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Lysyl Oxidase: A Lung Adenocarcinoma Biomarker of Invasion and Survival

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BACKGROUND: Lung adenocarcinoma invasion and metastasis arises from autocrine and paracrine signaling events between tumor epithelial cells and the stromal microenvironment that is mediated in part by transforming growth factor-β (TGF-β) signaling. The copper-dependent amine oxidase lysyl oxidase (LOX) plays a role in extracellular matrix structure and is up-regulated in invasive type II TGF-β receptor-deficient cells. The authors hypothesized that LOX expression is associated with extent of invasion and survival in patients with lung adenocarcinoma. **METHODS:** LOX immunohistochemical staining was examined in 166 surgically resected lung adenocarcinomas and results were correlated with clinicopathological features and survival. **RESULTS:** High-intensity LOX staining was found to be associated with the linear extent of invasion (Spearman correlation coefficient = 0.2; $P = .01$). There was an association between high LOX staining and decreased 5-year survival observed within the entire cohort (log-rank $P < .001$) and among the patients with stage I disease (n = 119; $P < .001$). Cox proportional hazards regression analysis confirmed that LOX was a significant prognostic indicator of increased risk of 5-year mortality for all patients (hazard ratio [HR], 2.55; 95% confidence interval [95% CI], 1.51-4.30 [$P < .001$]) and for patients with Stage I disease (HR, 3.51; 95% CI, 1.77-6.99 [$P < .001$]). LOX expression was found to be independently associated with risk of death after adjustment for relevant covariates (HR, 2.29; 95% CI, 1.33-3.94 [$P = .003$]). **CONCLUSIONS:** Higher expression of LOX is associated with invasion and is an independent predictor of poor prognosis in patients with early stage lung adenocarcinoma. ***Cancer*** **2011;117:2186–91.** *© 2010 American Cancer Society.*

KEYWORDS: lysyl oxidase, lung adenocarcinoma, invasion, transforming growth factor-β, stroma.

Lung cancer is the second most common cancer in the United States and the leading cause of cancer death, with 219,440 new cases and 159,390 deaths reported in 2009.[1] The overall 5-year survival rate correlates with stage at diagnosis, and ranges from 49% for localized disease to 2% for metastatic disease.[2] The standard of care for patients with early stage lung cancer is surgical resection, with consideration of adjuvant chemotherapy for patients with stage IIA, stage IIB, and selected stage IB tumors.[3] However, approximately half of resected tumors recur with metastatic disease within 5 years, suggesting that tumor cell invasion, dissemination, and micrometastases occur before surgical resection and that the TNM classification alone is insufficient to definitively predict which resected tumors are more likely to recur with metastatic disease.[4] Recent research has been directed toward identifying and validating pathological and molecular markers that can refine lung cancer prognostic assessment and guide patient selection for resection and adjuvant chemotherapy treatment.

The most common histologic type of lung cancer, adenocarcinoma, is a heterogeneous group of tumors, ranging in aggressiveness from the noninvasive bronchioloalveolar cancer (BAC) to microinvasive tumors, mixed-type tumors, and pure invasive adenocarcinoma.[5,6] The histological distinction between BAC and other adenocarcinomas is tissue invasion, an initial and required step for tumor metastasis that is absent in BAC. The mean 5-year survival rate for patients with stage IA BAC is 100%, versus 59% for patients with other stage IA adenocarcinomas.[7] Recent clinical reports have suggested that the prognosis and radiographic appearance of BAC is unique, and may support modifying the clinical approach to lung adenocarcinomas according to invasive subclass. Molecular analysis has identified down-regulation of transforming growth factor-β (TGF-β) receptor II (TGFβRII) expression as an early important event in progression of adenocarcinomas of the lung and other sites.[8-10] Among potential downstream mediators of invasion in TGFβRII

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repressed lung adenocarcinoma cells, we identified overexpression of the genes encoding lysyl oxidase (LOX), its family member LOXL2, and the chemokine CCL5.[8,11]

