Received: 5 December 2018 | Revised: 24 March 2019 | Accepted: 25 March 2019

DOI: 10.1111/cas.14010

ORIGINAL ARTICLE

Cancer Science WILEY

Lysyl oxidase induces epithelial-mesenchymal transition and predicts intrahepatic metastasis of hepatocellular carcinoma

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Funding information
Japan Society for the Promotion of Science, Grant/Award Number: 15K10191 and 16K19939

Hepatocellular carcinoma (HCC) has high recurrence rates even after curative hepatectomy. Drug therapy for recurrence of HCC is still limited; therefore, identifying new therapeutic targets is urgently needed. We searched for genes that would predict HCC recurrence from intrahepatic metastasis in an exhaustive DNA microarray database by searching genes associated with high early recurrence rate and having higher expression in the tumor area compared to background liver. We detected lysyl oxidase (LOX) and validated the clinical significance of LOX in 358 patients who underwent hepatectomy. Expression of LOX was evaluated by qRT- PCR, and immunohistochemical (IHC) staining. High LOX expression group had a significantly higher recurrence rate than the low LOX expression group (2-year recurrence rate was 64.0% vs 24.2%, $P < .0001$ for IHC) and poorer survival rate (5-year rate was 60.1% vs 86.2%, $P < .0001$ for IHC). Multivariate analysis showed that high LOX expression was an independent risk factor for early recurrence (IHC: HR, 2.52; $P < .0001$). Bioinformatic analysis showed that LOX expression was associated with hypoxia-inducible factor-1α (HIF-1α) and the hypoxia cascade, suggesting that HIF-1α or hypoxia regulates LOX expression and induces epithelial-mesenchymal transition (EMT). In vitro, LOX and HIF-1α were involved in migration and invasion capability. High LOX expression is associated with EMT markers and predicts early recurrence and poor survival in patients with HCC. These findings indicate that lysyl oxidase could be a potential therapeutic target for early recurrence of HCC.

KEYWORDS
early recurrence, epithelial-mesenchymal transition, hepatocellular carcinoma, intrahepatic metastasis, lysyl oxidase

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy of the liver. According to the International Agency for Research on Cancer (GLOBOCAN 2012),[1] the number of individuals who have liver cancer is approximately 780 000, the sixth highest (5.6%) among all cancer types. In addition, the total number of deaths from liver cancer is approximately 745 000, the second largest (9.1%) among all cancer types. Hepatectomy is an established curative treatment for hepatocellular carcinoma (HCC). However, even if patients undergo

curative treatment, they have a high frequency of HCC recurrence:[2-7] the recurrence rate of HCC after hepatectomy is greater than 10% per year, and reaches 70%-80% after 5 years. Furthermore, almost 90% of these cases have recurrence in the residual liver. Therefore, prevention of HCC recurrence after hepatectomy is urgent.

Two major types of recurrence in HCC are known: multicentric (MC) carcinogenesis and intrahepatic metastasis (IM). MC recurrence is considered a new HCC, independent of the first HCC, and attributable to background liver disease with a high risk of carcinogenesis that relapses after a long period, later than 2 years from hepatectomy. In contrast, IM recurrence is metastasis from the primary site, which relapses earlier than 2 years after hepatectomy. Molecular or clinical biomarkers that predict MC recurrence have been reported, and chemoprevention drugs against the background liver disease for MC recurrence, such as antiviral drugs, have been developed.[8-12] Molecular signatures of MC recurrence that can predict late recurrence after hepatectomy for HCC, and molecularly targeted drugs for the chemoprevention of MC recurrence, have also been reported.[13-20] However, no target gene for the molecular chemoprevention of IM recurrence has yet been identified.

In the present study, we carried out an exhaustive search to find a molecular biomarker that could predict IM recurrence and serve as a target gene for chemoprevention of IM recurrence, and discovered lysyl oxidase (LOX). LOX is a copper-dependent amine oxidase that catalyzes the final enzymatic step required for cross-linking of collagen and elastin molecules in the extracellular matrix.[21] In malignant tumors, LOX is involved in the remodeling of cancer stroma, and affects cancer metastasis or accelerates dedifferentiation of cancer cells.[22-24] High LOX expression is reportedly associated with cancer malignancy and cancer metastasis, and reflects patient prognosis in several types of cancer.[9-14,25-28] In the context of HCC, it has been reported that high LOX expression is associated with a marker of tumor angiogenesis, but the association between LOX expression and patient outcome, especially HCC metastasis of IM, is still unclear.[28,29] We aimed to comprehensively analyze the role of LOX in HCC by interrogating a large transcriptome database and to validate the impact of LOX expression in the recurrence of HCC from IM.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue specimens

The population enrolled in the present study consisted of 358 HCC patients who underwent hepatic resection. All patients were treated at the Department of Gastroenterological Surgery, Kumamoto University Hospital, between 2004 and 2014. The patients underwent imaging studies, such as ultrasonography, dynamic computed tomography (CT), enhanced magnetic resonance imaging (MRI) and CT angiography, for diagnosis and staging of HCC before surgery. The final diagnosis was confirmed pathologically in resected specimens. This study was a retrospective, non-interventional, observational study, approved by the institutional ethics committee of Kumamoto University Hospital and carried out in accordance with the 1975 Declaration of Helsinki. HCC tissue was obtained at the time of surgical resection, snap-frozen, and stored at −80°C. RNA or cDNA was extracted from frozen sample tissue as described below. Formalin-fixed paraffin-embedded (FFPE) tissues were used for H&E staining and immunohistochemical (IHC) staining.

