Association of Expression Aberrances and Genetic Polymorphisms of Lysyl Oxidase with Areca-Associated Oral Tumorigenesis

Tzong-Ming Shieh,1 Shu-Chun Lin,1 Chung-Ji Liu,1,2 Shu-Shin Chang,1 Ti-Hsuan Ku,1 and Kuo-Wei Chang1,3

Abstract

Purpose: Areca nut use is the major cause of oral squamous cell carcinoma (OSCC) in Southern Asians. Areca nut contains a high level of free copper ions. Lysyl oxidase (LOX) is a copper-activated enzyme critical for extracellular matrix organization. Contradictory evidence has been put forward to suggest that LOX may be either an oncogenic or a suppressive element. This study investigated the oncogenic significance of LOX in areca-associated OSCC.

Experimental Design: The expression assays and polymorphism analysis were done to know the clinicopathologic implications of LOX status in OSCC. Knockdown and overexpression experiments were conducted to know the phenotypic effects of LOX on OSCC cells.

Results: Up-regulation of LOX mRNA and LOX protein expression in OSCCs relative to adjacent oral mucosa was found. Precancerous lesions had the highest LOX mRNA expression. Areca nut extract up-regulated LOX expression in oral epithelial cells. Knockdown of LOX induced cellular migration and invasion, but it reduced the anchorage-independent growth and xenographic tumorigenesis of OSCC cells. The reduction of migration and invasion by LOX overexpression was partially rescued by blockage of LOX activity. The Arg158Gln polymorphism was associated with earlier clinical stage of OSCC. Wild-type LOX overexpression induced anchorage-independent growth in OSCC cells, but this was not for LOX Arg158Gln overexpression.

Conclusion: LOX exerts oncogenic roles in areca-associated OSCC. This potential could be affected by the existence of LOX propeptide domain or genetic polymorphism.

Oral squamous cell carcinoma (OSCC) is a worldwide disease and the third most common malignancy in developing countries. Its high incidence in Southern Asians is due to the popularity of the habit of areca (betel) nut chewing (1). Areca nut extract was known to induce oncogenic signals in oral epithelial cells (1, 2). Lysyl oxidase (LOX) is synthesized in cells as a preproenzyme. A 50-kDa LOX proenzyme is secreted into the extracellular matrix (ECM) and cleaved between Gly168 and Asp169 by a peptidase to yield a 32-kDa active enzyme and the extracellular matrix (ECM) and cleaved between Gly168 and Asp169 by a peptidase to yield a 32-kDa active enzyme and an 18-kDa propeptide (3, 4). Five LOX family members have been identified encoding LOX and various LOX-like proteins (LOXL1, LOXL2, LOXL3, and LOXL4; ref. 5). All LOX family members show a highly conserved COOH-terminal region, such as the copper-binding site and other active domains (3–5). LOX plays a central role in ECM organization. It also localizes inside the nucleus (6). In a previous screening, we identified a high level of LOX expression in OSCC tissue (7). Areca nut has been shown to contain a high amount of soluble copper. The increased level of soluble copper found in the oral fluids of chronic areca nut chewers may relate to the pathogenesis through LOX activation (8).

The mRNA expression of LOX and other LOX family members (especially LOXL2) has been observed in breast cancer cells with a highly metastatic phenotype (9, 10). A recent study has confirmed that LOX expression is regulated by a hypoxia-inducible factor and is associated with hypoxia and poor prognosis in human breast and head and neck squamous cell carcinoma (11). In addition, inhibition of LOX eliminates the metastasis of orthotopic breast cancer in mice (11). LOXL2 is also associated with the epithelial-mesenchymal transition that seems to favor tumor progression (10). A remarkable reduction of LOX mRNA expression has been found in head and neck squamous cell carcinoma (12), cutaneous squamous cell carcinoma, and basal cell carcinoma (13), but the enzyme was up-regulated around invading tumor cells. Abrogation of LOX expression has been shown to enhance the proliferation and invasion of HaCaT cells (13). Previous studies have suggested that there are multifunctional roles for LOX at the various stages of tumorigenesis or in different types of tumors (3, 4, 9, 14, 15).