LOX is a copper-dependent amine oxidase encoded by members of a 5-gene family that includes LOX and 4 LOX-like proteins (LOXL1-4). LOX is synthesized as a 48-kilodalton (kD) pre-pro-enzyme and then N-glycosylated and secreted from the cell as a 50-kD pro-enzyme. The pro-enzyme is cleaved extracellularly by bone morphogenetic protein-1 (BMP-1) into the mature 32-kD LOX protein and an 18-kD pro-peptide (LOX-PP). The enzyme covalently cross-links collagen and elastin in the extracellular matrix, thereby increasing tensile strength and maintaining tissue integrity.[12] LOX up-regulation occurs in the setting of fibrosis (eg, in scleroderma and cirrhosis), whereas congenital deficiency of LOX results in connective tissue disease, including Menkes disease and cutis laxa.[13,14]

As a component of the extracellular matrix, LOX plays a role in facilitating tumor-stromal interactions that are important for tumor progression and metastasis. Recent studies have shown that LOX mRNA expression is increased in tumors or malignant cell lines of the breast, central nervous system, head and neck, and prostate; renal cell; melanoma; and osteosarcoma, as summarized in Payne et al.[14] To our knowledge, the role of LOX in lung adenocarcinoma progression has not previously been examined. In the current study, we assessed LOX expression in a cohort of lung adenocarcinomas to determine its association with invasion and survival.

MATERIALS AND METHODS

Tissue was obtained from the surgical specimens of 178 consecutive patients who underwent anatomic resection of lung adenocarcinomas at New York-Presbyterian Hospital/Columbia University Medical Center between 1997 and 2000. The specimens were collected according to an approved Columbia University Medical Center Institutional Review Board. The pathologic stage and linear extent of invasion for each specimen was determined by a panel of 3 surgical pathologists.[6] Tumors were restaged using the seventh edition of the American Joint Committee on Cancer staging manual.[15] The patients had a minimum of 5 years of clinical follow-up including vital status, which was confirmed by personal contact with each patient's physician and by review of the Social Security Death Index. Complete follow-up information was available for 173 of 178 (97%) of patients.

Tumor tissues were fixed in formalin and embedded in paraffin in a tissue microarray (TMA) conformation (Beecher, Sun Prairie, Wis). Lung tumor TMAs were constructed using two 1-mm diameter cores from 2 different areas of each tumor (total of 4 cores for each tumor) and the blocks were sectioned at 5-μm thickness. For immunohistochemistry, the blocks were deparaffinized in xylene, rehydrated through a graded ethanol series, and washed in phosphate-buffered saline. Antigen retrieval was performed in 10 mM of citrate buffer at pH 6.0 for 40 minutes. The specimens were incubated with primary antibody or negative control antibody followed by biotinylated linking antibody and streptavidin peroxidase. Slides were stained with chromagen and counterstained with hematoxylin. Rabbit anti-LOX (a polyclonal antibody against rat LOX peptides 293-309, kindly provided by Dr. Herbert Kagan, Boston University, Boston, Mass) was used as the primary antibody; LOX antibodies are currently commercially available from several sources. This antibody binds 50-kD pro-LOX and 32-kD mature LOX but not the isolated LOX-pro–peptide, which is cleaved between Gly162 and Asp163.[12] Negative controls were performed with antirabbit immunoglobulin G antibody (Dako Corporation, Carpinteria, Calif). Prostate epithelium/stroma was used as a positive control.[16] LOX immunostaining in tumor cell cytoplasm was scored as negative (0) or positive on a scale of 1 (faint) to 3 (strong). The pattern was predominantly cytoplasmic, with rare nuclear staining noted in tumors with strong cytoplasmic staining, as has been reported by others.[17,18] In positive cases, staining was uniform throughout the tumor cores; thus, an assessment of percent staining within tumors was not performed. For statistical analysis, the results were grouped into low (0-1, absent or faint staining) or high (2-3, moderate or strong staining). Tumor cells and stromal components (extracellular matrix, fibroblasts, and endothelial cells) were scored separately. LOX immunostaining in nonmalignant lung tissue was generally negative, with rare faint staining observed in alveolar type II cells and intra-alveolar macrophages. All samples were scored by consensus between 2 readers blinded to clinical outcome and extent of invasion.

