**LOXL1 and LOXL4 Are Epigenetically Silenced and Can Inhibit Ras/Extracellular Signal-Regulated Kinase Signaling Pathway in Human Bladder Cancer**

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**Abstract**

Promoter hypermethylation is one of the common mechanisms leading to gene silencing in various human cancers. Using a combination of pharmacologic unmasking and microarray techniques, we identified 59 candidate hypermethylated genes, including LOXL1, a lysyl oxidase-like gene, in human bladder cancer cells. We further showed that LOXL1 and LOXL4 are commonly silenced genes in human bladder cancer cells, and this silence is predominantly related to promoter methylation. We also found LOXL1 and LOXL4 gene methylation and loss of expression in primary bladder tumors. In addition, somatic mutations were identified in LOXL4, but not in LOXL1 in bladder cancer. Moreover, reintroduction of LOXL1 and LOXL4 genes into human bladder cancer cells leads to a decrease of colony formation ability. Further studies indicated that the overexpression of LOXL1 and LOXL4 could antagonize Ras in activating the extracellular signal-regulated kinase (ERK) signaling pathway. Thus, our current study suggests for the first time that lysyl oxidase-like genes can act as tumor suppressor genes and exert their functions through the inhibition of the Ras/ERK signaling pathway in human bladder cancer. [Cancer Res 2007;67(9):4123–9]

**Introduction**

With more than 120,000 new cases a year in Europe and the United States, bladder cancer remains the second most common genitourinary neoplasm and the fifth most common cancer in the United States (1–3). Like other human cancers, loss of function of tumor suppressor genes (TSG) and activation of oncogenes are two major alternations that promote bladder cancer initiation and progression. Gene mutations and loss of heterozygosity (LOH) are well-known genetic changes leading to the inactivation of TSGs. In recent years, the epigenetic change of gene promoter hypermethylation has been intensively studied and confirmed to be an important molecular mechanism contributing to TSG inactivation along with other genetic alternations in human cancer (4–6).

The significance in identifying novel gene promoter methylation targets is 2-fold. First, methylated genes are potential candidate TSGs. Subsequent investigation of the function of these methylated genes could increase our understanding of tumor initiation and progression. Second, the methylation genes could serve as potential DNA markers for cancer diagnosis or even early detection of cancer (6). Specifically for bladder cancer, the gene methylation pattern, combined with the use of urine or serum samples, can be an alternative method for cancer diagnosis and have profound clinical applications (6–10).

There are numerous methods for large-scale screening for the detection of methylated genes in the human genome. However, most of them are either technically complex (restriction landmark genomic scanning, methylation-sensitive representational difference analysis; refs. 11, 12), or they can only screen a small number of genes (CGI array, arbitrarily primed PCR; refs. 13–16). Our laboratory has developed a simple technique that combines microarray and pharmacologic unmasking (5-aza-2’-deoxycytidine treatment of cancer cells). This technique can screen up to 32,000 genes in one experiment and is now widely used in methylation screening for all different types of cancers (17, 18).

In this study, we used this combination technique in bladder cancer for the first time and identified 59 candidate hypermethylated genes. Among these genes, LOXL1, a lysyl oxidase-like gene, has been further studied. We showed that LOXL1 and LOXL4, but not other lysyl oxidase genes, are frequently silenced in human bladder cancer cell lines, and that this silence is predominantly related to promoter methylation. We further confirmed promoter methylation and loss of expression of LOXL1 and LOXL4 genes in human bladder tumors. We also found that in bladder tumors, LOXL4 but not LOXL1 harbored somatic mutations and polymorphisms, which are clustered in exon 8 that encodes for the scavenger receptor cysteine-rich (SRCR) domain. Moreover, reintroduction of LOXL1 and LOXL4 could antagonize the oncogene Ras in activating the extracellular signal-regulated kinase (ERK) signaling pathway in bladder cancer cells and NIH3T3 cells containing an active Ras mutation. Finally, we found that LOXL1 and LOXL4 genes predominantly localized in the cytoplasm, and overexpression of LOXL1 and LOXL4 genes leads to a dramatic decrease of colony formation in human bladder cancer cells. All together, our data indicate that LOXL1 and LOXL4 are potential TSGs, and that their expressions are predominantly silenced through epigenetic mechanisms in human bladder cancer.
Materials and Methods

