Significance of Lysyl oxidase-like 2 gene expression on the epithelial-mesenchymal status of hepatocellular carcinoma

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Abstract. In the present study, we investigated the role of lysyl oxidase-like 2 (LOXL2), the correlation between LOXL2 and epithelial to mesenchymal transition (EMT) and the effects of using β-aminopropionitrile (BAPN) to inhibit LOXL2 with the aim of reducing tumor progression in hepatocellular carcinoma (HCC). The expression level of LOXL2 was evaluated in HCC and adjacent non-cancerous tissues using quantitative reverse transcription polymerase chain reaction and clinicopathological analyses. The effects of BAPN on cell proliferation, migration and invasion were investigated in vitro. Additionally, LOXL2 expression was assessed in the culture supernatants of HCC cell lines. Our results revealed that LOXL2 expression was higher in HCC cell lines and tissues. There was a significant correlation between EMT status and LOXL2 levels (P=0.004). BAPN reduced migration and invasion in HCC cells. HCC patients with high levels of LOXL2 expression had relatively shorter disease-free survival (P=0.009) and overall survival (P=0.035). The expression level of LOXL2 was similar between cell supernatants and HCC cell lines. A multivariate analysis demonstrated that portal vein invasion (P=0.015), venous invasion (P=0.026), serum AFP (α-fetoprotein) levels (P=0.019) and LOXL2 expression (P=0.009) were independent prognostic factors. Our results indicated that a higher level of LOXL2 may contribute to tumor progression, indicating that LOXL2 has clinical value as a therapeutic target in HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most diagnosed cancer and the second most frequent cause of cancer-related deaths in males worldwide. In women, it is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer-related deaths (1). The prognosis remains poor in HCC patients since most systemic therapies for this disease are ineffective. Therefore, new treatment options, including molecular-targeted therapies, are urgently needed.

LOX (lysyl oxidase) proteins belong to the LOX family, which comprises five different enzymes (i.e., lysyl oxidase, lysyl oxidase-like 1, lysyl oxidase-like 2, lysyl oxidase-like 3 and lysyl oxidase-like 4), some of which have been identified as oncogenes and regulators of cell growth (2-4). LOX and LOXL1-4 are extracellular matrix-modifying enzymes that catalyse crosslinking in collagen and elastin (5). Among these proteins, LOXL2 has been reported to play a crucial role in metastasis in a variety of malignancies (6-9). Increased LOXL2 levels lead to tumor progression and metastasis, probably by promoting tumor cell invasion and the remodelling of the tumor microenvironment (2,10-14).

Previous studies revealed that LOXL2 was associated with epithelial-mesenchymal transition (EMT) in tumor specimens obtained from patients (9,15). Peinado et al (15) reported that LOXL2 mediated the induction of EMT by repressing E-cadherin, indicating that LOXL2 contributes to tumor progression. EMT was originally proposed as a process of organogenesis that was characterized by the combined loss of expression of epithelial cell junction proteins, such as E-cadherin, and the gain of expression of mesenchymal markers, such as vimentin (16-18).
In the present study, we demonstrated that LOXL2 is involved in the invasion of HCC and is expressed at higher levels in HCC than in normal tissues. The clinical significance and biological involvement of LOXL2 in the progression of HCC has not yet been determined. Therefore, we investigated HCC specimens and the effect of LOXL2 overexpression. Since LOXL2 plays important roles in tumor cell motility and invasiveness, we analyzed the association between LOXL2 expression and prognosis in addition to several clinicopathological factors.

Since Zhu et al have already reported that the upregulation of LOX was associated with poor prognosis on HCC patients (19), we further investigated cell proliferation, invasive potential and wound healing in LOXL2-inhibited HCC cell lines by BAPN, which is a potent and irreversible inhibitor of lysyl oxidase (20,21). Furthermore, we revealed that LOXL2 promoted HCC invasion and is correlated with EMT and is therefore, an attractive therapeutic target.

Materials and methods

Cell lines and culture conditions. Human HCC cell lines (SNU-182, SNU-387, SNU-398, SNU-423 and SNU-449) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The HCC cell lines SK-HEP-1, PLC/PRF/5 and Hep3B were kindly provided by Barrie Bode, Professor and Chair of the Department of Biological Sciences at Northern Illinois University (DeKalb, IL, USA). HuH-1, HuH-2 and HuH-7 cell lines were provided by Jake Liang (NIDDK, National Institutes of Health, Bethesda, MD, USA) and the HLE and HLF cell lines were provided by Sato J (National Institutes of Biomedical Innovation, Health and Nutrition, Japan). FOCUS, an another hepatoma cell line, was provided by Jack Wands (Brown University, Providence, RI, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich), which has been reported as an inhibitor of LOXL2 or control was added at the optimized concentrations (23,24). After 24 h, frames were removed, and wounds were made in the plates. Cell migration towards the wound was monitored at 24 and 48 h.