Statistical Analysis

Statistical analysis was performed using SPSS statistical software (version 14.0; SPSS Inc, Chicago, Ill). Correlation was assessed with the Spearman rho correlation coefficient and 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 values $< .05$ were considered statistically significant.

Table 1. Patient Demographics and Histology

Demographics	All Patients N=166	Stage I Patients n=119
Age, mean ± SD, y	66 ± 9	67 ± 9
Female	100 (60%)	79 (66%)
Tumor size, mean ± SD, cm	2.8 ± 1.5	2.8 ± 1.8
5-y survival rate	64%	72%
Pathologic stage		
IA	73 (44%)	73 (61%)
IB	46 (28%)	46 (39%)
IIA	11 (6%)	
IIB	13 (8%)	
IIIA	18 (11%)	
IIIB	3 (2%)	
IV	2 (1%)	
Tumor invasive subclass		
Pure BAC	10 (6%)	10 (8%)
Microinvasive (<6 mm invasion)	22 (13%)	22 (19%)
Mixed-type (≥6 mm invasion)	79 (48%)	60 (50%)
Invasive	55 (33%)	27 (23%)
LOX immunostaining		
0 (low)	32 (19%)	27 (22%)
1 (low)	94 (57%)	63 (53%)
2 (high)	37 (22%)	27 (23%)
3 (high)	3 (2%)	2 (2%)

SD indicates standard deviation; BAC, bronchioloalveolar cancer; LOX, lysyl oxidase.

RESULTS

Of the 178 specimens available, 7 were excluded because of lack of tumor tissue on the microarray or technically inadequate immunostaining, and 5 specimens were excluded because of loss to follow-up; 166 specimens were included in the statistical analysis. Clinical parameters of the entire cohort and the subgroup of patients with stage I disease are summarized in Table 1. The 5-year survival rate for the cohort was 64%. We detected high LOX expression in 40 of 166 specimens (24%) (Figure 1). High LOX tumor cell expression increased with invasiveness, from none of the BACs to 14% of microinvasive tumors, 18% of mixed-type tumors, and 38% of invasive tumors (Pearson chi-square $P = .01$) (Table 2). The mean maximal linear extent of invasion was 1.82 cm for specimens with low LOX expression and 2.62 cm for specimens with high LOX expression. High tumor LOX expression was positively correlated with invasion (Spearman correlation coefficient [r] = 0.2; $P = .01$). In the stroma, LOX staining was absent in fibroblasts and the extracellular matrix, and was faintly positive in endothelial cells in 45% of cases. Stromal staining was not associated with invasion or survival.

LOX expression in tumor cells was significantly associated with 5-year survival (71% for low LOX expression vs 43% for high expression; log-rank $P < .001$) (Figure 2). Univariate Cox proportional hazards regression analysis confirmed an association between higher LOX expression and an increased risk of death at 5 years (hazard ratio [HR], 2.55, 95% confidence interval [95% CI], 1.51-4.30 [$P < .001$]). A multivariate regression model was generated using relevant covariates (age, gender, stage of disease, and invasive size), and high LOX expression remained a predictor of poor 5-year survival (HR, 2.29; 95% CI, 1.33-3.94 [$P = .003$]) (Table 3).

We examined the association between LOX expression and survival when the analysis was restricted to stage I tumors (n = 119). Twenty-nine (24%) of the stage I tumors demonstrated high LOX expression. LOX expression was associated with 5-year survival in the subgroup of patients with stage 1 disease (80% for low LOX expression vs 48% for high expression; log-rank $P < .001$) (Figure 3). Cox proportional hazards regression indicated that higher LOX expression in patients with stage I tumors correlated with an increased risk of death at 5 years (HR, 3.51; 95% CI, 1.77-6.99 [$P < .001$]). On multivariate regression analysis, after accounting for age, gender, stage of disease (IA vs IB), and invasive size, LOX expression was found to be a significant independent predictor of 5-year mortality (HR, 3.18; 95% CI, 1.52-6.65 [$P = .002$]) (Table 4).