Tissue sample and cell lines. Seven different bladder cancer cell lines and one immortalized bladder cell, HUC1, were purchased from American Type Culture Collection and cultured in recommended media. All bladder tumor samples and matched adjacent normal tissues were from the Johns Hopkins Hospital. The tissues were collected immediately after surgery and kept in −80°C for long-term storage. The DNA was extracted using regular phenol-chloroform and ethanol precipitation. The RNA was extracted using TRIzol (Invitrogen), and 2 μg RNA was used for reverse transcription reaction.

DNA bisulfite modification and bisulfite sequencing. DNA bisulfite modification was done using a protocol described previously (17). Briefly, 2 μg DNA was denatured in 0.2 mol/L of NaOH for 20 min at 50°C. The denatured DNA was then diluted in 500 μL of freshly prepared solution of 10 mmol/L hydroquinone and 3 mol/L of sodium bisulfite and incubated for 3 h at 50°C. The DNA was then purified through a column and suspended in 100 μL TE buffer. Bisulfite-modified DNA was amplified using 10× PCR buffer [166 mmol/L (NH₄)₂SO₄, 670 mmol/L Tris (pH 8.8), 67 mmol/L MgCl₂, 0.7% 2 mercaptoethanol, 1% DMSO] and primer sets that were designed according to sequence change after bisulfite treatment. The sequences of the primers are shown as Supplementary Data S4. All PCR products were sequenced with an internal primer using ABI big-dye cycle sequencing kit (Applied Biosystems).

5-Aza-2'-deoxycytidine treatment and reverse transcription-PCR. Bladder cancer cells were treated with 5 μmol/L 5-aza-2'-deoxycytidine (5-Aza-dC) every 24 h for 3 days, TSA (300 nmol/L) was added for the final 24 h. RNA was extracted using the TRIzol reagent. Reverse-transcription reaction was done using single-strand cDNA Synthesis Kit (Invitrogen). Sequences of PCR primers are provided as Supplementary Data S4.

Exon-PCR and mutation analysis. Mutation screenings of 7 exons of LOXL1 and 15 exons of LOXL4, including intron-exon boundaries, were done by PCR amplification and direct sequencing of both strands for DNA samples of all cells and tumors. The primers used for PCR and sequencing analysis are described as Supplementary Data S5.

Plasmids construction and immunofluorescence. LOXL1 and LOXL4 full-length open reading frame sequence were amplified using cDNA with high-fidelity enzyme. The PCR products were purified with gel purification kit and directly cloned into pcDNA3.1D/V5-His-Topo vector using pcDNA3.1 Directional Topo Expression kit (Invitrogen). Positive clones were confirmed by direct sequencing and Western blotting with anti-V5 antibody (1:2,500; Invitrogen). Immunofluorescence assay was done using the protocol described previously (19). Anti-V5 antibody (1:5,000) was used as primary antibody, and Alex 548 anti-mouse antibody (1:800; Invitrogen) was used as secondary antibody. Approximately 1 μg/mL Hoechst 33342 was used for nucleus staining. The images were taken using a Zeiss confocal microscope.

Colony formation assay. Cells seeded in six-well plates were transfected with 1 μg LOXL1, LOXL4, or pcDNA3.1D/V5-His-Topo vector. After 24 h, cells were reseded in 10-cm dishes with a density of 2,000 cells per dish and 5,000 cells per dish in the presence of G418 (500 μg/mL) for 2 to 3 weeks.

Transfection and Western blotting. Cells seeded in 10-cm dishes were transfected with 5 μg LOXL1, LOXL4, and pcDNA3.1D/V5-His-Topo vector. Cell lysates were collected after 48 h and blotting with either anti-V5 antibody to confirm the expression of LOXL1 or LOXL4 or anti-p-ERK antibody. For 5637 cells, transfection was done with the same amount of LOXL1, LOXL4, and HRAS G12V constructs. The enhanced chemiluminescence kit (Amersham Biosciences) was used for the detection of signals.