For the invasion assays, the cells were trypsinized in 0.25% trypsin containing EDTA. Subsequently, the cells were suspended and then implanted in a Transwell insert with 8-µm pores that was previously coated with 50 µl Matrigel (Corning Life Sciences, Tewksbury, MA, USA) and 200 µl of medium containing 5% FBS was added to the bottom chamber. Migration was allowed for 24 h and a cotton swab was then used to remove the non-migrated cells in the upper chamber. The filters were then individually fixed with 4% polysorbate and dyed with Diff-Quik stain (Dade Behring Holdings, Inc., Deerfield, IL, USA). Migrated cells were counted in five random fields in each chamber under a light microscope (Olympus DP-70; Olympus Corp., Tokyo, Japan).

Cell proliferation assay. Cell proliferation of the HLF and SK-HEP-1 cell lines was evaluated in vitro using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assays. Control and transfected cells were seeded in 96-well plates at an initial cell density of 5,000 cells/well. The cells were cultured in DMEM containing 5% FBS for 24, 48, 72 and 96 h. At the indicated time-point, the culture medium was then used to remove the non-migrated cells in the upper chamber. Cell migration was allowed for 24 h and a cotton swab was then used to remove the non-migrated cells in the upper chamber. The filters were then individually fixed with 4% polysorbate and dyed with Diff-Quik stain (Dade Behring Holdings, Inc., Deerfield, IL, USA). Migrated cells were counted in five random fields in each chamber under a light microscope (Olympus DP-70; Olympus Corp., Tokyo, Japan).

Cell culture supernatants. The culture supernatants of HCC cell lines, including SK-HEP-1, PLC/PRF/5, Hep-3B, HLE, HLF, HuH-2 and HuH-7 cells, were investigated using a CircuLex Human LOXL2 ELISA kit (Medical & Biological Laboratories, Co., Ltd., Nagoya, Japan). A total of 1x10⁶ cells were transfected and cultured in DMEM containing 10% FBS for 24 or 48 h. After 48 h, the culture supernatant was separated using a centrifuge and the amount of LOXL2 protein in the culture supernatant was assessed using an ELISA kit.

Statistical analysis. Differences in numerical data between two groups were evaluated using Fisher's exact test or χ² test. Survival rates were calculated using the Kaplan-Meier method and the difference in survival curves was analyzed using the log-rank test. Independent prognostic factors were analysed
using the Cox proportional hazards regression model. Data are expressed as the mean ± SD. P<0.05 is considered to indicate a statistically significant difference. The data were analysed using JMP version 11 software (JMP; SAS Institute, Cary, NC, USA).

**Results**

The expression of LOX and LOXL family members in human HCC cell lines. Real-time PCR analyses were used to determine the mRNA expression levels of LOX and LOXL family members in human HCC cell lines (Fig. 1A). Furthermore, the mRNA expression levels of E-cadherin and vimentin were assessed to determine the extent of EMT and to evaluate the correlation between the EMT status and the expression levels of LOX and LOXL family members. A positive correlation between EMT status and LOXL2 expression was observed in human HCC cell lines (Fig. 1B).

**LOXL2 expression in resected HCC specimens and its clinico-pathological features.** Subsequently, the expression of LOXL2 was assessed in cancerous and normal liver tissues obtained from 150 resected HCC specimens. LOXL2 expression levels were significantly higher in cancerous tissues than in normal tissues (Fig. 2).

The demographics of the 150 patients that were subjected to HCC resection are listed in Table II. The enrolled patients were assigned to two groups (high and low expression) according to the average mRNA level of LOXL2. The high and low expression groups included 40 and 110 patients, respectively. No significant correlations were found between LOXL2 expression levels and clinicopathological parameters (Table III).
Survival analysis of the expression of LOXL2 in HCC-resected patients. To determine whether LOXL2 affects survival in patients with HCC, we analysed disease-free survival (DFS) and overall survival (OS). For DFS, the median survival time (MST) of the patients with low and high LOXL2 expression was 135.3 and 30.3 months, respectively (P=0.009; Fig. 3A). For OS, the MST of patients with low and high LOXL2 expression was 91.6 and 44.6 months, respectively (P=0.035; Fig. 3B). These results revealed that there was a significant difference in survival time in resected HCC patients.