DISCUSSION

Signaling between the stroma and epithelium, via direct contact and secreted mediators, is essential for tissue growth and differentiation and for tumor initiation and disease progression. Malignant transformation in the epithelium is accompanied by alterations in the stroma, characterized by increased collagen production, disorganized growth, and exuberant proliferation.[19] Distinct changes in gene expression occur within the stromal compartment of a tumor, such that gene sets generated from the stromal compartment are predictive of outcome in patients with breast and digestive cancers.[20,21] TGF-β plays a major role in tumor-stromal interactions. Excessive TGF-β activity is present in stromal, inflammatory, and cancer cells within a tumor, and the metastatic phenotype can develop when the epithelium overcomes the growth-inhibitory

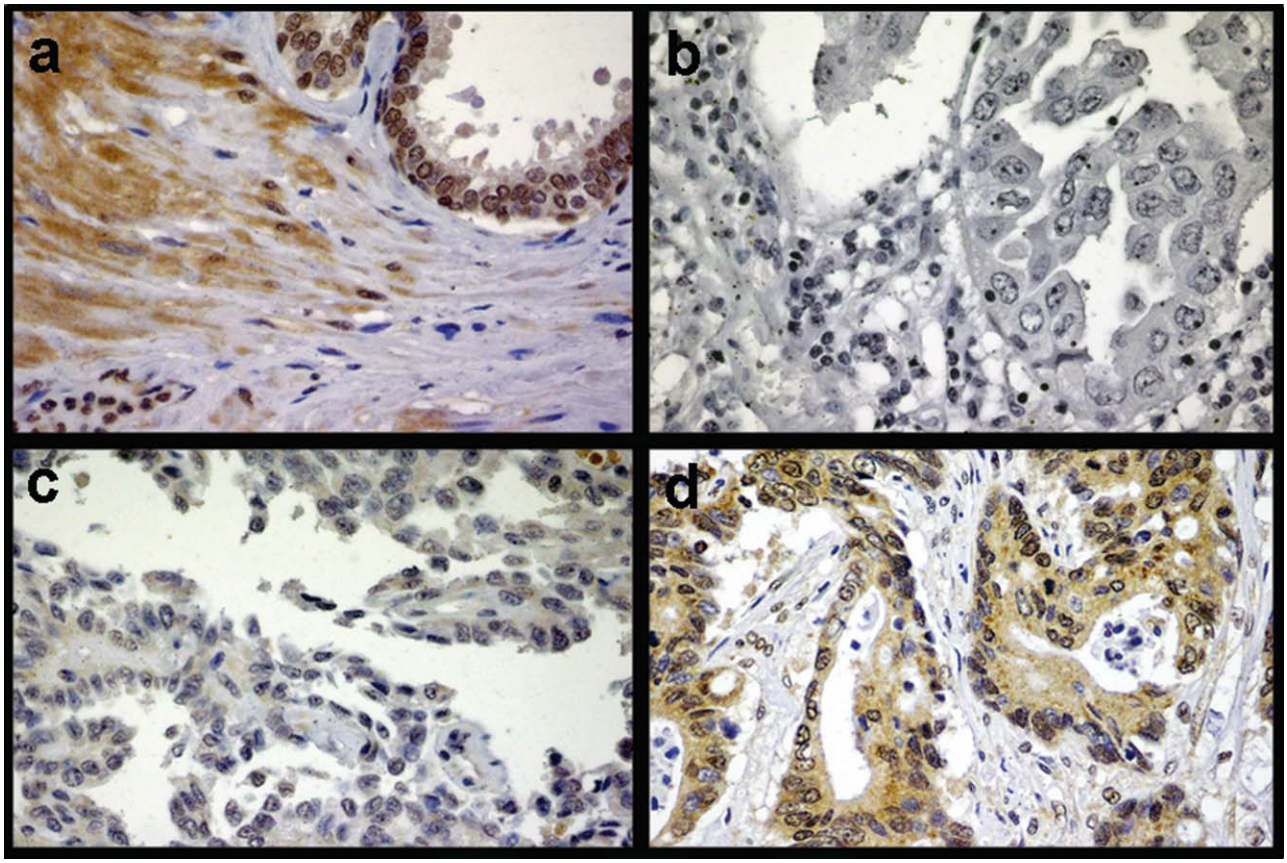

Figure 1. Lysyl oxidase (LOX) immunostaining is shown in lung adenocarcinoma and controls. (a) Positive control prostate epithelium is shown. (b) Negative control (antirabbit immunoglobulin G) lung adenocarcinoma is shown. (c) Low LOX immunostaining is shown in mixed-type lung adenocarcinoma. (d) High LOX immunostaining is shown in invasive lung adenocarcinoma (magnification ×150).

