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Differential expression of the *LOX* family genes in human colorectal adenocarcinomas

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Abstract. Lysyl oxidase (LOX) is an amine oxidase that catalyzes the cross-linking of collage or elastin in the extracellular matrix, regulating the tensile strength and structural integrity of connective tissues. Recently, four paralogues (*LOXL*, *LOXL2*, *LOXL3* and *LOXL4*) of *LOX* have been identified in humans, each containing the functional domains required for the amine oxidase activity toward collagen and elastin. Paradoxical roles of the LOX family members have been reported in various neoplastic tissues as tumor suppressors or promoters depending on tumor status and type. To address expression of the *LOX* family genes in colorectal adenocarcinomas, we performed real-time PCR analysis with matched tumor/normal tissue specimens from 104 patients. The expression of the *LOX* family genes was not statistically associated with tumor location, stage, growth type, or differentiation status. However, upregulation of *LOX*, *LOXL2* and *LOXL4* was significantly correlated with absence of lymphovascular invasion (P=0.012, 0.014 and 0.005, respectively), suggesting that the oxygen tension in or around the tumors may be an important regulator for the differential expression of *LOX*, *LOXL2* and *LOXL4* in colorectal cancer. Additionally, expression of *LOX*, but not the other *LOX* family genes, was significantly upregulated in patients with a diffuse cytoplasmic expression pattern of CEA, indicating that *LOX* upregulation may be associated with increased invasiveness and metastatic potential in colorectal cancer.

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Introduction

Lysyl oxidase (LOX) is a copper-dependent amine oxidase responsible for the development of lysine-derived cross-linkages in collagen and elastin in the extracellular matrices. LOX oxidatively deaminates the ε amino groups of peptidyl lysine residues to α-aminoadipic δ-semialdehydes, and then the resulting aldehyde groups undergo spontaneous condensation with unreacted ε amino groups or neighboring aldehyde groups to form cross-linkages within collagen and elastin fibers (reviewed in ref. 1). The LOX-mediated cross-linking of collagen and elastin is an essential step in maintaining structural integrity of connective tissues.

Four lysyl oxidase-like genes, *LOXL*, *LOXL2*, *LOXL3* and *LOXL4*, have been identified in humans (2-5). The copper binding domain and the lysyl-tyrosyl-quinone (LTQ) residues that are required for the amine oxidase activity are strictly conserved at the C-terminus of all LOX family members. In addition to amine oxidase activity toward collagen and elastin, LOX has also been noted for several novel functions in tumorigenesis, cellular senescence and chemotaxis (reviewed in ref. 6). *Lox* expression was greatly downregulated in NIH 3T3 cells transformed by *H-RAS* but recovered in interferon-mediated revertants (7,8). Blocking *Lox* expression led to contact inhibition, inability to proliferate in soft agar and tumor formation in nude mice, proposing a possible role of Lox as a tumor suppressor (7,8). Additionally, increased LOX levels were detected at the invasion front adjacent to the infiltrating tumor cells in immunohistochemical analysis of breast and oral carcinoma, suggesting a defense mechanism by LOX against tumor infiltration (9).

However, perplexing expression patterns of *LOX* and its human paralogues have been observed in various neoplastic tissues and cells. *LOX* upregulation was detected in breast and renal cell carcinomas (10,11), while *LOX* downregulation was observed in prostate, bronchogenic, gastric, and head and neck squamous cell carcinomas (HNSCC) (12-15). *LOXL2* expression was also downregulated in HNSCC (15) but upregulated in colon and esophageal and breast carcinomas

(16,17). In addition, *LOXL3* and *LOXL4* showed upregulation in breast cancer and HNSCC, respectively (17,18). Of particular interest, upregulation of the *LOX* family genes have been reported in more invasive and metastatic tumors. The expression of *LOX* and *LOXL2* was upregulated in highly invasive metastatic cells of breast carcinoma, while little to no expression was detected in non-metastatic cells of breast carcinoma (19). *LOXL4* showed higher expression in invasive HNSCC tumors than primary ones (20). Additionally, *LOXL2* showed enhanced immunostaining in the cytoplasm or perinuclear areas of laryngeal squamous cell carcinoma (21). Further, the enhanced expression of *LOXL2* was significantly correlated with increased local recurrence and decreased survival (21). These findings, taken together, suggest that the *LOX* family members may play paradoxical roles in suppression or progression of tumorigenesis depending on tumor status and type.