The clinical variables associated with HCC prognoses were evaluated using COX regression models. A univariate analysis revealed that the following prognostic factors were significant for overall survival: tumor multiplicity, pathological stage, portal vein invasion, venous invasion, serum AFP level and LOXL2 expression. A multivariate analysis demonstrated that portal vein invasion (HR, 2.363, 95% CI, 1.19-4.566, P=0.015), venous invasion (HR, 1.673, 95% CI, 1.051-2.710, P=0.026), serum AFP level (HR, 2.363, 95% CI, 1.098-2.871, P=0.019), and LOXL2 expression (HR, 1.782, 95%CI, 1.003-3.117, P=0.009), were independent prognostic factors (Table IV).

Table III. Correlation between LOXL2 expression and clinicopathological characteristics of HCC patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>LOXL2 high (N=40)</th>
<th>LOXL2 low (N=110)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (mean ± SD)</td>
<td>62.3±10.1</td>
<td>62.3±10.1</td>
<td>0.716</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>131/19</td>
<td>131/19</td>
<td>0.283</td>
</tr>
<tr>
<td>Etiology (HBV vs. HCV vs. HBV+HCV vs. others)</td>
<td>28/97/2/23</td>
<td>28/97/2/23</td>
<td>0.853</td>
</tr>
<tr>
<td>Histological type of tumor (mod/well/poor)</td>
<td>111/30/9</td>
<td>111/30/9</td>
<td>0.782</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>4.8±3.1</td>
<td>4.8±3.1</td>
<td>0.117</td>
</tr>
<tr>
<td>Tumor mutiplicity (solitary vs. multiple)</td>
<td>113/37</td>
<td>113/37</td>
<td>0.627</td>
</tr>
<tr>
<td>Pattern of tumor growth (expansive vs. infiltrative)</td>
<td>124/26</td>
<td>124/26</td>
<td>0.153</td>
</tr>
<tr>
<td>Formation of fibrous capsule (present vs. absent)</td>
<td>113/37</td>
<td>113/37</td>
<td>0.424</td>
</tr>
<tr>
<td>Septal formation (present vs. absent)</td>
<td>99/51</td>
<td>99/51</td>
<td>0.185</td>
</tr>
<tr>
<td>Child-Pugh classification (A/B)</td>
<td>135/21</td>
<td>135/21</td>
<td>0.322</td>
</tr>
<tr>
<td>Liver damage (A/B)</td>
<td>107/43</td>
<td>107/43</td>
<td>0.301</td>
</tr>
<tr>
<td>Pathological T category (T1/T2/T3/T4)</td>
<td>15/81/38/16</td>
<td>15/81/38/16</td>
<td>0.853</td>
</tr>
<tr>
<td>Portal vein invasion [(+] vs. [-])</td>
<td>26/124</td>
<td>26/124</td>
<td>0.627</td>
</tr>
<tr>
<td>Venous invasion [(+] vs. [-])</td>
<td>9/141</td>
<td>9/141</td>
<td>0.153</td>
</tr>
<tr>
<td>Serum AFP level (±SD) (ng/ml)</td>
<td>62.3±10.1</td>
<td>62.3±10.1</td>
<td>0.578</td>
</tr>
</tbody>
</table>

HBV, hepatitis B virus; HCV, hepatitis C virus; Well, well-differentiated adenocarcinoma; Mod, moderately-differentiated adenocarcinoma; Poor, poorly-differentiated adenocarcinoma; AFP, α-fetoprotein.
Migration and invasion assay of LOXL2 suppressed cell lines.

A functional analysis of LOXL2 expression was performed in HCC cell lines using BAPN. LOXL2 was overexpressed in HLF and SK-HEP-1 cells and the results of the MTT assays revealed that BAPN did not suppress proliferation in HCC cells. In both cell lines, even when the BAPN concentration was increased, the proliferation rate was not affected (Fig. 4). In wound healing assays, wound closure was suppressed by BAPN in both cell lines (Fig. 5A) and in invasion assays, the invasion ability was significantly suppressed by BAPN (Fig. 5B).

