma was correlated with depth of tumor invasion (P < 0.0001) and lymph node metastasis (P < 0.0001) (Table IA). There was no significant association with age, sex or tumor differentiation. Kaplan–Meier survival analysis of 274 gastric carcinoma specimens revealed a correlation between higher LOXL2 expression levels and shorter overall survival times (P < 0.0001). Furthermore, multivariate analysis revealed that LOXL2 was an independent prognostic factor for gastric cancer (Table IB).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Risk ratio (95% CI)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Age, year</td>
<td>1.105 (0.993–1.107)</td>
<td>0.128</td>
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<tr>
<td>Gender</td>
<td>0.784 (0.573–1.073)</td>
<td>0.128</td>
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<tr>
<td>PT (primary tumor)</td>
<td>0.796 (0.664–0.954)</td>
<td>0.014*</td>
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<tr>
<td>PN (lymph node metastasis)</td>
<td>1.503 (1.264–1.786)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>PM (distant metastasis)</td>
<td>1.807 (1.022–3.194)</td>
<td>0.042</td>
</tr>
<tr>
<td>Differentiation</td>
<td>0.073</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Moderate</td>
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<tr>
<td>Low</td>
<td>34</td>
<td>73</td>
</tr>
</tbody>
</table>

| Score of LOXL2 expression | 1.509 (1.285–1.771) | <0.0001* |

*Statistical analysis was performed by a χ² test, P values <0.05 were considered to be statistically significant.
Fig. 1. LOXL2 expression correlates with invasive capacity of gastric carcinoma cell lines. (A) Establishment of cell lines with high- or low-invasive abilities. Top, transwell invasion assays. Bottom, western blot of LOXL2 using anti-LOXL2 antibody (R&D Systems). (B) LOXL2 expression correlates with invasive capacity of gastric carcinoma cell lines. Top, transwell invasion assays. Bottom, western blot of LOXL2. (C) Western blot analysis of our purified anti-LOXL2 antibody specificity and LOXL2 protein expression in gastric carcinoma cell lines. Proteins collected from the cell layer (CL) and conditioned media (CM) were used for western blot analysis. CM from cell lines was concentrated 20-fold by Centricon centrifugation and 20 μl of the concentrated CM was used for western blot. (D) Immunoprecipitation assay showed that our purified anti-LOXL2 antibody specifically recognized human LOXL2 protein. Top, LOXL2 protein immunoprecipitated by anti-LOXL2 antibody (own production) was detected by western with anti-LOXL2 antibody (R&D Systems). Normal rabbit IgG immunoprecipitation is used as negative control. Bottom, LOXL2 protein immunoprecipitated by anti-LOXL2 antibody (R&D Systems) was detected by western with anti-LOXL2 antibody (own production). Normal mouse IgG immunoprecipitation is used as negative control. (E) Western blot analysis of our purified anti-LOXL2 antibody specificity in His-tagged LOXL2-transfected N87 cells. LOXL2 expression is detected only in proteins isolated from His-tagged LOXL2-transfected N87 cells using our purified anti-LOXL2 antibody (left) or anti-His antibody (right). (F) Western blot analysis of our purified anti-LOXL2 antibody specificity in LOXL2 RNAi BGC823 cells. LOXL2 expression is downregulated in LOXL2 RNAi BGC823 cells relative to control cells. (G) Our purified anti-LOXL2 antibody specificity was tested against commercial rhlLOXL2 proteins.