Among all the single nucleotide polymorphism sites in the LOX coding region, G473A has the highest frequency (15). The mutation changes the Arg at residue 158 to Gln (LOX-Arg158Gln), and the site is near the peptidase cutting sites of LOX. From the above evidence, it is clear that the role of LOX...
as either an oncogenic or suppressive factor is still wide open. This study was based on a hypothesis that LOX is oncogenic for OSCC and a polymorphism in LOX gene could have influences on the oncogenic potential of LOX. Tissue and functional studies were proposed to prove this hypothesis.

Materials and Methods

Cell culture. The OSCC cell lines OECM-1, SCC25, SAS, and OC3 as well as normal human oral keratinocyte (NHOK) and normal human oral fibroblasts (NHOF) were cultured using previous protocols (1, 7). NHOK and NHOF transfected with human papillomavirus E6 and E7 exhibited an extended life span and were designated as NHOK-E and NHOF-E. The preparation of areca nut extract (ANE) was done using the protocols previously used (1). Wortmannin and β-aminopropionitrile (β-APN) were purchased from Sigma-Aldrich.

Subjects. The sampling of tissues was approved by the institutional review board. For mRNA analysis, 65 OSCC and noncancerous matched tissue (NCMT) pairs were used. Samples were frozen or first embedded in OCT and then frozen immediately in liquid nitrogen. All OSCC cases had been confirmed to contain a neoplastic fraction >70%. The stromal components of NCMTs were carefully removed by shaving. In total, 16 OSCCs and NCMT pairs, 10 corresponding neck lymph node metastasis (LNM) lesions, and 12 oral precancerous lesions together with their appropriate matched NCMTs were subjected to microdissection to obtain pure epithelial components using laser capture microdissection (16). The clinicopathologic variables of the OSCC cases are described in Table 1. For polymorphism analysis, 205 primary OSCC cases (30-82 years old; 51.8 ± 9.8) without previous treatment and 216 male areca chewer controls (24-82 years old; 49.5 ± 11.1) were selected from people who came for a physical checkup but who had no neoplastic operation, immune disorder, or oral lesion. All subjects were male areca chewers.

LOX knockdown. LOX small interfering RNA (siRNA; Supplementary Table S1) and scramble siRNA (purchased from Ambion) were transfected into cells using siPORT amine transfection reagent and the protocols provided by the manufacturer (Ambion). The short hairpin RNA vector for the knockdown of LOX (shLOX-i; TRCN0000045911) was obtained from the RNA interference consortium (Academia Sinica, Taipei, Taiwan). shLuc-i was the vector as a control. The lentiviruses carrying shLOX-i or shLuc-i were generated using protocols recommended by provider.

LOX overexpression. Full-length cDNAs of LOXWT and LOX-Arg158Gln were amplified using specific primers (Supplementary Table S1). The amplicons were cloned into the pDNR-Dual donor vectors first (Clontech) and then cloned into pLPS-3EGFP (Clontech) by Cre recombinase. After selection, the constructs were checked by restriction enzyme digestion mapping, confirmed by sequencing, and named pLPS-LOXWT and pLPS-LOXArg158Gln, respectively. These plasmids and a vector alone control were transfected into SAS for overexpression.

Statistical analysis. Nonparametric analysis, including Mann-Whitney test for unpaired analysis and Wilcoxon signed-rank test for paired analysis, Fisher’s exact test, logistic regression, analysis of the odds ratio (OR), and 95% confidence interval (95% CI) tests, was done using Prism 5.0 (GraphPad Software) or Statistical Package for the Social Sciences 12.0 (SPSS, Inc.) statistical software. Differences between the variants were considered significant when P < 0.05.

The methodologies for other analysis are described in supplement.