Characterization of the expression of LOXL2 in HCC patients and correlation with EMT status. The mRNA expression levels of E-cadherin and vimentin were evaluated using real-time PCR in cancerous tissues obtained from 150 resected-HCC patients and then, the EMT status was determined based on the vimentin to E-cadherin ratio, as described in Materials and methods section. Although there was no significant correlation between LOXL2 expression and vimentin, there was a significant correlation between LOXL2 expression and both E-cadherin levels and EMT (Fig. 6).

LOXL2 levels in cultured supernatants. Finally, we assessed the expression levels of LOXL2 in the culture supernatants of HCC cell lines. The results of these assays are displayed in Fig. 7. In HLE, HLF and SK-HEP-1 cells, the expression levels of LOXL2 were high, similar to those of LOXL2 in HCC cell lines.

**Table IV. Univariate and multivariate analyses of prognostic factors on overall survival.**

<table>
<thead>
<tr>
<th>Univariate analysis</th>
<th>HR</th>
<th>95% CI</th>
<th>P-value</th>
<th>HR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
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<tr>
<td>Age, years (≥65 vs. &lt;65)</td>
<td>1.219</td>
<td>0.772-1.933</td>
<td>0.393</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Sex (male vs. female)</td>
<td>1.294</td>
<td>0.659-2.927</td>
<td>0.478</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size (high vs. low)</td>
<td>1.596</td>
<td>0.987-2.662</td>
<td>0.057</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor multiplicity (multiple vs. solitary)</td>
<td>1.735</td>
<td>1.066-2.767</td>
<td>0.027*</td>
<td>1.621</td>
<td>1.066-2.767</td>
<td>0.083</td>
</tr>
<tr>
<td>Pattern of tumor growth (expansive vs. infiltrative)</td>
<td>1.036</td>
<td>0.579-2.019</td>
<td>0.911</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formation of fibrous capsule (absent vs. present)</td>
<td>1.583</td>
<td>0.941-2.575</td>
<td>0.082</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Septal formation (absent vs. present)</td>
<td>1.344</td>
<td>0.826-2.144</td>
<td>0.229</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Child-Pugh classification (B vs. A)</td>
<td>1.544</td>
<td>0.828-2.682</td>
<td>0.163</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver damage (B vs. A)</td>
<td>1.388</td>
<td>0.856-2.208</td>
<td>0.179</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathological stage (III, IV vs. I, II)</td>
<td>1.816</td>
<td>1.140-2.871</td>
<td>0.012*</td>
<td>0.094</td>
<td>0.487-1.664</td>
<td>0.747</td>
</tr>
<tr>
<td>Portal vein invasion [(+] vs. (-)]</td>
<td>2.216</td>
<td>1.197-3.579</td>
<td>0.011*</td>
<td>2.363</td>
<td>1.09-4.566</td>
<td>0.015*</td>
</tr>
<tr>
<td>Venous invasion [(+] vs. (-)]</td>
<td>2.656</td>
<td>1.219-5.135</td>
<td>0.016*</td>
<td>1.673</td>
<td>1.051-2.71</td>
<td>0.026*</td>
</tr>
<tr>
<td>Serum AFP level (high vs. low)</td>
<td>1.673</td>
<td>1.051-2.710</td>
<td>0.029*</td>
<td>1.760</td>
<td>1.098-2.871</td>
<td>0.019*</td>
</tr>
<tr>
<td>LOXL2 expression (high vs. low)</td>
<td>1.672</td>
<td>1.017-2.681</td>
<td>0.043*</td>
<td>1.782</td>
<td>1.003-3.117</td>
<td>0.009*</td>
</tr>
</tbody>
</table>

*Statistically significant; HR hazard ratio; CI confidence interval; AFP α-fetoprotein; follow-up duration was 51.9 months (range, 2.6-204 months).
Figure 4. Proliferation assay in human HCC cell lines. (A and B) In both cell lines, even when the BAPN concentration was increased, the proliferation rate was not affected.

Figure 5. Wound healing and invasion assays in HCC cell lines. (A) BAPN significantly decreased migration in the HLF and SK-HEP-1 cells (P<0.05). (B) BAPN significantly inhibited invasion in the SK-HEP-1 cells (P<0.05).