Statistical analysis. Statistical analysis was done using Student’s t test.

Results

LOXL1 is one of the candidate methylated genes identified using pharmacologic unmasking and microarray techniques. Three bladder cancer cell lines, SCaBER, T24, and UM-UC-3, were treated with the demethylating agent 5-Aza-dC (5 μmol/L) for 3 days to reactivate genes that are epigenetically silenced. Changes in gene expression were analyzed using microarray chips containing 12,599 genes (Affymetrix U95Av2). Treatment with 5-Aza-dC resulted in the up-regulation (defined as a 3.0-fold increase) of more than 500 unique genes in each of the three cell lines. Among them, 132 genes were reactivated by demethylation treatment in at least two cell lines. We further examined the expression status of these 132 genes in the expression profiles from normal bladder tissue and the immortalized uroepithelial cell line, HUC-1. Fifty-nine genes which showed abundant expression in normal bladder tissue but were commonly silenced in at least two bladder cancer cell lines were thus identified as specifically silenced genes in bladder cancer (Supplementary Data S1 and S2).

Among these 59 genes, LOXL1 is of particular interest. LOX was the first gene identified in this family and was confirmed to be related to the Ras-induced cell reversion procession (20–22). Recently, several family genes (LOXL1, LOXL2, LOXL3, and LOXL4) were cloned and have been shown to possess various functions, including developmental regulation, tumor suppression, cell motility, and cellular senescence (23, 24). We thus hypothesized that LOXL1 and possibly other LOX family genes might be epigenetically silenced and act as TSGs in bladder cancer.

LOXL4 are the most frequently silenced genes in the lysyl oxidase gene family in bladder cancer cells. To confirm our hypothesis, we first did reverse transcription-PCR to check the expression pattern of all family members in a panel of bladder cancer cells. As shown in Fig. 1A, the loss of expression was seen in six out of eight bladder cell lines for LOXL1 (75%), five out of eight cell lines for LOXL4 (62.5%), and four out of eight cell lines for LOXL3 (12.5%). These findings confirmed our previous microarray data and established that LOXL1 and LOXL4 are the most frequently silenced genes in bladder cancer.
for LOX (50%). LOXL2 and LOXL3, however, retain their expression in the majority of the cell lines. In contrast, all five genes retained expression in normal bladder tissue, and three genes (LOXL1, LOXL2, and LOXL3) also retained expression in HUC1 cells. We therefore focused on the LOXL1 and LOXL4 genes because they were the most frequently silenced in bladder cancer cell lines.

We next treated these cells with the demethylating agent 5-Aza-dC. Both LOXL1 and LOXL4 genes were robustly reactivated in all the cell lines, which previously showed none or low expression of these genes (Fig. 1B). We did not observe additional reactivation effect with TSA (300 nmol/L) on these cell lines (data not shown). In addition, we did direct bisulfite DNA sequencing to investigate the promoter hypermethylation status of the LOXL1 and LOXL4 genes in human bladder cancer cells (Fig. 2A). We found that the methylation pattern of these two genes perfectly matched their expression pattern in human bladder cancer cell lines (Fig. 2B). All together, our data clearly indicate that LOXL1 and LOXL4 genes frequently lose their expression in human bladder cancer cell lines, and that this silence is predominantly related to their promoter hypermethylation status.

**LOXL1 and LOXL4 are frequently hypermethylated and lose expression in primary bladder tumors.** We next investigated LOXL1 and LOXL4 gene methylation in 64 primary bladder tumor samples and 12 normal tissues using bisulfite sequencing. LOXL1 methylation was found in 42 out of 60 primary tumors (70%) and 4 out of 12 (33.3%) normal appearing controls (Student’s t test, P = 0.037; Fig. 3A). LOXL4 was methylated in 26 out of 63 primary tumors (40%), and no methylation was found in normal appearing controls (Fig. 3B). All together, 58 out of 63 (92%) tumors displayed either LOXL1 or LOXL4 methylation in their promoter regions, and 10 out of 58 cases (17%) showed both LOXL1 and LOXL4 methylation (Fig. 3C).