Results

LOX mRNA expression in OSCC cells. OECM-1, SCC25, and OC3 cells showed mRNA expression of LOX and LOXL3 at a much higher level than NHOK cells, but the expression of other LOX family members was not consistent across these cell lines (Fig. 1A, top). LOX mRNA expression was also high in SAS cells. Therefore, LOX would seem to have consistently higher expression in OSCC cells than normal epithelial cells. Reverse transcription-PCR (RT-PCR) analysis further confirmed low or undetectable LOX mRNA expression in multiple primary NHOKs. However, a higher LOX mRNA expression was detected in NHOK-E and NHOF-E (Fig. 1A, middle). Western

Table 1. LOX expression and clinicopathologic variables

<table>
<thead>
<tr>
<th></th>
<th>LOX mRNA expression (n = 65)</th>
<th>LOX protein expression (n = 58)</th>
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<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
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<tr>
<td>Well</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Moderate or poor</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>P</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>0.64 (0.23-1.77)</td>
<td>0.44 (0.14-1.31)</td>
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<tr>
<td>Tumor size</td>
<td></td>
<td></td>
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<tr>
<td>T1-T3</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>T4</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>P</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>1.22 (0.46-3.26)</td>
<td>0.80 (0.28-2.32)</td>
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<tr>
<td>LNM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absence</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td>Presence</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
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<tr>
<td>OR (95% CI)</td>
<td>2.96 (1.07-8.20)</td>
<td>1.88 (0.65-5.45)</td>
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<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-III</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>IV</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>P</td>
<td>1.00</td>
<td></td>
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<tr>
<td>OR (95% CI)</td>
<td>1.08 (0.40-2.89)</td>
<td>0.59 (0.14-1.82)</td>
</tr>
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</table>

NOTE: Fisher’s exact test.
blot analysis confirmed the LOX expression in OSCC cell lines in relation to a NHOK (Fig. 1A, bottom). OECM-1 cells showed a slightly higher level of LOX mRNA expression than SAS cells. However, it showed 3.1- and 2.7-fold higher LOX protein expression and activity than SAS cells, respectively (Fig. 1B). SAS and OECM-1 cells treated with 100 to 1,000 µmol/L h-APN showed a progressive reduction of LOX activity (Fig. 1C). Up-regulation of LOX mRNA expression at various dosages of ANE was found in distinctive NHOKs (Fig. 1D). Other associated analyses for ANE regulation are described in supplements.

**LOX and LOXL2 expression in oral tissues.** RT-PCR analyses for LOX and LOXL2 were extended to the tissue pairs. Supplementary Figure S2 shows representative LOX analysis showing LOX mRNA expression in OSCC samples and corresponding NCMT samples. The LOX mRNA expression (LOX/GAPDH) in 65 OSCCs and NCMTs pairs was 0.97 ± 0.08 for OSCC and 0.70 ± 0.09 for NCMT (Fig. 2A), which indicated a statistically significant difference between these two groups. Supplementary Figure S2 also shows representative LOXL2 analysis. LOXL2 mRNA expression (LOXL2/GAPDH) was 3.01 ± 0.62 for OSCC and 1.89 ± 0.55 for NCMT across tissue pairs (Fig. 2B), which also indicates a statistically significant difference between these two groups. The median values for the relative mRNA expression in LOX and LOXL2 were used as cutoffs. OSCCs with a low LOX mRNA expression level have a significantly higher propensity to show neck LNM (Table 1). LOX mRNA expression was not associated with any other tested variables. LOXL2 mRNA expression was not associated with any variable (Supplementary Table S3).

Epithelial cells from the various oral tissues were retrieved by
laser capture microdissection. The results of triplicate quantitative RT-PCR analysis are shown in Fig. 2C and D. LOX and LOXL2 mRNA expressions in the OSCC samples were significantly higher than their paired NCMT samples. Oral precancerous lesion seemed to have the highest LOX mRNA expression (Fig. 2C).