2.2 | Immunohistochemical staining and scoring

Immunohistochemical staining was carried out on 3-μm sections obtained from FFPE sections of HCC as previously described. In brief, sections were deparaffinized and rehydrated, and LOX antigen was activated with proteinase K. TWIST antigen was activated with buffer solution of pH 6 and autoclaving for 60 minutes, whereas E-cadherin antigen, Vimentin antigen, and Slug antigen were activated with buffer solution of pH 9 and microwaving for 20 minutes. The sections were incubated with anti-LOX antibody (1:100, Ab31238; Abcam, Cambridge, UK), anti-TWIST antibody (1:50, sc-15393; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-Slug antibody (1:30, c1967; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C, followed by incubation with secondary antibody (EnVision rabbit, Dako, Tokyo, Japan) for 30 minutes at room temperature. The sections were incubated with anti-E-cadherin antibody (1:400, #3195; Cell Signaling Technology) and anti-Vimentin antibody (1:50, sc-6260; Santa Cruz Biotechnology) overnight at 4°C, followed by incubation with secondary antibody (EnVision mouse, Dako, Tokyo, Japan) for 30 minutes at room temperature. Slides were developed with DAB (Wako Tablet, 040-27001 [5 mg], Dako, Tokyo, Japan) and counterstained with hematoxylin. Intensity and area of immunoreactivity were each scored 0-3.

Proportion of positive cells was graded as follows: 0 (no positive tumor cells); 1 (1%-35% positive tumor cells); 2 (36%-70% positive tumor cells); and 3 (71%-100% positive tumor cells). Staining intensity was scored according to the following criteria: 0 (no staining, negative); 1 (weak, light brown); 2 (moderate, yellow brown); and 3 (strong, brown). The staining index (ranging from 0 to 9) was calculated by multiplying the score for the proportion of positive cells by that for staining intensity. Scores from 0 to 5 and from 6 to 9 defined the low and high LOX expression groups, respectively.

2.3 | Quantitative reverse transcription-polymerase chain reaction

Quantitative RT-PCR was carried out to determine LOX mRNA levels in HCC tissue cells. Total RNA was isolated from cells or tissue using Trizol (Invitrogen, Carlsbad, CA, USA), and the concentration of purified RNA was measured by comparing A_{260} and A_{280} using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was generated from the total RNA using a ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions, and subsequently used as a PCR template. qRT-PCR was carried out as described previously.[30-32] Transcript levels were measured in a duplicate set of reactions for each gene and calculated as fold change relative to control siRNA-transfected cells.

Relative hypoxanthine phosphoribosyltransferase 1 levels were used for normalization. Sequences of primers used in this study are listed in Table S1.

2.4 | Cell culture

SK-hep1 cell line was purchased from ATCC (Manassas, VA, USA). Sk-hep1 cells of density of 4.0×10^4 cells/mL were cultured in Eagle's minimal essential medium (E-MEM)/10% FBS medium at 37°C in a humidified 5% CO_2 atmosphere for 2 days and used for downstream analysis.

2.5 | Cell transfection

According to the manufacturer's instructions, siRNAs targeting LOX (Silencer Select siRNA, LOX, ID: s8254, s8255, s8256; Thermo Fisher Scientific, Tokyo, Japan) and hypoxia-inducible factor-1α (HIF-1α; Silencer Select siRNA, HIF-1α, ID: s6539, s6540, s6541; Thermo Fisher Scientific, Tokyo, Japan), and control siRNAs were transfected to the cells using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Yokohama, Japan).

Before transfection, Sk-hep1 cells were seeded to achieve 70%-80% confluence. After 24 or 48 hours of transfection, the supernatant was removed and washed with PBS twice, and RNA of Sk-hep1 cells was extracted.

2.6 | Cell Counting Kit-8 assay (growth assay)

We evaluated cell growth using a CCK-8 Kit (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's protocols. Sk-hep1 cells were inoculated in a 96-well plate at 3.0×10^3 cells in 100 μL/well and the plate was incubated overnight in a humidified incubator at 37°C with 5% CO_2. Each well of the plate also received 10 μL CCK-8 solution at the indicated time points (0, 1, 2, 3 days). Absorbance was measured at 450 nm using a microplate reader after incubating the plate for 1.5 hours. The absorbance of each sample was measured in triplicate.

2.7 | Migration and invasion assays

We carried out a migration assay of Sk-hep1 cells in six-well plates. Each well was coated with 200 μL BD Matrigel (BD Biosciences, San Jose, CA, USA). Sk-hep1 cells (4.0×10^4 cells/mL) were plated and allowed to adhere for 12 hours. The six-well plates were imaged with a KEYENCE BZ-X700 all-in-one fluorescence microscope equipped with a CO_2 and temperature controlled chamber and time-lapse tracking system (KEYENCE, Osaka, Japan). Phase contrast images were taken every 10 minutes for 24 hours and converted to video files using a BZ-X Analyzer (KEYENCE). We analyzed the videos for cell migration with video editing analysis software VW-H2MA (KEYENCE) and subsequently processed the tracking data with Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) to create *xy* coordinate plots and distance measurements. Evaluation of migration distance was carried out by randomly selecting three cells at each well, tracking for 15 seconds, and graphing the average value of the three moving distances.

For the invasion assay, BioCoat Matrigel invasion chambers were used (24-well plates, 8-μm pores; BD Biosciences) according to the manufacturer's protocol. Sk-hep1 cells (4.0×10^4 cells/mL) were suspended in E-MEM and seeded into the upper chamber. E-MEM supplemented with a carrier solution (PBS) was placed in the lower chamber. After 24 hours of incubation, the cells on the upper surface were removed by a cotton swab, and the cells on the lower surface of the membrane were fixed with 100% methanol for 2 minutes. Then, the cells were stained with Toluidine blue for 2 minutes and rinsed with water. The number of cells that migrated through the membrane were counted in five microscopic fields (40× magnification) per membrane.

2.8 | Bioinformatic and statistical analysis

Exhaustive analysis to search for target genes for IM chemoprevention and recurrence prediction was carried out using the public database GSE10141, which comprises a cohort consisting of HCC patients who underwent curative surgery and uniform follow up after surgery and includes RNA from both cancerous tissue and the adjacent background liver. Network enrichment analysis was carried out using GSEA (gene set enrichment analysis: http://software.broadinstitute.org/gsea/index.jsp) and two other HCC public databases, GSE14520 and GSE9843, which include HCC patient samples from curative surgery. Statistical analysis was carried out using R-3.1.1 (https://www.r-project.org/) and JMP10 (SAS, Tokyo, Japan) programs.