Figure 6. The correlation between the mRNA levels of LOXL2 and E-cadherin or vimentin in HCC tissues. (A) E-cadherin mRNA expression was significantly lower in the high-LOXL2 expression group (P<0.001). (B) There was no significant difference between the mRNA expression levels of vimentin and LOXL2. (C) The EMT ratio was significantly higher in the high-LOXL2 expression group (P=0.004).
LOX is a key enzyme that controls extracellular matrix, collagen and elastin maturation, however its role is not limited to these functions. It also plays a critical role in cancer development and invasion. Previous studies have reported that LOX is downregulated in various malignancies, including basal and squamous cell, colon, oesophageal, gastric, head and neck squamous cell, pancreatic, and prostatic cancer (21,25-27). In particular, LOXL2 has been reported to be an important molecule during metastasis in a variety of malignancies (6-9). Several studies have reported that LOXL2 is associated with tumor development and invasion in HCC (19,28,29). In the present study, we demonstrated that LOXL2 levels are certainly correlated with EMT status in our cell line libraries as previously reported, and that LOXL2 inhibition by BAPN reduced the migration and invasion ability of HCC cells.

Whether the LOX family is clinically implicated in cancer development and progression has remained controversial (30). Increased expression of LOXL2 leads to tumor progression and metastasis, probably because LOXL2 promotes tumor cell invasion and the remodelling of the tumor microenvironment. Previous studies have revealed that LOXL2 was associated with EMT in tumor specimens obtained from patients (9,15). Peinado et al (15) reported that LOXL2 mediated the induction of EMT by suppressing E-cadherin, indicating that LOXL2 contributes to tumor progression. EMT was originally proposed as a process involved in organogenesis that was characterized by the combined loss of expression of epithelial cell junction proteins, such as E-cadherin, and gain of expression of mesenchymal markers, such as vimentin (16-18). Therefore, targeting the LOX family to control EMT has been suggested as a new therapeutic strategy for treating cancer (14).

We investigated the proliferative activities and invasive and migratory potential in HCC cells using BAPN, a potent irreversible lysyl oxidase inhibitor. Several previous studies have used BAPN to inhibit LOXL2 both in vitro and in vivo (10,14,31). In the present study, we found that HCC cell proliferation was not inhibited by BAPN, whereas migration and invasion were significantly inhibited. We think this may have occurred because LOXL2 has been reported to regulate intracellular and extracellular proteins (5,32), and induce fibrosis, tumor invasion and metastasis (6-9,13,15,28).

To better understand the clinical association between LOXL2 and EMT in HCC prognosis, we assessed E-cadherin and vimentin expression levels as markers of epithelial and mesenchymal cells, respectively. Previous studies have revealed that patients with high expression levels of E-cadherin had better overall survival than those with low E-cadherin levels. Therefore, E-cadherin may be a prognostic marker in HCC patients (33,34). In a previous study, we reported that EMT status was a critical prognostic factor in pancreatic (17), gastric (35) and colorectal cancer (36). When EMT status was determined using the vimentin/E-cadherin ratio (17), patients could be divided into epithelial and mesenchymal groups. The patients in the mesenchymal group had significantly poorer survival than those in the epithelial group. Furthermore, there was significant correlation between the expression of LOXL2 and vimentin. However, there was a significant correlation between LOXL2 expression and E-cadherin levels or EMT in the 150 surgically resected specimens examined in this study. In a previous study, tumor metastasis was induced as a result of the intracellular LOXL2-induced oxidation of the transcription factor Snail, which subsequently induced EMT (11,15,37). These findings are consistent with our results, indicating that our method of predicting HCC prognosis could be useful. In order to eliminate the possibility of BAPN side-effects, we think that these knocked-down experiments should be confirmed by siRNA experiments.

Several limitations of the present study should be acknowledged. Firstly, the study was retrospectively designed, although the results were in accordance with previous studies (28,38). Secondly, we only assessed the mRNA expression. An analysis of a larger cohort based on the protein expression is required to confirm the present exploratory study. In addition, further studies are required to investigate the background mechanisms involved in the association between LOXL2 and EMT in HCC.

In conclusion, we have demonstrated that LOXL2 levels are a critical prognostic factor that is correlated with EMT in HCC patients. Further investigations are necessary to determine the relationship between EMT and LOXL2 and its value as a novel therapeutic target.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon the reasonable request.
Authors' contributions
GN and SY conceived and designed the study. SY and YK provided administrative support. GN, SY, MH, MS, HT, HS and TF provided the study materials and patients. GN, SY, ST, YN, MK, NI, CT and DK collected and assembled the data. GN, SY, GN, MK and MF analysed and interpreted the data. GN, SY, MH, MS and YK wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate
All experimental protocols were approved by the Ethics Committee of Nagoya University Hospital (Nagoya, Japan).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References