LOX immunohistochemistry was done on 21 OSCC and NCMT pairs and 37 unpaired OSCC. The antibody detected cytosolic, nuclear, and extracellular immunoreactivity (Fig. 2E). No immunoreactivity was detected in sections served as negative controls. The LOX immunoreactivity in NCMT was mainly localized in the stratum spinosum (Fig. 2E, a and c), which was similar to that reported in epidermis (13). LOX immunoreactivity was heterogeneously distributed in OSCC (Fig. 2E, b), which was dispersed across squamous (Fig. 2E, b and d), basoloid (Fig. 2E, e), or anaplastic (Fig. 2E, f) tumor cells. In tissue pairs, 57.6 ± 5.3% epithelial cells in NCMT exhibit LOX immunoreactivity, including that was cytosolic or nuclear, whereas it was 92.5 ± 2.8% in OSCC. This difference was statistically significant (Fig. 2F). The median value for the percentage of immunoreactive cells in OSCC was used as cutoffs to differentiate tumor with high LOX protein expression and the opposite status. LOX protein expression was not associated with any variable. No significant difference was noted between NCMT and OSCC in nuclear LOX immunoreactivity. Nuclear LOX immunoreactivity was not associated with any variable in OSCC.

LOX knockdown reduced anchorage-independent growth and tumorigenesis of OSCC cells. To validate the LOX-specific effects, a siRNA against LOX was used. This siRNA was able to

![Fig. 2. LOX and LOXL2 expression in oral tissues. A and B, LOX and LOXL2 mRNA expression in paired oral tissues, respectively. C and D, quantitative RT-PCR analysis of LOX and LOXL2 mRNA expression in various stages of oral carcinogenesis, respectively. The relative expression level was achieved by normalization with paired NCMT. Horizontal lines, mean values. OPL, oral precancerous lesion. E, LOX immunohistochemistry. a and c, representative NCMT. b and d, corresponding OSCC of (a) and (c), respectively. Note the scattered and intense LOX immunoreactivity mainly distributed in stratum spinosum in (a) and (c), respectively. b and d, well-differentiated OSCC. e, moderately differentiated OSCC. f, poorly differentiated OSCC. Nuclear and cytosolic LOX immunoreactivity was present in (a), (c), (d), and (e). Arrows, representative cells with nuclear and cytosolic immunoreactivity. b and f, had only cytosolic immunoreactivity. LOX immunoreactivity was eminent in stromal tissue in (a), (b), (c), (d), and (e), whereas it was absent in sclerosing stroma in (f). Magnifications, ×100 (a, b, c, e, and f) and ×200 (d). F, percentage of LOX-immunoreactive cells in paired oral tissues. *, P < 0.05; **, P < 0.01; ***, P < 0.001, Wilcoxon signed-rank test.](image-url)
reduce LOX mRNA expression, protein expression (Fig. 3A, top), and activity in SAS (Fig. 3B). But the knockdown effects in OECM-1 were weaker (Fig. 3B), which were most likely due to lower transfection efficiency and the presence of high endogenous LOX activity in OECM-1 cells. Knockdown of LOX significantly induced mobility in the SAS and OECM-1 cell lines by about 4- and 2-fold relative to controls, respectively. It also induced SAS invasion by 3.5-fold (Fig. 3C). Knockdown of LOX had no influence on cell growth (Supplementary Fig. S3A). However, knockdown of LOX significantly reduced the anchorage-independent growth of SAS cells by ~30% (Fig. 3D).

Infection of lentiviruses carrying shLOX-i and shLuc-i (control) followed by selection for 3 days was done on SAS cells. The integration of lentivirus in host genome was confirmed by Taqman LATE-RT-Q-PCR analysis (data not shown). shLOX-i significantly reduced LOX protein expression (Fig. 3A, bottom) and activity (Fig. 3B, right). It also significantly reduced xenographic tumorigenesis of SAS cells and the tumor weight in nude mice (Fig. 3E).