Fig. 2. Overexpression of LOXL2 protein in gastric cancer is correlated with tumor aggressiveness and prognosis. (A) Example case that LOXL2 is overexpressed in gastric tumors by immunohistochemical staining on the tissue microarray. There were three normal tissues and three cancer tissues in each case. Bottom, magnification of tissues in black frames. Statistical analysis of LOXL2 expression in tissue microarray is shown in supplementary Figure 1A (available at Carcinogenesis Online). (B) Example case that LOXL2 was increased in lymph node metastases compared with primary cancer tissues. There were three cancer tissues and three lymph node metastases tissues in each case. Bottom, magnification of tissues in black frames. Statistical analysis of LOXL2 expression in tissue microarray is shown in supplementary Figure 1B (available at Carcinogenesis Online). (C) Kaplan–Meier analysis shows subjects with high LOXL2 expression level have greater risk for death. Primary gastric cancer tissues with moderate to strong LOXL2 expression (score = 2 and 3) were classified as LOXL2 positive. Patients with LOXL2-positive tumors (n = 131) had significantly worse survival than did those with LOXL2-negative/weak tumors (n = 143; P < 0.01, log-rank test).
LOXL2 positively regulates tumor cell invasion and metastasis. (A) Top, western blot analysis after stable transfection of shRNA plasmid. Bottom, transwell invasion assays (left) and adhesion assays (right). (B) Progression of LOXL2 knockdown cells and control cells after subcutaneous injection. Top, growth curve \((n=6)\). Bottom, tumor burden 7 weeks postinjection \((n=6)\). (C) Hematoxylin and eosin (HE) staining panels show LOXL2 knockdown significantly inhibits xenograft tumor invasion into surrounding tissue. Arrows indicate area of muscular invasion. (D) Lung metastasis of each group at 7 weeks after subcutaneous injection. Statistical plot shows the metastasis loci counted on the whole lung surface per mouse. (E) Left, western blot analysis after stable transfection of LOXL2 expression vector. Right, transwell invasion assays. Bars, standard error; *\(P<0.05\).
Secreted LOXL2 promotes invasion in PAMC82-P3 and BGC823 cells via the Src/FAK pathway but not the Snail/E-cadherin pathway. LOXL2 has both intracellular and secreted forms. A previous report demonstrated that LOXL2 could intracellularly interact with Snail to downregulate E-cadherin expression and then induces invasive behavior in cancer cells (18). We examined Snail and E-cadherin expression in PAMC82-P3 and BGC823 cells and observed that RNAi of LOXL2 led to a decrease in Snail expression and an increase in E-cadherin expression, whereas anti-LOXL2 antibody treatment had no such effects (Figure 5A). Therefore, we hypothesize that secreted LOXL2...
Fig. 5. Secreted LOXL2 promotes invasion by activating Src and FAK. (A) Effects on protein expression mediated by LOXL2 knockdown and anti-LOXL2 antibody (100 μg/ml) treatment in PAMC82-P3 and BGC823 cells. (B) Effects on protein expression (top) and invasion (bottom) mediated by rhLOXL2 or anti-LOXL2 antibody addition in BGC823 cells. Tumor cells were cultured in 10% serum until 50–70% confluent and then serum starved overnight. Western blot and invasion assays were measured 24 h after incubation with 3 μg/ml rhLOXL2 or 12 h after incubation with high dose of anti-LOXL2 antibody. (C) Effects on
may promote gastric cancer cell invasion and metastasis via a signaling pathway rather than N-cadherin.

The Src/FAK pathway has been reported to play a key role in tumor metastasis (28). We observed that treatment of PAMC82-P3 and BGC823 cells with the anti-LOXL2 antibody did not lead to a change in FAK expression. However, the Tyr418 phosphorylation of FAK markedly decreased when the secreted LOXL2 was neutralized by the anti-LOXL2 antibody. Additionally, RNAi of LOXL2 gave rise to a decrease in Tyr418 phosphorylation of FAK relative to shEGFP-transfected control (Figure 5A). Because it has been reported that phosphorylation of FAK at Tyr418 could be induced by Src (29), we examined the Src expression and activity for its role in the LOXL2-signaling pathway. Using the phosphorylation of Tyr418 as an indicator of Src activity, we observed that Src activation in anti-LOXL2 antibody-treated PAMC82-P3 and BGC823 cells was reduced as compared with untreated or IgG-treated control cells (Figure 5A). RNAi of LOXL2 consistently gave rise to a decrease in Src phosphorylation compared with shEGFP-transfected control. However, Src protein expression was not affected by anti-LOXL2 antibody treatment or LOXL2 knockdown (Figure 5A).