The purpose of this study was to explore the pathological relevance of the *LOX* family genes to clinical parameters of cancer by assessing expression of *LOX* and its paralogues in matched tumor/normal tissues from patients with colorectal adenocarcinoma. In our studies, *LOX*, *LOXL2* and *LOXL4* showed statistically significant upregulation in tumors without lymphovascular invasion (LVI), suggesting that the oxygen tension in or around the tumor tissues is an important regulator for the differential expression of these genes. We also report that *LOX*, but not the other *LOX* family members, was significantly upregulated in tumors with diffuse cytoplasmic *CEA* expression compared to those with apicoluminal expression, indicating a possible functional role of LOX in increased invasiveness and metastatic potential.

Materials and methods

Tissue samples. Patients diagnosed with hereditary non-polyposis colorectal cancer or familial adenomatous polyposis and its attenuated variants were excluded, as were those pre-operatively treated with chemoradiotherapy. After informed consent, tissues were acquired from primary tumors (5 cm proximal to the tumor margin) and normal tissues of 104 Korean colorectal adenocarcinoma patients who underwent curative operation at the Asan Medical Center (Seoul, Korea). All the tissue specimens were confirmed by histologic identification. Of these patients, 58 were male and 46 were female, with a mean age of 60 years (range: 33-82 years). Among the patients, 28 had cancers of the right colon (cecum-splenic flexure of transverse colon) and 76 had cancers of the left colon (splenic flexure of transverse colon-rectum). Using the tumor staging system of the American Joint Committee on Cancer (AJCC), 28 patients were in stage I, 37 were in stage II, 33 patients were in stage III and 6 were in stage IV. The median follow-up period was 19 months (range: 4-31 months). This study was conducted with the approval of the Institutional Review Board for Human Genetic and Genomic Research at the Asan Medical Center in accordance with the Helsinki declaration.

Reverse transcriptase (RT)-PCR. Total RNA was extracted from the matched tumor and normal tissues of the patients, using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Total cDNA was synthesized with 0.5 μg of the extracted RNA, using *M-MLV* Reverse Transcriptase (Promega) and oligo (dT)15 (Promega) in a 20 μl reaction. PCR amplification was performed using *ExTag* polymerase (Takara) with primers specific to each member of the *LOX* family. The primer sequences and annealing temperatures are listed in Table I. The PCR primers were derived from the 3' UTR sequences of the *LOX* family genes, where little sequence conservation was observed among the *LOX* family members. The reaction mixture was subjected to 30 cycles of 94˚C for 20 sec, 55-59˚C for 20 sec and 72˚C for 30 sec with a pre-denaturation at 94˚C for 4 min and a final extension at 72˚C for 7 min. *GAPDH* was used for an internal control. The amplified PCR products were then analyzed by electrophoresis on 2% agarose gels. Relative quantitation of the PCR amplicons was performed using Molecular Imaging Software, version 4.5 (Kodak).

Real-time quantitative PCR. The primers used for RT-PCR were also used for real-time PCR analysis. The TaqMan probes were labeled with a 5' FAM reporter and a 3' BHQ quencher (Integrated DNA Technologies). Real-time PCR amplification was performed in duplicate using the Chromo 4™ real-time system (MJ Research). PCR was performed in a total volume of 10 μl, containing 1 μl of cDNA, 5 μl of 2 x Premix EX Tag™ (Takara), 10 pmole of each primer and a TaqMan probe. The probe sequences are listed in Table I. The reaction mixture was subjected to 35 cycles of 95˚C for 15 sec and 55-59˚C for 60 sec with a pre-denaturation at 95˚C for 15 min. Commercially available *GAPDH* primers and probes (Applied Biosystems) were used for an internal control. A cDNA-free reaction was also included as a negative control in each reaction. The relative expression of the *LOX* family genes was determined using the $\Delta\Delta$Ct method. The Ct value was defined as the threshold PCR cycle at which the amplified PCR product was first detected.

Immunohistochemistry. Paraffin-embedded tissue cores of carcinomas were used for the construction of tissue blocks for tissue microarray using a precision instrument (Beecher Instrument Inc.) Tissue array blocks containing core cylinders were subjected to immune staining with a monoclonal antibody to CEA (American Type Culture Collection) and labeled by the streptavidin-biotin method using the LSAB® kit (Dako). For CEA, apicoluminal localization or lack of staining was interpreted as negative, whereas diffuse cytoplasmic staining was considered positive. All immunohistochemical staining results were confirmed by two separate pathologists.

Statistical methods. Differential expression of *LOX* and its paralogues was compared with levels of various clinical and molecular parameters by cross-table analysis using Fisher's exact test. The potential parameters were verified by multivariate analysis using logistic regression. Numerical values for independent variables were tested by unpaired Student's t-test or ANOVA with least-squares deviation verification. All statistical analyses were performed using SPSS software (ver.13, SPSS Inc.). A P-value <0.05 was considered statistically significant.