3 | RESULTS

3.1 | Selection of a gene that is strongly associated with early recurrence of HCC after curative surgery

As a first step toward discovering target genes for the prediction of early recurrence, we exhaustively investigated the correlation between each expressed gene and the rate of early recurrence using GSE10141, a microarray database of the cancerous area and background liver tissue of HCC cases. We analyzed the association of each gene and the rate of early recurrence within 2 years using the Cox regression model and extracted genes with a high hazard ratio (HR >3) for early recurrence to select genes that might be useful for the prediction of early recurrence and targetable genes for the prevention of early recurrence. Moreover, we extracted genes that were significantly more highly expressed in the tumor area than in the adjacent surrounding liver ($P < .01$ by paired *t* test), and selected genes that had a high hazard ratio for early recurrence and were highly expressed in the tumor area to identify candidate drug therapy targets that might work selectively against the cancer cells and not against normal liver cells. As a result, we detected LOX (Figure 1 and Table S2).

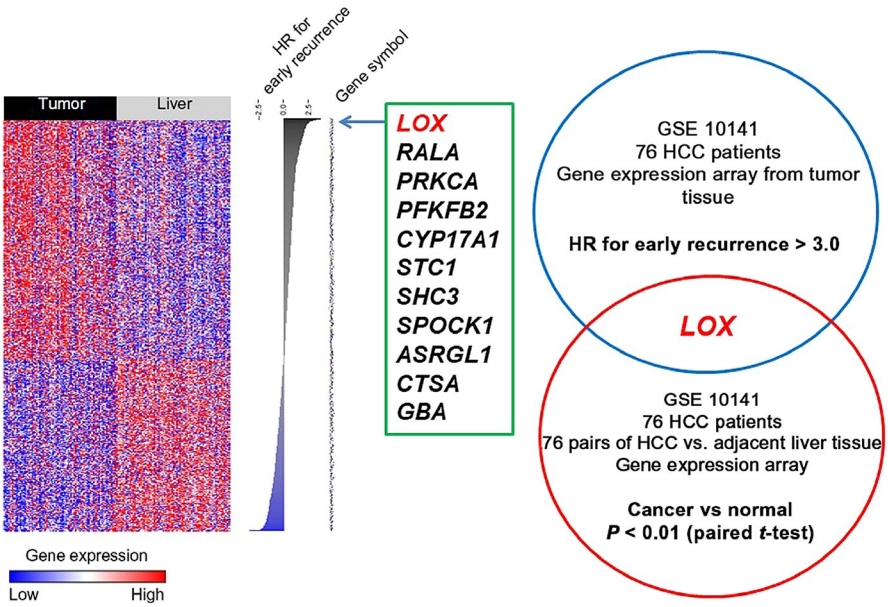

FIGURE 1 Correlation between each gene and the rate of early recurrence using GSE10141. We searched for genes with a high hazard ratio for early recurrence to extract genes potentially useful for the prevention of early recurrence. In addition, we extracted genes that were highly expressed in the tumor area to select genes potentially useful as drug therapy targets. HCC, hepatocellular carcinoma; LOX, lysyl oxidase

3.2 | Validation of the role of LOX as a predictor of HCC recurrence from IM

We hypothesized that high LOX expression in HCC is a predictor of early recurrence, suggesting recurrence from IM, and then first validated the hypothesis using public databases. We used GSE10141 and GSE9843, which are cohorts from HCC patients and samples that underwent liver resection, and we assigned the patients into two groups based on LOX expression. We assigned the patients with the higher 25 percentile as the high LOX group, and those with the lower 75 percentile as the low LOX group. The high LOX group had a significantly high rate of early recurrence (earlier than 2 years) in both GSE10141 ($P < .001$) and GSE9843 ($P = .026$) (Figure S1).

3.3 | LOX mRNA expression is higher in HCC tissue and associated with a high rate of early recurrence and poor overall survival in a validation cohort of HCC patients

To further validate the clinical role of LOX mRNA expression, we used another cohort comprising 149 HCC patients who underwent

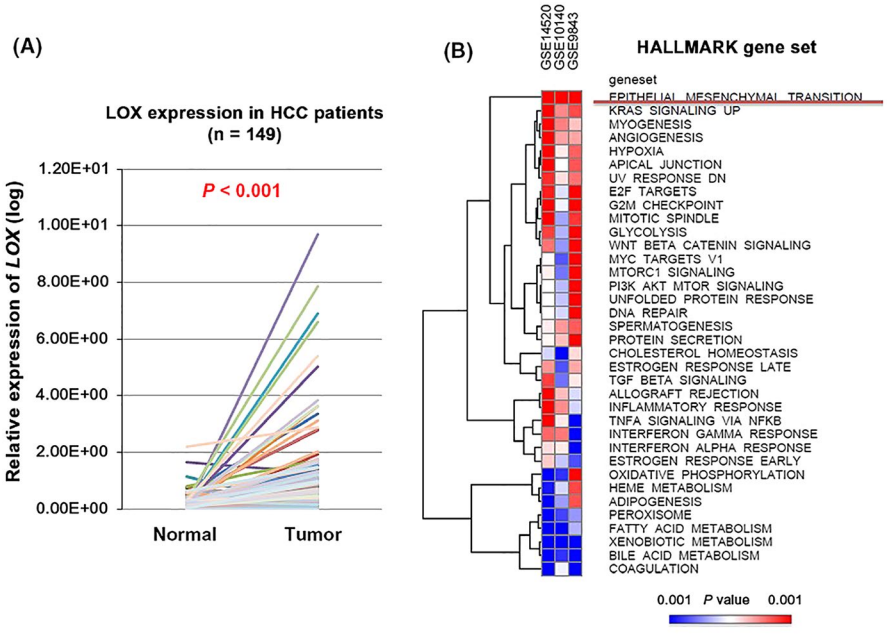

FIGURE 2 A, Lysyl oxidase (LOX) expression between the tumor lesion (Tumor) and the adjacent liver tissue (Normal) measured by qRT-PCR (n = 3). B, Gene set enrichment analysis (GSEA) using the HALLMARK gene set in a transcriptome matrix from the three cohorts GSE10140, GSE14520 and GSE9843. Red color indicates a positive correlation and blue color indicates a negative correlation between LOX expression and the gene set enrichment. HCC, hepatocellular carcinoma

curative hepatic resection at Kumamoto University Hospital (Figure S2). We first confirmed the expression of LOX mRNA in HCC tumor lesions and the adjacent surrounding liver tissue and found that LOX expression was significantly higher in the tumor lesion than in the adjacent liver ($P < .001$) (Figure 2).