In addition, we also treated LOXL2-expressing gastric carcinoma N87 cells with 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine (PP2) (Src kinase inhibitor), which led to a significant reduction in invasion by these N87 cells (Figure 5B). Moreover, the presence of recombinant human lysyl oxidase-like 2 (rhLOXL2) increased Src phosphorylation and the invasion potentials of N87 and BGC823-shLOXL2-1# cells. And the rhLOXL2-induced invasion and Src activation could both be suppressed by anti-LOXL2 antibody or PP2 (Figure 5B and C). To further demonstrate our conclusion that secreted LOXL2 acts through Src but not Snail pathway, we have also shown that under Snail knockdown condition, LOXL2 still can regulate cancer cell invasion (Figure 5D).

To explore whether LOXL2 affects Src phosphorylation and Snail expression in human malignant tissues, we examined Src (p-Tyr418) and Snail by immunohistochemical assay in 60 samples of primary gastric carcinoma tissues. The Spearman’s correlation coefficient between LOXL2 expression and the expression of Src (p-Tyr418) and Snail were 0.677 (P < 0.001) and 0.077 (P = 0.558), respectively (supplementary Table S1 and Figure S9 are available at Carcinogenesis Online). These data indicate that LOXL2-mediated metastasis in gastric tumors is via upregulation of Src phosphorylation.

**Secreted LOXL2 regulates Src activation is hydrogen peroxide dependent**

LOXL2 produces hydrogen peroxide (H2O2) on regeneration of catalytic activity (6,7) and it has recently been shown that the presence of H2O2 can promote Src activation (11). Therefore, we investigated whether secreted LOXL2 regulates Src activation through H2O2. We found that treatment of the N87 and BGC823-shLOXL2-1# cells with H2O2 led to an increase in Src activation (Figure 5E). Moreover, we also demonstrated that Src activation of N87 and BGC823-shLOXL2-1# cells were also increased by the addition of rhLOXL2, respectively. And the rhLOXL2-induced Src activation in both cell lines could be suppressed by treatment with catalase (which catalyzes the decomposition of H2O2 into molecular oxygen and water) (Figure 5E). These data suggested that secreted LOXL2 regulates Src activation is H2O2 dependent.

**Discussion**

Metastasis is a multifactorial process, including tumor cells capable of escaping their normal microenvironment, traversing into and out of lymphatic or blood vessels and proliferating in new ‘soil’ (30). Implicit in these stages, invasion is the critical ability for tumor cells to metastasis (30). During invasion, malignant cells reside on or within two major types of extracellular matrix: the basement membrane or the stromal matrix (31). Basement membrane is one of the most important barriers against cancer cell invasion (31). Therefore, for this study, we used BD Matrigel, a solubilized base membrane preparation from the Engelbreth-Holm-Swarm mouse sarcoma, as a model basement matrix to mimic gastric carcinoma invasion in vivo. Using a transwell chamber, we isolated the highly invasive subpopulation PAMC82-P3 from parent cell line PAMC82. This in vitro selection provides a useful approach to isolate cell variants with different potentials of invasion and metastasis.

Microarray analysis was used to determine which genes might be involved in invasion. LOXL2 is one of the most interesting genes shown by the microarray data to be dysregulated. LOXL2 belongs to an amine oxidase family whose members have been implicated in cross-link formation in stromal collagens and elastin, cell motility and tumor progression (32). So far, few studies on the expression of LOXL2 in human tumor samples are available (33). Studies have reported LOXL2 upregulation in colon and esophageal tumors (17), oral squamous cell carcinomas (33) and laryngeal squamous cell carcinomas (33), although another study reported the decreased expression of LOXL2 in head and neck squamous cell carcinomas and ovarian tumors (14,15). The expression of LOXL2 in gastric cancer remained unclear. In the current study, we showed that LOXL2 is significantly overexpressed in gastric tumor tissue and that this expression is significantly correlated with tumor invasion, lymph node metastasis and tumor-node-metastasis stage, indicating that LOXL2 is involved in gastric carcinoma invasion and metastasis. In addition, multivariable Cox hazards regression analysis showed that LOXL2 was an independent prognostic factor and might provide additional prognostic information that is not discernible with current clinical and pathological parameters alone. This finding confirmed the observation in previous studies that LOXL2 is implicated in the tumor progression (18).