We next checked the expression ratio in 14 cases of primary bladder tumor samples and 6 cases of normal appearing tissues. LOXL1 expression was lost or dramatically decreased in 7 out of 14 primary tumor samples (50%). Likewise, LOXL4 was found to have low or no expression in 5 out of 14 cases (35.7%). Lost expression of both of LOXL1 and LOXL4 was found in four cases. In contrast, LOXL1 expression was retained in all six normal appearing tissues, and only one case showed lost expression of LOXL4 (Fig. 4A).

We next checked the expression and methylation pattern of LOXL1 and LOXL4 genes in four paired normal appearing tissues and tumor samples. We found that LOXL1 lost expression in two tumor samples compared with their normal appearing tissue, whereas LOXL4 lost expression in one case of tumor, with another case having a dramatic decrease in expression (Fig. 4B). We further compared the methylation profile with the expression pattern. We found that the two paired samples without expression change are both unmethylated, whereas one case (1386) is methylated in tumor (loss of expression), but not in the normal appearing tissue (with expression). These data further support the notion that methylation is the main mechanism leading to the loss of expression of LOXL1 and LOXL4 in bladder cancer.

**Somatic mutations were identified in LOXL4 but not in LOXL1 in bladder cancer.** To further evaluate whether there are other genetic alterations in LOXL1 and LOXL4 genes in human bladder cancer, we sequenced each exon of both genes in 8 bladder

![Figure 2](image-url)  
Figure 2. LOXL1 and LOXL4 are both methylated in bladder cancer cell lines. A, representative figures showed position of CpG islands and methylation of LOXL1 and LOXL4 genes. Arrows, the methylated CpGs. B, expression and methylation of LOX genes in bladder cancer cell lines.

![Figure 3](image-url)  
Figure 3. Methylation of LOXL1 and LOXL4 in primary bladder tumors and normal appearing samples. A, LOXL1 is methylated in 70% primary bladder tumors and 33.3% normal appearing samples. B, LOXL4 is methylated in 40% primary bladder tumor samples but not in normal appearing samples. C, 92% of primary bladder tumors have either LOXL1 or LOXL4 gene methylation, and 17% tumor have both methylation of LOXL1 and LOXL4.
cancercells, 30 cases of normal appearing tissue, and 30 cases of tumors. No somatic mutations were identified in all seven exons of LOXL1. However, for LOXL4, we found an A1214C (exon 8) heterogeneous polymorphism in 2 of the 8 cell lines and 10 of 30 normal and tumor DNA samples. In addition, homogeneous polymorphisms in the same position were found in three other cell lines and in five tumors, but not in the normal appearing samples. Among these five, two cases have paired normal appearing tissues (Supplementary Data S3). Thus, these homogeneous polymorphisms are possibly cancer related. In addition, two somatic mutations, just two base pairs 5’ of the polymorphism, were identified in bladder tumors. T1212C leads to a nonsense mutation (Asn404Asn), whereas T1212G leads to missense mutation (Asn404Lys; Table 1; Supplementary Data S3). Notably, all the polymorphisms and somatic mutations were located in exon 8 encoding for the SRCR domain.

**LOXL1** and **LOXL4** antagonize Ras activation of the ERK signaling pathway in bladder cancer cells. Although the reverse correlation of the LOX gene and ras oncogene was observed 20 years ago, no further studies were done to investigate the mechanisms behind this phenomenon. Because we have already found that LOXL1 and LOXL4 genes are frequently silenced and methylated in bladder cancer, it was reasonable to hypothesize that the overexpression of LOXL1 and LOXL4 can block Ras-induced cell signaling in bladder cancer. We initially used the mouse fibroblast NIH3T3 cells and ras-transformed NIH3T3 cells as a model to test our hypothesis. As shown in Fig. 5A, ras-transformed NIH3T3 cells have much stronger p-ERK expression than wild-type NIH3T3 cells. We observed significant inhibition of p-ERK activation when we transfected either LOXL1 or LOXL4, but not their vector into ras-transformed NIH3T3 cells (Fig. 5A).