LOX polymorphism for OSCC. In male areca chewers, the frequency of the A allele in the control and OSCC was 18.8% and 17.8%, respectively. No statistically significant difference was found between these different subsets (Table 2). It was also found that the OC3, OECM-1, SAS, and SCC25 cell lines carried the homozygous wild-type LOX genotype. An association among LOX polymorphism, LNM, and stage was investigated. The G/A genotype was significantly lower in OSCC with LNM relative to OSCC without LNM (Table 2). The A allele was significantly less frequent in advanced OSCC relative to OSCC at an earlier stage (Table 2). In addition, the frequency of the G/A genotype or the G/A plus A/A genotype was significantly lower in stage IV tumors relative to tumors within earlier stages (Table 2). Overall, genotyping suggested that there was an association between the A allele/G/A genotype and a lower tumor grade and less propensity for LNM.

LOXArg158Gln has less induction of SAS anchorage-independent growth than LOXWT. Plasmids pLPS-LOXWT, pLPS-LOXArg158Gln, and pLPS-3EGFP (control) were transfected into SAS cells with the aim of investigating the phenotypic effects of the two genotypes. Figure 4A shows similar transfection rates for the tested plasmids as revealed by EGFP mRNA expression. An increase in LOX mRNA expression and
LOX-EGFP was seen at 48 h after transfection (Fig. 4A). Both cytosolic and nuclear EGFP were seen in the LOX-transfected cells. Cells showing either exogenous LOXWT or LOX-Arg158Gln were found to cause a significant induction in LOX activity, which could be reduced by 800 μmol/L β-APN (Fig. 4B). Relative to the control, the migration and invasion of the LOX-transfected cells was remarkably reduced (Fig. 4C and D). Attenuation of LOX activity by β-APN partially reverted the reduction (Fig. 4C). There was no noticeable growth difference between SAS cells transfected with LOXWT and those transfected with LOXArg158Gln over a culture period of 96 h (Supplementary Fig. S3B). However, it was

### Table 2. LOX polymorphism in different clinical settings

<table>
<thead>
<tr>
<th>Allelotype</th>
<th>Control (%)</th>
<th>OSCC (%)</th>
<th>OR (95% CI)</th>
<th>Control (%)</th>
<th>OSCC (%)</th>
<th>OR (95% CI)</th>
<th>Control (%)</th>
<th>OSCC (%)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>81.2</td>
<td>82.2</td>
<td>Reference</td>
<td>80.7</td>
<td>84.6</td>
<td>Reference</td>
<td>77.2</td>
<td>86.1</td>
<td>Reference</td>
</tr>
<tr>
<td>A</td>
<td>18.8</td>
<td>17.8</td>
<td>0.77 (0.74-1.50)</td>
<td>19.3</td>
<td>15.4</td>
<td>0.28 (0.79-2.30)</td>
<td>22.8</td>
<td>13.9</td>
<td>0.01 (1.15-3.24)</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>67.6</td>
<td>67.8</td>
<td>Reference</td>
<td>63.8</td>
<td>74.4</td>
<td>Reference</td>
<td>57.8</td>
<td>75.7</td>
<td>Reference</td>
</tr>
<tr>
<td>G/A</td>
<td>27.3</td>
<td>28.8</td>
<td>0.79 (0.69-1.63)</td>
<td>33.9</td>
<td>20.5</td>
<td>0.04 (1.02-3.92)</td>
<td>38.9</td>
<td>20.9</td>
<td>0.00 (2.67-1.41-5.06)</td>
</tr>
<tr>
<td>A/A</td>
<td>5.1</td>
<td>3.4</td>
<td>0.50 (0.55-3.90)</td>
<td>2.4</td>
<td>5.1</td>
<td>0.44 (0.39-8.62)</td>
<td>3.3</td>
<td>3.5</td>
<td>0.75 (0.27-6.19)</td>
</tr>
<tr>
<td>G/A + , A/A</td>
<td>32.4</td>
<td>32.2</td>
<td>0.99 (0.66-1.51)</td>
<td>36.2</td>
<td>25.6</td>
<td>0.10 (0.91-3.20)</td>
<td>42.2</td>
<td>24.3</td>
<td>0.00 (2.47-1.34-4.55)</td>
</tr>
</tbody>
</table>