Akiri et al. have reported that LOXL2 could promote tumor fibrosis and tumor progression (16). However, biological evidence based on in vitro and in vivo experiments has not yet clarified the relationship between LOXL2 and cancer metastasis. In the current study, to our knowledge for the first time, we have shown that LOXL2 positively regulates invasion and adhesion of gastric cancer cells in vitro and induced a metastatic phenotype in vivo. LOXL2 is a copper-dependent enzyme known to function both intracellularly and extracellularly (9,17). A recent study has revealed that intracellular LOXL2 could interact with Snail to prevent Snail degradation and then promote tumor progression (18). Whether the secreted LOXL2 can function to promote tumor invasion and metastasis remains unclear. In our studies, the increasing concentrations of our laboratory made anti-LOXL2 antibody led to a dose-dependent decrease in invasion and adhesion. Those results shown for the first time that secreted LOXL2 play an important role in tumor invasion and metastasis and targeting secreted LOXL2 might be an effective strategy for suppressing gastric tumor metastasis. Therefore, what we are most interested in is whether secreted LOXL2 is a valuable therapeutic target. To evaluate the therapeutic usefulness of inhibiting LOXL2, animals were dosed with the anti-LOXL2 antibody. Treatment of mice with increasing concentrations of antibody led to a dose-dependent inhibition in tumor growth. Furthermore, mice treated with a high dose of the anti-LOXL2 antibody displayed significantly fewer lung metastases. Taken together, these promising results support further development of anti-LOXL2 antibody for the therapy of human gastric carcinoma metastasis.
However, the molecular mechanism by which secreted LOXL2 regulates cell invasion and adhesion remains unclear. RNAi of LOXL2 led to an increase in E-cadherin expression, confirming that intracellular LOXL2 can interact with Snail to influence cellular behavior (18). However, this does not explain how an anti-LOXL2 antibody can decrease gastric cancer cell metastasis in vitro and in vivo as intracellular LOXL2 is inaccessible to the antibody, which cannot penetrate an intact cell membrane. In concordance with the hypothesis, we found that Snail and E-cadherin expressions were not affected by anti-LOXL2 antibody treatment. LOXL2 produces H₂O₂ on regeneration of catalytic activity and it has recently been shown that the presence of H₂O₂ can promote Src and FAK activation. Furthermore, FAK has been previously shown to play an important role in adhesion formation and invasion (28,34). Thus, secreted LOXL2 may promote cell invasion and adhesion via activation of the Src/FAK pathway. To test this hypothesis, we studied FAK expression and activation in cells after LOXL2 RNAi- or antibody-mediated LOXL2 neutralization and found that phosphorylation of Tyr⁷⁷⁶ in FAK was downregulated. Then, we measured the effect of LOXL2 modulation on Src activity and observed that Src activation was suppressed in PAMC82-P3, BGC823 and LOXL2-ectopic expression N87 cells, which were treated with the anti-LOXL2 antibody. RNAi of LOXL2 gave a consistent decrease in Src phosphorylation. The closely correlated LOXL2 expression and Src (p-Tyr⁴¹⁸) levels was further evaluated by immunohistochemical assay in 60 gastric cancer tissues. However, LOXL2 overexpression failed to correlate with Snai expression in the gastric cancer tissues. Finally, we found that secreted LOXL2 regulates Src activation is H₂O₂ dependent.

In summary, we have established a gastric carcinoma invasion model: a highly invasive subline of tumor cells was established in which LOXL2 is overexpressed. Further investigation revealed that LOXL2 was significantly correlated with gastric cancer invasion and metastasis and was a valuable prognostic marker. Our studies have demonstrated that secreted LOXL2 promotes this invasive phenotype via the Src/FAK pathway. Furthermore, anti-LOXL2 antibody therapy showed that LOXL2 is a valuable therapeutic target for preventing and treating gastric cancer metastases.

Supplementary material
Supplementary Figures S1–S9 and Table S1 can be found at http://carcin.oxfordjournals.org/

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