We then checked the status of Ras and p-ERK in a panel of bladder cancer cell lines (Fig. 5B). T24 and UM-UC-3 are two cell lines harboring both a Ras mutation and an enhanced ERK activation. 5637 and SW780 are two cell lines with wild-type Ras with a dramatically low ERK activation. To check whether LOXL1 and LOXL4 genes can antagonize the Ras-activating ERK signaling pathway in bladder cells, we cotransfected the Ras gene containing the G12V mutation and LOXL1 or LOXL4 gene into 5637 cells without detectable ERK activation. Mutant Ras alone can significantly activate ERK signaling, and this activation can be inhibited by both LOXL1 and LOXL4 reintroduction, with LOXL4 being less effective than LOXL1 (Fig. 5C).

Finally, we transfected LOXL1 and LOXL4 into J82 and UM-UC-3 cell lines. As shown in Fig. 5D, these two cell lines both have enhanced ERK activation but with a different Ras status. We found that LOXL1 can markedly inhibit ERK activation in both cell lines, whereas LOXL4 can only marginally inhibit ERK activation in these cells. Thus, consistent with the results from 5637 cells, LOXL1 and LOXL4 have different capabilities in inhibiting the ERK signaling pathway.

**Table 1. Somatic mutations and polymorphisms of LOXL4 gene in bladder cancer**

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<tr>
<th>Exon</th>
<th>NT</th>
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<tr>
<td>Heterogeneous polymorphisms</td>
<td>Exon 8</td>
<td>A1214C</td>
<td>Asp&lt;sup&gt;405&lt;/sup&gt;Ala</td>
<td>SRCR*</td>
<td>10</td>
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<td>Homogeneous polymorphisms</td>
<td>Exon 8</td>
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<tr>
<td>Somatic mutations</td>
<td>Exon 8</td>
<td>T1212G</td>
<td>Asn&lt;sup&gt;404&lt;/sup&gt;Lys</td>
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<td>Exon 8</td>
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*Scavenger receptor cysteine-rich (SRCR) domains.
† These NT changes have also been detected in two paired samples.
LOXL1 and LOXL4 are predominantly located in the cytoplasm and can inhibit colony formation in bladder cancer cells. Lysyl oxidase has been shown to have both intracellular and intranuclear locations in liver and kidney cells (23, 25). Until now, the cellular locations, as well as the functions of the new lysyl oxidase-like proteins in cancer cells, have not been studied. To clarify whether LOXL1 and LOXL4 gene cellular locations are consistent with their function to inhibit the Ras/ERK signaling pathway, we transfected the LOXL1 and LOXL4 genes into the bladder cancer cell line UM-UC-3, which contains a Ras mutation and, accordingly, an enhanced ERK activation (Fig. 6B). We found that both LOXL1 and LOXL4 expression can lead to a 40% to 50% decrease in anchorage-dependent colony formation in UM-UC-3 cells. Similar results were also obtained when we used the J82 cell line, which contains wild-type Ras but enhanced ERK activation (data not shown). Taken together, these data indicate that LOXL1 and LOXL4 could serve as TSGs in bladder cancer.

Discussion

Lysyl oxidase (LOX) is a copper-containing amine oxidase that belongs to a heterogeneous family of enzymes that oxidize primary amine substrates to reactive aldehydes. LOX has been traditionally known for one function, the extracellular catalysis of lysine-derived cross-links in fibrillar collagens and elastin (23, 26). We have shown here for the first time that two novel members of this amine oxidase family, LOXL1 and LOXL4, are frequently silenced, and this silence is predominantly related to promoter hypermethylation in human bladder cancer. Beyond that, LOXL1 and LOXL4 can inhibit anchoring-dependent colony formation and can antagonize the Ras effect in activating the ERK signaling pathway in bladder cancer cells. Thus, our study indicates that LOXL1 and LOXL4 can serve as TSGs in human bladder cancer. Recently, LOX was reported to be methylated in human gastric cancer (26%) by MSP; ref. 27). In contrast to that report, we showed that LOX was not the most frequent silenced LOX gene family member in bladder cancer. Although the same promoter region showed hypermethylation in human bladder cancer cells, we did not observe hypermethylation of LOX in primary bladder tumors (data not shown). Thus, LOX gene methylation might be restricted to some specific tissues.