Table 2. Tumor LOX Expression Is Associated With Invasive Subclass

Tumor	Low LOX Staining, No. of Cases (% Each Subclass)	High LOX Staining, No. of Cases (% Each Subclass)
Pure BAC	10 (100%)	0 (0%)
Microinvasive	19 (86%)	3 (14%)
Mixed-type	65 (82%)	14 (18%)
Invasive	34 (62%)	21 (38%)

LOX indicates lysyl oxidase; BAC, bronchioloalveolar cancer.

effect of TGF-β.[22] Consistent with this, repression of TGFβRII leads to an invasive phenotype in multiple cancer types, including those of the lung, breast, and colon.[8-10] We detected up-regulation of LOX gene expression in TGFβRII knockdown lung adenocarcinoma cells.[8] In the current study, we demonstrated that LOX expression is associated with lung adenocarcinoma progression as measured by invasiveness, and with survival.

Recent reports have indicated that the mature LOX enzyme mediates tumor progression both in a tumor cell autonomous fashion and in the tumor microenvironment. An increase in LOX or LOXL expression, as measured by mRNA, protein, and/or enzymatic activity, is associated with tumor progression and poorer survival in patients with breast, esophageal, prostate, renal cell, and head and neck cancers.[14,21-25] Inhibition of LOX under hypoxic conditions was reported to reduce tumor invasion and metastases, without any change in primary tumor growth, in a murine model of breast cancer.[23] In addition to local effects on invasion, secreted LOX can circulate to distant sites and contribute to the formation of the premetastatic niche by stimulating collagen cross-linking in the extracellular matrix of a target organ and promoting the adherence and invasion of circulating tumor cells and bone marrow-derived cells that stimulate angiogenesis.[24] It is interesting to note that our results demonstrate a correlation between invasion and tumor cell LOX expression but no association between invasion and extracellular or

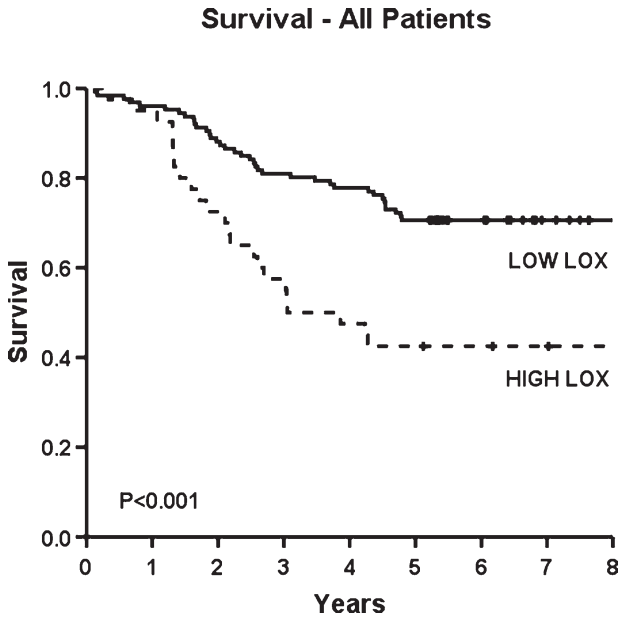

Figure 2. Survival for all patients is shown. High lysyl oxidase (LOX) expression is associated with increased 5-year mortality (log-rank $P < .001$).