Patients were separated based on LOX expression and grouped into a high LOX group (n = 38) consisting of patients in the higher 25% for LOX expression, and a low LOX group (n = 111) of patients in the lower 75%. The high LOX group had a significantly higher rate of early recurrence (2-year recurrence rate was 64.3% vs 42.7%, $P = .024$) and a worse survival rate (3-year rate was 62.7% vs 82.0%, $P = .0441$) than the low LOX group (Figure S3A). Moreover, in comparisons of clinicopathological factors between the high and low LOX groups, there were significantly more portal vein invasion (vp)-positive patients and poorly differentiated carcinoma in the high LOX group ($P = .004$ and .002, respectively) (Table S3). Factors associated with early recurrence were also evaluated by univariate Cox regression analyses (Table S4). Univariate analysis showed that AFP-L3 score higher than 10 ng/mL, PIVKA-II score higher than 100 mAU/mL, multiple tumors, tumor size larger than 50 mm, Fc-positive, Fc-inf-positive, vp-positive, poorly differentiated carcinoma and high LOX expression were all significantly associated with early recurrence ($P < .05$).

3.4 | LOX protein expression is associated with early recurrence and overall survival in patients with HCC

To evaluate the role of LOX protein expression, TWIST protein expression and E-cadherin protein expression, we carried out IHC analysis in the Kumamoto University Hospital cohort, which included 358 patients with FFPE samples. In comparing high LOX expression and low LOX expression samples, several samples of Slug and Vimentin were also stained (Figure 3A). Overall sample selection is summarized in Table S5.

The high LOX group had a significantly higher rate of early recurrence (2-year recurrence rate was 64.0% vs 24.2%, $P < .0001$) and poorer survival rate (5-year rate was 60.1% vs 86.2%, $P < .0001$) than the low LOX group (Figure 3B). Moreover, high LOX-L2 expression of the LOX family in HCC was associated with early recurrence (Figure S4).

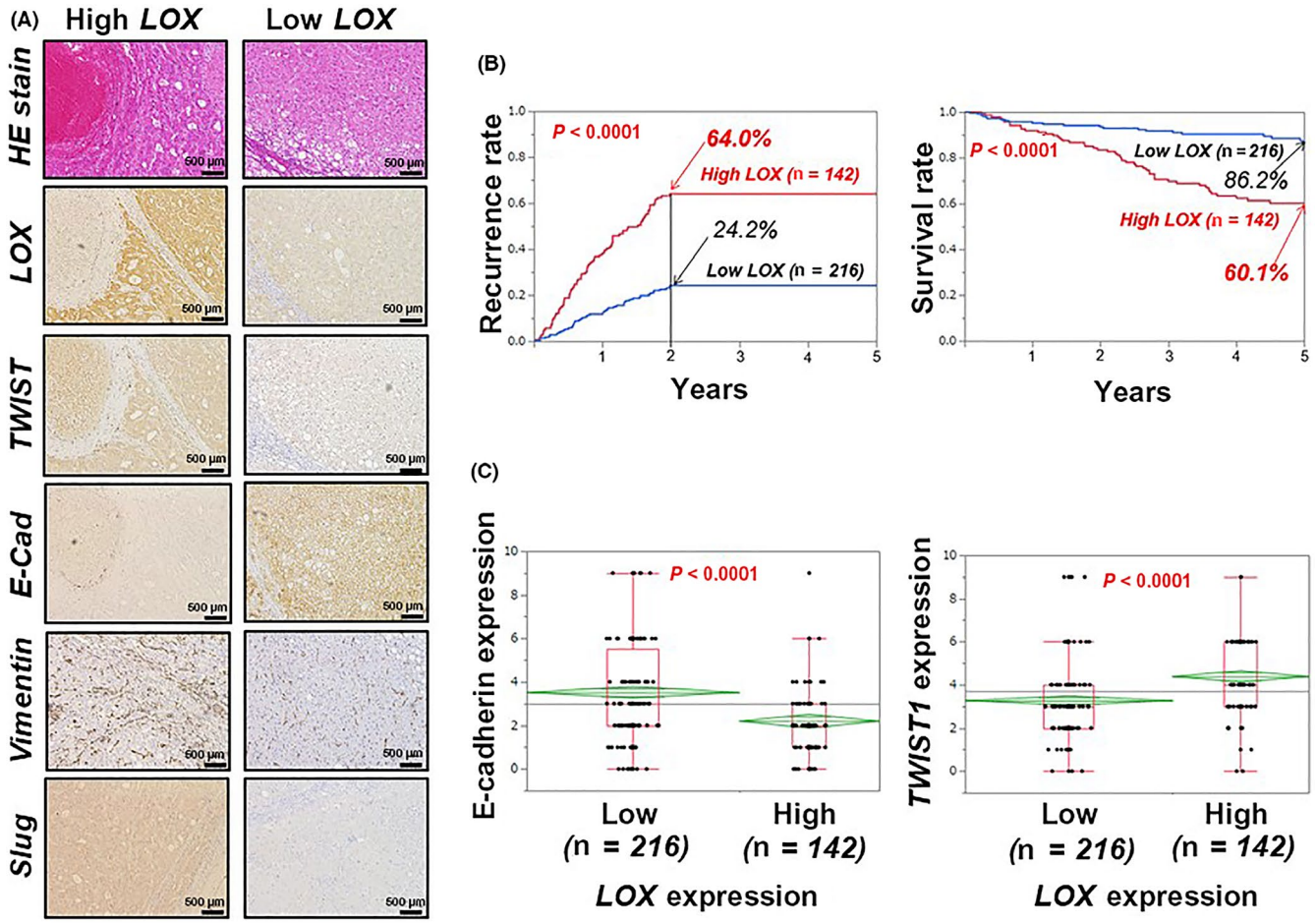

FIGURE 3 Immunohistochemical staining of each expressed gene. A, Representative pictures of the expression of TWIST, E-cadherin (E-Cad), Vimentin and Slug in high lysyl oxidase (LOX) expression (left panels) and low LOX expression hepatocellular carcinoma (HCC) cases (right panels). B, Analyses of early recurrence rate and survival rate in the high and low LOX expression groups. C, Correlation between expression of LOX and expression of E-cadherin (left) or TWIST (right)