It is of interest that we identified a homogeneous polymorphism (A1214C) of the LOXL4 gene in five tumor cases but not in normal tissue. Further study is needed to clarify whether this kind of polymorphism is related to any pathologic characteristic of bladder cancer. It is necessary to point out that all the polymorphisms and somatic mutations were identified in exon 8 of the LOXL4 gene, which encodes for the SRR domain. SRCR domains are conservative disulfide-rich extracellular domains shared by LOXL2, LOXL3, and LOXL4, but not by LOX and LOXL1. They are also found in several extracellular receptors and may be involved in protein-protein interactions. Interestingly, Xu et al. (28) reported...
that two out of seven missense mutations located in the SRRC domain of MSTR1 (macrophage scavenger receptor 1) gene in families affected with hereditary prostate cancer, suggesting that germ line mutations and sequence variants of the MSTR1 gene were associated with prostate cancer risk. Taken together, the hotspot of nucleotide change clustered in the SRRC domain might influence the primary function of LOXL1 and MSTR1 genes and lead to alterations of downstream signaling pathways in bladder and prostate cancer.

There is some controversy in the literature regarding the function of the different members of the lysyl oxidase family in human cancers. It was reported that LOX, LOXL1, and LOXL2 were overexpressed in metastatic breast cancer. LOX-expressing MCF7 cells displayed increased invasiveness in an *in vitro* invasiveness assay (29). LOXL2 overexpression was also reported to promote tumor fibrosis and tumor progression in *in vivo* (30). More recently, LOX was reported to be essential for hypoxia-induced metastasis (31). In contrast to above, LOX levels are reduced in several cancer cell lines as well as in ras-transformed cell lines (21, 32, 33). The LOX gene was reported to be methylated in human gastric cancer (27). Our current study indicates that at least two members of the lysyl oxidase family, LOXL1 and LOXL4, can serve as TSGs in bladder cancer. We believe that this kind of functional variation in LOX family members is structure dependent and tissue specific. On the one hand, all LOX and LOX-like proteins (LOXL1, LOXL2, LOXL3, and LOXL4) have highly conserved copper-binding and lysyl-trosylquinone cofactor sites (23, 24). There are also some conserved domains such as multiple SRRC domains shared only in LOXL2, LOXL3, and LOXL4 (34, 35). Thus, these structural similarities and differences might account for their varied functions in cells. On the other hand, it is not unusual that different gene family members play different active roles in different tissues due to the specific environment and different upstream signals. TP53 is a well-known TSG, whereas the family member DeltaNp63z is often overexpressed and oncogenic in many tumor types (4, 36–40). LOX was initially known as rrg (ras recognition gene; ref. 20). In many naturally occurring and oncogene-induced (ras) tumors, LOX is down-regulated (21), whereas LOX is one of the main genes induced in concomitance with the reversion process (22, 41). H-ras–transfected NIH3T3, induced to revert by IFNα/γ, would return to their transformed phenotype upon transfection with an antisense LOX. Until now, it has not been shown that the newly identified lysyl oxidase gene family members (LOXL1 and LOXL4) can directly inhibit the Ras/ERK signaling pathway. Giving the primary importance of the ras gene in carcinogenesis, the Ras/ERK signaling pathway has attracted considerable attention as a target for anticancer therapy. Several major approaches have been undertaken thus far: (a) the inhibition of Ras protein expression through ribozymes, antisense, or miRNA; (b) the prevention of membrane localization of Ras; and (c) the inhibition of downstream effectors of Ras function such as the use of Raf kinase inhibitors and mitogen-activated protein/ERK kinase inhibitors (37, 42–44). Our current study provides new evidence that LOXL1 and LOXL4 can antagonize Ras function and inhibit the ERK signaling pathway in bladder cancer. LOXL1 and LOXL4 status may thus be important in determining sensitivity to the Ras/ERK signaling pathway inhibitors in bladder cancer.

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**References**


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