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equences of primers and probes used for RT-PCR and real-time PCR.

Gene	Primer or Probe	Sequence	Annealing temperature
LOX	Forward primer	5'-GAACGCTTAAGTCATCATTACTTG-3'	56˚C
	Reverse primer	5'-TGGCTCATTCATTTAGATAATACTGA-3'	
	Probe	5'-TTGCACTACAATTTCAAAAGGAACATG-3'	
LOXL	Forward primer	5'-AATCTCTCCCCTTCCAAAGCAG-3'	57˚C
	Reverse primer	5'-AGCTCTGTGGTATGGCCAAAGT-3'	
	Probe	5'-AAACCACAGGGATTCCGGACGCCA-3'	
LOXL2	Forward primer	5'-GTCTGTGTTTCCTTTGACCCA-3'	55˚C
	Reverse primer	5'-GATTATGACTCCTGTTCCGTTACT-3'	
	Probe	5'-TTGTGATCTCCTTAGATTGCTTCTCCCA-3'	
LOXL3	Forward primer	5'-ATGTCAGCAACCGCACTCAT-3'	57˚C
	Reverse primer	5'-AGTCAGGTGCTGCTCTTTGT-3'	
	Probe	5'-ATGGGAATCTTTGCTCCCAGGCCCA-3'	
LOXL4	Forward primer	5'-AGGGCCTCTGCCAAGGAAATAA-3'	59˚C
	Reverse primer	5'-ATCAAGCAGGAATGGTGGCCTT-3'	
	Probe	5'-TACCTGGCAGGCAGTCTGCTCTCTCT-3'	

Table II. Comparative analysis of the *LOX* family gene expression with clinicopathologic parameters.

Clinicopathological parameters (No. of patients)	Percentage of tumors with a $2^{\Delta\Delta Ct}$ value >1-fold (P-value)				
	LOX	*LOXL*	*LOXL2*	*LOXL3*	*LOXL4*
Location, right vs. left rectum (28/76)	42.9/43.4 (0.57)	42.9/46.1 (0.474)	50/50 (0.587)	46.4/51.3 (0.413)	42.9/42.1 (0.559)
Largest diameter, ≤4 cm vs. >4 cm (39/65)	35.9/47.7 (0.166)	33.3/52.3 (0.046)[a]	43.6/53.8 (0.209)	46.2/52.3 (0.343)	35.9/46.2 (0.206)
AJCC stage, I + II vs. III + IV (53/51)	50.9/35.3 (0.079)	49.1/41.2 (0.271)	54.7/45.1 (0.216)	56.6/43.1 (0.12)	47.2/37.3 (0.205)
Growth type, expanding vs. infiltrative (86/18)	44.2/38.9 (0.443)	47.7/33.3 (0.198)	52.3/38.9 (0.219)	50.0/50.0 (0.602)	45.3/27.8 (0.133)
Differentiation, (91/13) WD or MD vs. PD or mucinous	42.9/46.2 (0.526)	45.1/46.2 (0.585)	49.5/53.8 (0.500)	49.5/53.8 (0.500)	41.8/46.2 (0.495)
Synchronous adenoma, absence vs. presence (64/40)	48.4/35 (0.126)	43.8/47.5 (0.431)	53.1/45.0 (0.273)	53.1/45.0 (0.273)	43.8/40.0 (0.432)
Lymphovascular invasion, absence vs. presence (75/29)	50.7/24.1 (0.012)[a]	50.7/31.0 (0.056)	57.3/31.0 (0.014)[a]	52.0/44.8 (0.331)	50.7/20.7 (0.005)[a]

[a]Statistically significant p-values; WD, well differentiated; MD, moderately differentiated and PD, poorly differentiated.

Results

Expression of the LOX family genes in colorectal tumors. Expression of the *LOX* family genes was assessed by the comparative ΔΔCt method in which ΔCt was defined as a difference in the Ct values between one of the *LOX* family genes and the *GAPDH* control [$\Delta Ct = (Ct)_{LOX} - (Ct)_{GAPDH}$]. ΔΔCt was defined as a difference in the ΔCt values between a normal tissue and a tumor tissue of the same patient [$\Delta\Delta Ct = (\Delta Ct)_{normal} - (\Delta Ct)_{tumor}$]. The N-fold difference in the expression of the *LOX* family genes was expressed as $2^{\Delta\Delta Ct}$. $2^{\Delta\Delta Ct}$ values over 1-fold indicate upregulation of the tested *LOX* family gene in a tumor tissue compared to a normal counterpart of the same patient. Mean values of $2^{\Delta\Delta Ct}$ were 2.79±0.69 (mean ± SEM) for *LOX*, 2.88±0.65 for *LOXL*, 5.99±1.74 for *LOXL2*, 5.70±1.6 for *LOXL3* and 4.21±0.86 for *LOXL4*. The $2^{\Delta\Delta Ct}$ values of all the *LOX* family genes were closely correlated between any two of them (R=0.494-0.959,