TABLE 1 Comparisons of clinicopathological factors between high and low LOX expression groups immunohistochemistry staining

Factor	High LOX (n = 142)	Low LOX (n = 216)	*P* value
Age, y, median (IQR)	68.5 (61-75)	68 (61-74)	.96
Gender (male/female) (%)	118 (83.1%)/24 (16.9%)	156 (72.2%)/60 (27.8%)	.02
HBsAg (+) (%)	32 (22.5%)	56 (25.9%)	.46
HCVAb (+) (%)	66 (46.5%)	97 (44.9%)	.77
AFP (ng/mL), median (IQR)	14.5 (5.5-289)	10.2 (4.2-89.1)	.052
AFP-L3 (%), median (IQR)	1.5 (0.5-35.4)	0.5 (0.5-8.7)	.006
PIVKA-II (mAU/mL), median (IQR)	128 (27.5-1877)	69.5 (22-588)	.08
ICG R15 (%), median (IQR)	11.2 (6.5-23.9)	11.7 (8.1-16.5)	.30
No. of tumors, median (IQR)	1 (1-2)	1 (1-1.8)	.25
Tumor size (mm), median (IQR)	36 (25-60)	32 (22-50)	.03
Eg/Ig (%)	131 (92.2%)/11 (7.8%)	210 (97.2%)/6 (2.8%)	.04
Fc (+) (%)	127 (89.4%)	177 (81.9%)	.07
Fc-inf (+) (%)	110 (77.5%)	156 (72.2%)	.26
vp (+) (%)	34 (23.9%)	45 (20.8%)	.49
Histological type (well + mod/poor)	105 (73.9%)/37 (26.1%)	178 (82.4%)/38 (17.6%)	.056
Hepatic fibrosis, median (IQR)	3 (1-4)	3 (1-4)	.64

AFP, alpha-fetoprotein; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP; Eg/Ig, expansive growth/invasive growth; Fc, capsule formation; Fc-inf, cancerous infiltration of the capsule; HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C antibody; ICG 15R, indocyanine green retention rate after 15 min; IQR, interquartile range; LOX, lysyl oxidase; PIVKA-II, protein induced by vitamin K absence or antagonist-II; vp, portal vein invasion.

In addition, in comparisons of clinicopathological factors between the high and low LOX groups, there were significantly more male ($P = .02$), high AFP-L3 ($P = .006$), large tumor size ($P = .03$) and Ig-positive ($P = .04$) patients in the high LOX group (Table 1). Factors associated with early recurrence were also evaluated by univariate and multivariate Cox regression analyses (Table 2). Univariate analysis showed that PIVKA-II score higher than 100 mAU/mL, multiple tumors, tumor size larger than 50 mm, poorly differentiated carcinoma, advanced hepatic fibrosis and high LOX expression were significantly associated with early recurrence. Multivariate analysis showed that PIVKA-II score higher than 100 mAU/mL (HR, 2.36; $P = .025$), and high LOX expression (HR, 2.52; $P = .015$) were independent risk factors for early recurrence.

3.5 | High LOX expression is associated with molecular EMT markers in multiple HCC patient cohorts

To identify the functional role of LOX that aggravates HCC malignancy, we carried out GSEA analysis using the HALLMARK gene set, which is the most sophisticated molecular database from MSigDB (molecular signatures database from the Broad Institute: http://software.broadinstitute.org/gsea/msigdb), in a transcriptome matrix from the three cohorts GSE10140, GSE14520 and GSE9843 (Figure 2). We found a strong association between high LOX expression and the epithelial-mesenchymal transition (EMT)-associated gene set (EPITHELIAL_MESENCHYMAL_TRANSITION) in all three cohorts ($P < .001$ in all three cohorts) as shown in Figure 2. We also validated the correlation between LOX expression and EMT markers in the Kumamoto University Hospital cohort by IHC staining and qRT-PCR. High LOX expression was significantly associated with high TWIST expression at the protein and mRNA levels ($P < .0001$ and .04, respectively), and with low E-cadherin protein expression ($P < .0001$) (Figure 3C and Figure S3B). Vimentin and Slug expression was significantly high in high LOX expression group compared to low LOX expression group at the protein level.

3.6 | Assessment of the factors upstream of LOX in multiple HCC cohorts

Considering the factors upstream of LOX, it has been reported that HIF-1α or hypoxia activates LOX expression in colon cancer and regulates distant metastasis.[33] We therefore next assessed the association between LOX expression and the expression of HIF-1α or hypoxia-related genes.

We selected the molecular gene sets from MSigDB (http://software.broadinstitute.org/gsea/msigdb) and analyzed the association among these molecular gene sets and LOX expression by GSEA in the transcriptome matrix of four HCC cohorts,

TABLE 2 Univariate and multivariate analyses for early recurrence

	Univariate analysis		Multivariate analysis	
Variable	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age >70 y	0.83 (0.53-1.28)	.39		
Gender (male)	1.31 (0.78-2.20)	.31		
HBsAg (+)	0.98 (0.59-1.61)	.92		
HCVAb (+)	1.22 (0.79-1.87)	.38		
ICG 15R ≥20	1.56 (0.89-2.74)	.13		
AFP >200	1.54 (0.94-2.53)	.09		
AFP-L3 >10	1.37 (0.85-2.19)	.19		
PIVKA-II >100	2.17 (1.40-3.36)	.0005	2.36 (1.10-5.05)	.025
No. of tumors >1	2.28 (1.05-4.92)	.035	2.26 (0.95-5.39)	.06
Tumor size >50 mm	1.66 (1.04-2.66)	.034	1.54 (0.69-3.45)	.29
Ig (+)	1.56 (0.59-4.13)	.38		
Fc (+)	1.63 (0.86-3.08)	.14		
Fc-inf (+)	1.29 (0.78-2.12)	.33		
SM (+)	1.74 (0.49-6.12)	.39		
vp (+)	1.30 (0.78-2.17)	.31		
Histological type (poor)	1.80 (1.07-3.01)	.025	1.86 (0.72-4.80)	.20
Hepatic fibrosis >2	1.79 (1.15-2.77)	.01	1.75 (0.79-3.85)	.17
High LOX expression	5.36 (3.37-8.54)	<.0001	2.52 (1.20-5.28)	.015