**NOTE:** Logistic regression, age-adjusted.

Fig. 4. LOX expression and phenotypic effects. A, left, mRNA analysis. Transfection resulted in up-regulation of LOX mRNA expression. The EGFP mRNA expression was used to measure transfection efficiency. Right, protein analysis. Exogenous LOX-EGFP and endogenous LOX were present in LOXWT- and LOXArg158Gln-transfected cells. B, remarkable induction in LOX activity at 24 h following LOX transfection relative to a vector alone (VA) control. β-APN reduced LOX activity in the transfected cells. C and D, remarkable reduction of mobility and invasion in transfected cells relative to vector alone, respectively. β-APN induced migration of transfected cells in (C). E, SAS cells with LOXWT expression exhibited significant induction of anchorage-independent growth relative to vector alone. Anchorage-independent growth was not significantly affected by LOXArg158Gln transfection. Top, representative fields; bottom, quantitation. Columns, mean of more than triplicate analysis; bars, SE, *, P < 0.05; **, P < 0.01; ***, P < 0.001, Mann-Whitney test.
noteworthy that the LOXWT-transfected cells exhibited a significant induction of anchorage-independent growth, whereas LOXArg158Gln-transfected cells did not show such an effect (Fig. 4E).

**Discussion**

We identified an up-regulation of LOX and LOXL2 mRNA expression in areca-associated OSCC tissues and cell lines relative to their normal counterparts. The findings substantiate a contribution of LOX and LOXL2 to the neoplastic process. Moreover, the combination of laser capture microdissection technology and quantitative RT-PCR analysis further validated the up-regulation of LOX and LOXL2 in tumor cells rather than the stromal cells in tumors. Absence of LOX expression has been noted in the vast majority of skin squamous cell carcinoma (13), but LOX is positive for most head and neck squamous cell carcinoma (11). A study has shown that there was remarkable down-regulation of LOX and LOXL2 mRNA expression in 26 head and neck squamous cell carcinoma tissues and cell lines (5). Differences in sample size, variation in the molecular changes in the different tumor subsets associating with various etiologic factors, and/or fluctuations in LOX mRNA expression at different stages of transformation may underlie the discrepancies between our data and earlier published works (11, 17). It is also possible that the RT-PCR analysis in the absence of real-time monitoring and the differences in antibody specificity caused the discrepancies in studies. When the neoplastic process was subjected to a precise dissection, LOX mRNA expression seemed to be highest in oral precancerous lesion during oral carcinogenesis. LOX may modulate different activities when its levels are modified in different cell/tissue context, the tumor type, and the tumor stage (3, 17). Although OSCC showing low LOX mRNA exhibited a higher potential for LNM, LOX protein up-regulation was found in the vast majority of OSCC. We therefore speculated a remarkable LOX up-regulation through posttranscriptional regulation in this tumor subset. Our preliminary analysis has suggested the LOXL3 up-regulation in OSCC (data not shown). Gorogh et al. (18) have shown the overexpression of LOXL4 in head and neck squamous cell carcinoma. Overall, the up-regulation in LOX gene family members was prevalent in OSCC.

Previous investigations focusing mostly on breast carcinomas have identified that LOX can induce migration and invasion of malignant breast epithelial cells (9, 11, 14). Blockage of LOX enzyme activity is also beneficial for controlling of breast cancer progression (11). Palamakumbura et al. (4) showed that the propeptide domain of LOX counteracted ras-associated transformation in fibroblasts. Min et al. (19) showed this propeptide counteracted the epithelial-mesenchymal transition exerted by HER-2/neu, which suppressed the invasion of breast cancer cells. In this study, we knocked down LOX expression and reduced LOX activity, and the analysis indicated that LOX expression might be associated with lower mobility and less invasion in OSCC cell lines. A recent study has specified that LOX was not sufficient to induce migration of normal breast epithelial cells. However, the interaction between LOX and placenta lactogen can synergistically promote breast epithelial migration (20). Providing that oral epithelial cells may lack LOX-interacting molecules that caused the silencing of LOX-associated migration regulation, the induction of migration or invasion could be the consequence of propeptide reduction through LOX knockdown. However, because LOX overexpression reduced migration and invasion, and β-APN partially rescued such reduction, it is likely that LOX activity plays inhibitory roles in the migration of OSCC cells. The findings were contradictory to studies in breast cancer cells (9–11, 14). Payne et al. (14) identified the activation of Src and focal adhesion kinase through LOX activity regulated the migration of breast cancer cells. Investigations are undergoing to elucidate the mechanisms of this unique phenotype using stable transfectants.