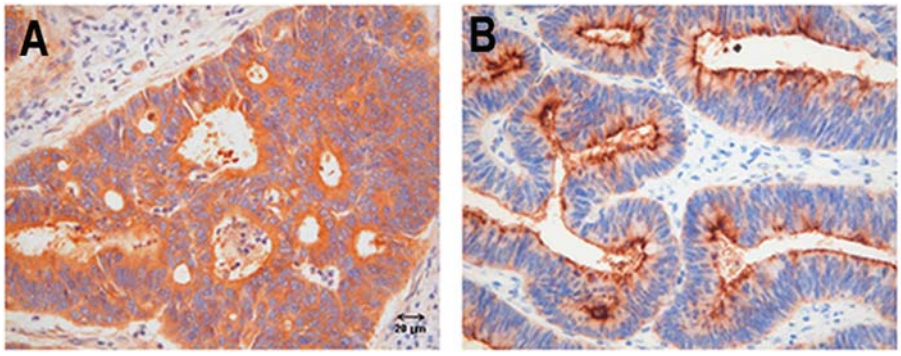

Figure 1. Typical examples of immunohistochemical staining with CEA showing a diffuse cytoplasmic distribution (A) or an apicoluminal pattern (B) in patients with colorectal adenocarcinomas.

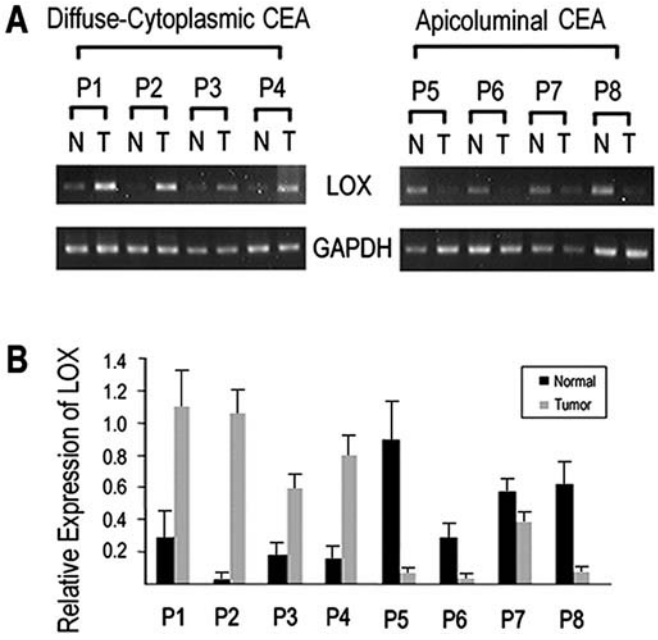

Figure 2. Relative expression of *LOX* compared to the cellular patterns of CEA immunoreactivity. (A) Typical examples of RT-PCR analysis of *LOX* with the matched tumor/normal tissue samples. P1 to P8 represent different patients with colon carcinoma. N and T stand for normal and tumor tissues of the matched pairs, respectively. P1 to P4 patients showed a diffuse cytoplasmic expression pattern of CEA, whereas P5 to P8 patients showed an apicoluminal expression pattern of CEA. (B) Quantitative analysis of *LOX* expression. The intensity of the PCR amplicons of *LOX* was expressed as a densitometric ratio to the intensity of the *GAPDH* amplicons. All assays were repeated in triplicate. The average is shown with error bars representing the standard deviation.

P<0.0001). In approximately half of the cases tested, the $2^{\Delta\Delta Ct}$ values were >1-fold (42.3% for *LOX*, 45.2% for *LOXL*, 50.0% for *LOXL2*, 50.0% for *LOXL3* and 42.3% for *LOXL4*). The $2^{\Delta\Delta Ct}$ values did not differ in gender and age.