AFP, alpha-fetoprotein; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP; Eg/Ig, expansive growth/invasive growth; Fc, capsule formation; Fc-inf, cancerous infiltration of the capsule; HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C antibody; ICG 15R, indocyanine green retention rate after 15 min; IQR, interquartile range; LOX, lysyl oxidase; PIVKA-II, protein induced by vitamin K absence or antagonist-II; SM, surgical margin; vp, portal vein invasion.

GSE10140, GSE14520, GSE9843 and GSE1898. We found five gene sets that indicate HIF-1α activation and 12 that indicate hypoxia; these gene sets were associated with high LOX expression, and the significance of each gene set and cohort is shown in Figure 4. We also analyzed the correlation between LOX and each downstream gene of the HIF pathway and the results are shown in Figure 4. LOX had significant correlation with some of the downstream genes such as DDIT4 (DNA damage inducible transcript 4), TLR1 (Toll like receptor 1) and TGFB3 (transforming growth factor beta 3).

3.7 | LOX regulates TWIST-induced EMT which is regulated by the HIF-1α pathway

Figure S5 shows the results of LOX mRNA expression in each cell line by qRT-PCR. We selected Sk-hep1 for the in vitro knockdown experiment because of the high LOX expression of Sk-hep1 cells. Furthermore, siRNAs targeting LOX and targeting HIF-1α adequately knocked down LOX and HIF-1α mRNA expression, respectively suggesting that the si RNAs works correctly.

Figure 5A shows the movement of cells in each well and quantification of the moving distance. When the knockdown of LOX or HIF-1α by RNA interference was applied to the Sk-hep1 cell line, the migratory capability was significantly decreased as compared with control (siLOX; $P = .014, .017$, siHIF-1α; $P = .013, .028$) (Figure 5A). In the invasion assay, when we applied the knockdown of LOX or HIF-1α by RNA interference to the Sk-hep1 cell line, the invasion capability was significantly decreased as compared with control (siLOX; $P = .02, <.001$, siHIF-1α; $P < .001$) (Figure 5B). In the CCK-8 assay, cell proliferation of Sk-hep1 was not significantly suppressed by the knockdown of LOX or HIF-1α compared with control (siLOX; $P = .30, .37$, siHIF; $P = .13, .49$) (Figure S6A). Furthermore, there was no morphological change in the knockdown of LOX or HIF-1α (Figure S6B).

Figure 5C shows the expression change of the EMT markers when we knocked down LOX or HIF-1α, or both LOX and HIF-1α. Message level of LOX expression was suppressed when LOX or HIF-1α were knocked down (all, $P < .001$). Conversely, knocking down of LOX did not suppress HIF-1α expression ($P = .33$), suggesting that HIF-1α is upstream of LOX. Furthermore, TWIST expression and Vimentin were suppressed by knocking down either LOX or HIF-1α or both LOX and HIF-1α (TWIST; $P = .04$, $P = .01$, $P = .005$, Vimentin; $P = .05$, $P = .02$, $P = .04$). Slug expression was significantly suppressed by the knockdown of HIF-1α ($P = .04$) and tended to be suppressed by LOX or both LOX and HIF-1α knockdown ($P = .053$, $P = .14$).