Both knockdown and overexpression experiments identified that LOX expression and LOX activity are positively associated with in vitro transformation and in vivo tumorigenesis. The findings were compatible with the facts that LOX was up-regulated in OSCC tissues and cells. Transformation is the integrated effects of various phenotypes, including proliferation, antiapoptosis, epithelial-mesenchymal transition, mobility, hypoxic survival, angiogenesis, autonomous growth, etc. Although LOX could reduce the migration and invasion of OSCC cells, the presence of LOX in tumors may be beneficial to the formation of suitable ECM environments for the integration of transformation signals (14). LOX activity may also facilitate other phenotypes to surpass the suppression of invasion (14, 17). Because the suppressive effects from LOX propeptide were rather clear (4, 19), the antagonism between LOX propeptide and LOX activity in various carcinogenic stage needs to be comprehensively investigated. Although LOX activity was found to be oncogenic, we were unable to identify the association between LOX protein expression and OSCC progression in tissues. The knockdown or overexpression systems that vigorously changed the LOX expression to a greater extent than tissue samples could underlie the discrepancy. It is also possible that the LOX activities driven by the overall LOX family genes play more important roles in transformation than that exerted by LOX gene only. Alternatively, the increase of propeptide associated with LOX up-regulation may hinder tumor progression in vivo (3).

ANE activates a range of signaling elements that underlie oral pathogenesis (1, 2). The up-regulation of LOX mRNA expression in many NHOKs by ANE might create an advantage for oncogenesis. Nearly all OSCC in this study were areca associated. The up-regulation of LOX in this OSCC subset and the high concentration of copper ion in areca (8) may highlight the importance of LOX for carcinogenesis in areca chewers. Preliminary analysis has shown that phosphatidylinositol 3-kinase/AKT activation could underlie such up-regulation. It would be interesting to investigate the involvement of hypoxia on LOX regulation in oral epithelial cells (11, 17). We investigated the effects of the functional LOXArg158Gln polymorphism on oral tumorigenesis (15). Relative to patients with the wild-type LOX, OSCC patients carrying the polymorphic allele have a lower risk of LNM and tumor progression. It is unclear whether the inhibition of tumor progression is caused by a more consolidated ECM barrier or by an unfavorable tumor cell phenotypes exerted by the LOXArg158Gln gene. To study this, we generated SAS cells expressing LOXWT and LOXArg158Gln. Both genotypes increased LOX activity to a similar level in their transformants. LOXWT-transfected cells had significantly increased anchorage-independent growth. However, LOXArg158Gln transfection did not result in a similar effect. This functional change in
polymorphic LOX seemed to substantiate the clinical findings that an OSCC having the LOXWT genotype is associated with greater tumor progression. Because there were only slight differences in LOX activity lying between LOXWT and LOXArg158Gln transfectants, whether this polymorphic change, which is localized in the propeptide, enhances the suppression effects of propeptide and thus attenuates oncogenic transformation of LOX requires further investigation. However, because the association between low LOX mRNA expression (Table 1) and LOXArg158Gln (Table 2) with nodal metastasis was only statistically borderline significant, extensive studies across different cohorts are required to further clarify the clinical implications.

We found that wild-type LOX was associated with a facilitation of oncogenic transformation in oral epithelial cells. In view of the possible phenotypic effects of the tested genetic polymorphism, the effects of the propeptide in counteracting LOX function, the diversity of the LOX family members, and the pluripotential of LOX, there is an urgent need for a comprehensive analysis that addresses LOX and the role in tumorigenesis of other family members.

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