Association with clinicopathological parameters. The patients showing >1-fold of $2^{\Delta\Delta Ct}$ were subjected to association studies with clinicopathological parameters (Table II). Upregulation of the *LOX* family genes was not significantly associated with location, AJCC stage, growth type, or differentiation status. In addition, the presence or absence of synchronous adenoma did not show any significant correlation with the upregulation of the *LOX* family genes either. However, in analysis with tumor diameter, *LOXL* showed a close correlation with larger tumors (>4 cm; P=0.046), while the other *LOX* family genes did not present any significant associations (Table II). Of particular interest, tumors without LVI were significantly correlated with upregulation of *LOX*, *LOXL2* and *LOXL4* (P=0.012, 0.014 and 0.005, respectively); however, association of LVI with *LOXL* and *LOXL3* was not statistically significant.

Association with carcinoembryonic antigen (CEA). We have previously reported that CEA, a well studied diagnostic marker of colorectal cancer, shows a diffuse cytoplasmic expression pattern rather than an apicoluminal expression pattern in more invasive colorectal tumors (22). In the present studies, the expression patterns of CEA depending on invasiveness were also confirmed in the colorectal tumor tissues (Fig. 1). In analysis of the *LOX* family genes with the CEA expression patterns, the $2^{\Delta\Delta Ct}$ values of *LOX* were significantly greater in the tumor tissues with diffuse cytoplasmic expression of CEA compared to those with apicoluminal expression (4.36±1.4 vs. 1.44±0.3, P=0.038) (Fig. 2). The other *LOX* family members, in contrast, did not show any significant associations with CEA expression patterns. None of the *LOX* family members showed any significant correlations with preoperative serum CEA levels (data not shown).

Discussion

Microarray studies have previously revealed that the expression of *LOX* is consistently upregulated in hypoxic human tumor cells (23). Further, LOX was identified to play an essential role in hypoxia-induced metastasis of breast and head and neck carcinomas (24). Hypoxia is caused by low oxygen tension in the surrounding tissues due to inadequate blood supply and hypoxic tumor cells are more metastatic and resistant to chemotherapy (25). In our results, upregulation of *LOX*, *LOXL2* and *LOXL4* was remarkably more frequent in the tumors without LVI than those with LVI. In tumors without LVI, blood supply to the tumor cells is limited, resulting in relatively lower oxygen tension around the tumor cells (26). Our results suggest that oxygen deprivation in the absence of LVI may enhance the expression of not only *LOX* but also *LOXL2* and *LOXL4* in colorectal tumors. A hypoxia-response element (HRE) present in the promoter region of the *LOX* gene was known to regulate the *LOX* upregulation through

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tion with hypoxia-inducible factor-1 (HIF-1) (24). udies on identification and functional characterization of HREs in the 5'-upstream regions of *LOXL2* and *LOXL4* or interactions with the *trans*-acting HIF-1 may reveal the regulatory mechanisms for the upregulation of these genes in hypoxic tumor cells.

CEA, a 180 kDa glycoprotein, is a member of the immunoglobulin supergene family that is comprised of at least 29 genes (27). Elevated serum levels of CEA are clinically associated with induction of metastasis and suppression of anoikis, a form of apoptosis caused by detachment from extracellular matrices, in colorectal carcinomas (28). CEA shows a diffuse-cytoplasmic expression pattern rather than an apicoluminal pattern in more invasive and metastatic neoplastic cells of colorectal tumors (22). In our studies, *LOX* upregulation was significantly greater in tumors with diffuse cytoplasmic CEA expression than those with absent or apicoluminal expression, further supporting the role for LOX in metastasis progression. CEA upregulation has been known to enhance the interaction of integrin and fibronectin, leading to increased adhesion of the CEA-expressing cells to the extracellular matrix and then resulting in suppression of anoikis (29). LOX also affects the integrin-fibronectin interaction through binding to fibronectin and the LOX-fibronectin interaction is involved in regulating proteolytic activation of the amine oxidase activity of LOX (30). These similar features of CEA and LOX in integrin signaling, together with our findings on the *LOX* upregulation in more invasive tumor tissues, suggest that LOX and CEA may exert a synergistic effect on the progression of metastasis, although the functional significance of their co-expression needs to be further elucidated.

In conclusion, upregulation of the *LOX* family genes was not significantly associated with location, stage, differentiation status, or growth type of colorectal adenocarcinomas. However, upregulation of *LOX*, *LOXL2* and *LOXL* was strongly correlated with the absence of LVI, suggesting that expression of these genes may be regulated by oxygen tension surrounding colorectal tumors. Due to limited follow-up periods, we could not elucidate the correlation of these *LOX* family genes with metastasis of colorectal tumors. Further longitudinal studies are required to address the correlation of the differential expression of the *LOX* family genes with local recurrence, survival and invasiveness in colorectal carcinomas.

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