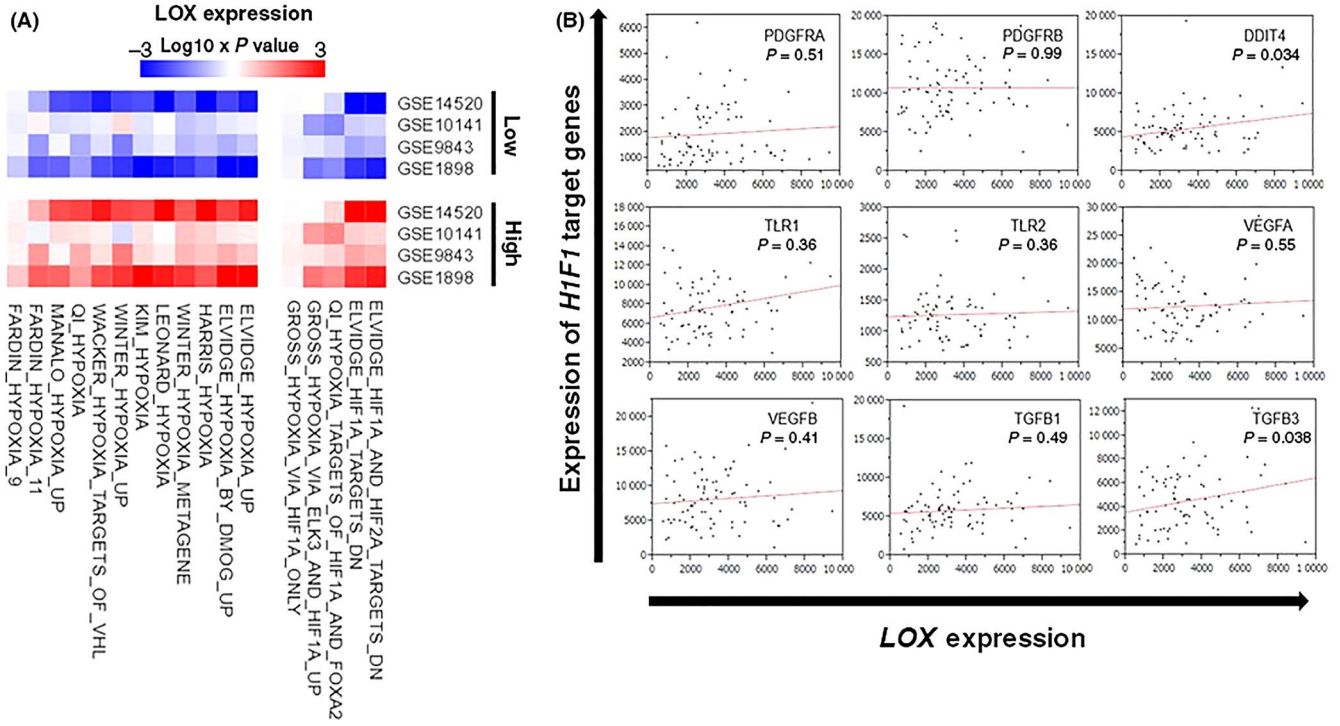

FIGURE 4 Association between lysyl oxidase (LOX) and hypoxia inducible factor (HIF)-1α or hypoxia related gene sets on the exhaustive DNA expression database. A, Results of gene set enrichment analysis (GSEA) using public databases (GSE14520, GSE10141, GSE9843 and GSE1898). Right panel shows the association between LOX expression and HIF1α-associated gene sets, and the left panel shows the association between LOX expression and hypoxia-associated gene sets. Red and blue color indicates a positive and negative correlation, respectively, and blue color shows the negative correlation between LOX expression and gene set enrichment. B, Correlation between LOX and each downstream gene of the HIF pathway in the dataset GSE10141

From this result, it was suggested that HIF-1α is located upstream of LOX and TWIST, and that Vimentin and Slug are located downstream of LOX.

4 | DISCUSSION

In the present study, by searching a large transcriptome database exhaustively, we identified LOX as a key molecule associated with early HCC recurrence within 2 years; this may be due to IM. We also validated the clinical role of LOX as a predictor of HCC recurrence from IM in an independent HCC cohort, and found that LOX is clinically associated with EMT markers and the HIF-1α/hypoxia cascade.

In HCC, early recurrence caused by IM has been a major problem that results in life-threatening tumor progression after curative surgery. Predicting recurrence from IM has therefore been an urgent problem for HCC treatment. In this study, we exhaustively searched for a molecular biomarker to predict the IM of HCC, and for a molecule that might serve as a drug target for the prevention of IM. This search yielded LOX, which is highly expressed in cancerous tissue compared with adjacent normal liver, suggesting that LOX can be the target of a molecular targeting drug that has less effect on normal liver tissue than on cancerous tissue.

It has been reported that LOX is associated with tumor malignancy, especially with distant metastasis that includes bone metastasis, in several types of cancers such as thyroid, breast, nasopharyngeal, gastric and pancreatic cancers.[23,24,27,34-37] With reference to HCC, some reports have shown an association between LOX and angiogenesis markers such as vascular endothelial growth factor (VEGF), but none has revealed an association with EMT or metastasis in HCC. We exhaustively examined the role of LOX in HCC using a large transcriptome matrix, which showed that high LOX expression is closely associated with EMT. Venning et al[38] reported that LOX crosslinks with collagen and forms an ECM in HCC tumors; LOX also regulates EMT by translocating TWIST into the nucleus of HCC cells. In the present study, we validated the correlation between LOX and EMT markers using clinical HCC samples, suggesting that high LOX expression results in EMT in HCC. In addition, there was no significant difference in the suppression of downstream genes between the single knockdown of each of LOX or HIF-1α and the double knockdown of both LOX and HIF-1α. The result suggests that LOX and HIF-1 exist on the same cascade. In contrast, Wong et al[39] reported that lysyl oxidase-like 2 (LOX-L2) is also associated with the metastasis of HCC by affecting the tumor microenvironment and creating a metastatic niche. We then also analyzed the clinical significance of LOX-L2 on HCC recurrence. LOX-L2 was significantly associated with early recurrence within 2 years ($P = .0008$) but not with overall survival ($P = .06$) (Figure S4), suggesting that LOX-L2 is involved in IM. This result supported the results of Wong et al showing that