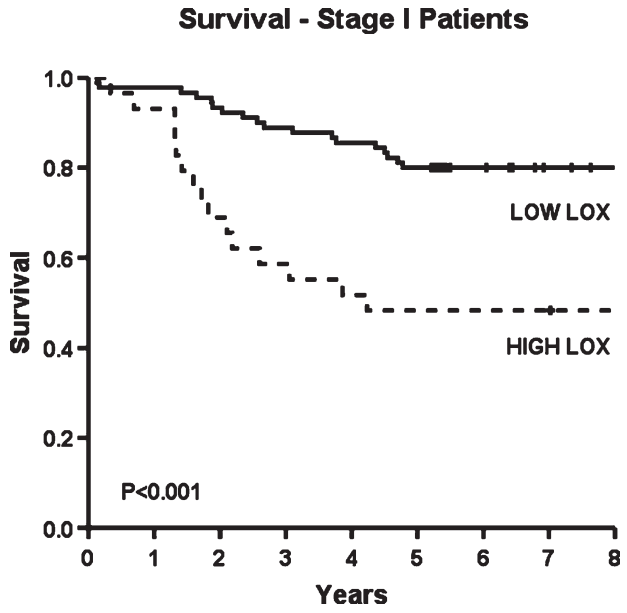

Figure 3. Survival for patients with stage I disease is shown. High lysyl oxidase (LOX) expression is associated with increased 5-year mortality in patients with stage I lung adenocarcinoma (log-rank $P < .001$).

Table 3. Five-Year Survival for Lung Adenocarcinoma: Effect of Clinical and Pathologic Factors

Factor	Univariate Analysis, HR (95% CI)	Multivariate Analysis, HR (95% CI)
LOX staining	2.55 (1.51-4.30) $P < .001$	2.29 (1.33-3.94) $P = .003$
Age	1.03 (1.00-1.06) $P = .036$	1.04 (1.02-1.07) $P = .002$
Female gender	NS	NS
Stage	1.55 (1.19-2.04) $P = .001$	1.65 (1.22-2.24) $P = .001$
Invasive size	1.23 (1.08-1.40) $P = .002$	1.16 (1.00-1.35) $P = .046$

HR indicates hazard ratio; 95% CI, 95% confidence interval; LOX, lysyl oxidase; NS, not significant.

Table 4. Five-Year Survival for Stage I Lung Adenocarcinoma: Effect of Clinical and Pathologic Factors

Factor	Univariate Analysis, HR (95% CI)	Multivariate Analysis, HR (95% CI)
LOX staining	3.51 (1.77-6.99) $P < .001$	3.18 (1.52-6.65) $P = .002$
Age	1.05 (1.01-1.10) $P = .014$	1.05 (1.01-1.09) $P = .024$
Female gender	NS	NS
Stage (IA vs IB)	4.00 (1.94-8.27) $P < .001$	3.77 (1.62-8.74) $P = .002$
Invasive size	1.21 (1.02-1.43) $P = .025$	NS

HR indicates hazard ratio; 95% CI, 95% confidence interval; LOX, lysyl oxidase; NS, not significant.

stromal compartment LOX protein expression, suggesting that cancer intracellular expression is representative of LOX activity in the tumor microenvironment and that the intracellular activity of LOX is important for the invasion process.

The results of the current study demonstrated that LOX protein is up-regulated in invasive lung adenocarcinoma and that LOX expression is independently associated with 5-year survival in patients with stage I adenocarcinoma. Taken together, these data indicate LOX tumor cell expression is an important biomarker of invasive morphology and clinical outcome in patients with early lung adenocarcinoma. The correlation between LOX staining and invasion is significant but not absolute because 62% of tumors with low LOX expression were found to have invasion on histology; however, LOX expression may provide important prognostic information that is independent of invasion. For example, of the microinvasive tumors with LOX staining, 2 of 3 patients died within 2 years, suggesting that LOX staining is an indicator of biological aggressiveness in a tumor with an otherwise favorable morphology. Once prospectively validated, these findings have promising clinical application in determining which early stage tumors are most likely to recur and thereby directing adjuvant treatment to those patients who would benefit most. In addition, LOX is an attractive target for personalized therapy. β-Aminoproprionitrile, an irreversible specific inhibitor

of LOX catalytic activity, has been shown to decrease metastases in a murine model of circulating breast cancer cells,[25] and other more selective therapies are currently in development.[26] Further investigation will be necessary to determine whether LOX inhibitors have potential as an adjunct to lung cancer therapy, particularly in those patients who demonstrate high expression of LOX in their tumors.

CONFLICT OF INTEREST DISCLOSURES

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