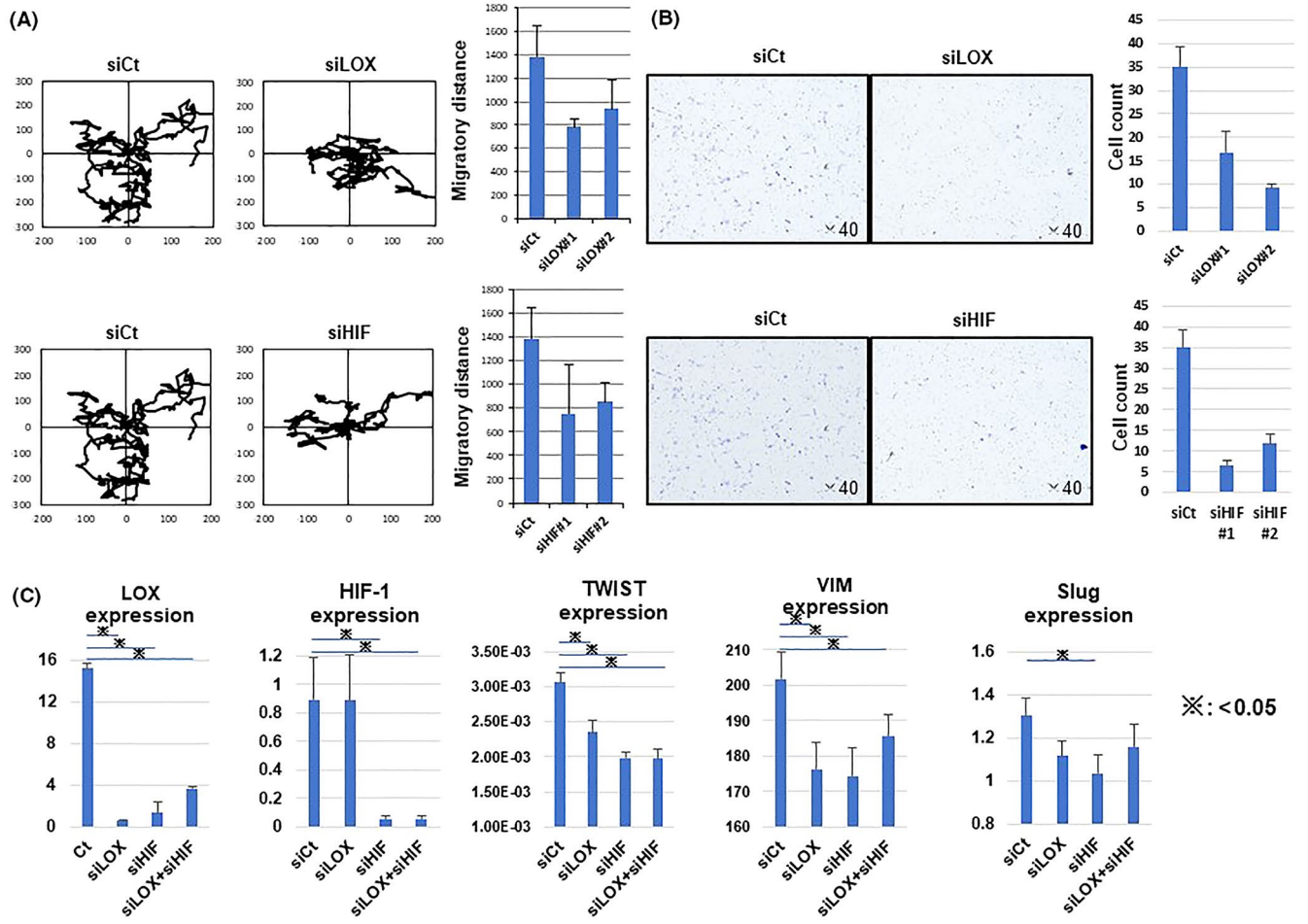

FIGURE 5 Investigation of the action of lysyl oxidase (LOX) and hypoxia inducible factor (HIF)-1α in vitro. A, Migration assay of the Sk-hep1 cell line when LOX or HIF-1α expression was knocked down by RNA interference (n = 3). B, Invasion assay of the Sk-hep1 cell line when LOX or HIF-1α expression was knocked downed by RNA interference (n = 3). C, Comparison of the expression of each gene (LOX, HIF-1α, TWIST, Vimentin and Slug) when LOX or HIF-1α or LOX and HiF-1α expression was knocked down by RNA interference (n = 3)

LOX-L2 also has an important role in the recurrence of HCC. We compared the expression of LOX in the background liver, LOX expression in the early recurrence group tended to be low compared with the other group which include the late recurrence and no recurrent patients ($P = .06$). In contrast, when we compared the all-recurrence group and no-recurrence group, LOX expression in the all-recurrence group tended to be high compared with the no-recurrence group ($P = .40$) (Figure S7). This result may suggest that early recurrence mainly occurred from IM recurrence. On the other hands, LOX expression in the background liver may influence tumorigenesis by MC and develop the late recurrence.

In addition, Erler et al and Reynaud et al[33,40-43] showed that LOX expression was increased by HIF-1α in the intratumoral hypoxic condition, and resulted in distant metastasis or bone metastasis. In the current study, comprehensive analysis using a large HCC transcriptome matrix showed that high LOX expression correlates with the expression of HIF-1α and the hypoxia pathway. These results suggest that EMT is promoted in HCC through HIF-1α and LOX under the intratumoral hypoxic condition during the process of IM. This hypoxic condition may be influenced by larger tumor size because

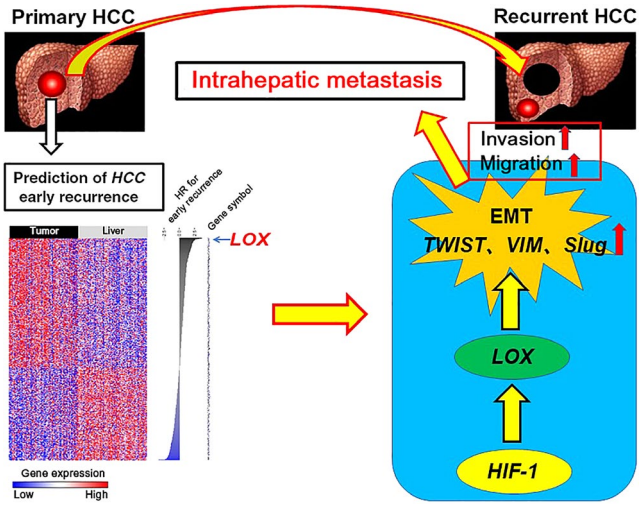

FIGURE 6 Schematic diagram of the regulation of lysyl oxidase (LOX) of epithelial-mesenchymal transition (EMT) and intrahepatic metastasis. By inducing EMT-related genes (ie, TWIST, Vimentin, Slug), LOX plays an important role in the regulation of EMT. HCC, hepatocellular carcinoma; HIF-1α, hypoxia inducible factor-1α

oxygen cannot reach the center of a large tumor. Our result that high LOX expression was associated with larger tumor size may support this hypothesis. In HCC, IFN treatment and direct acting antiviral (DAA) treatment for hepatitis C, nucleic acid analog treatment for hepatitis B, or retinoic acid are recognized preventive treatments for multicentric carcinogenesis based on the background liver disease,[8,9,44-48] but treatment for the prevention of recurrence from IM has not yet been developed. Llovet et al[5] showed that the adjuvant sorafenib did not decrease tumor recurrence.

According to our results, LOX is strongly correlated with recurrence from IM, and comprehensive gene analysis suggests that LOX induces EMT and leads to IM (Figure 6). As a future research topic, we believe that drug therapy targeting LOX should result in the suppression of recurrence from IM. In conclusion, we showed that LOX expression level predicts HCC recurrence from IM, and that LOX regulates EMT of HCC cells in conjunction with HIF-1α expression and hypoxia.

ACKNOWLEDGMENT

This work was supported by JSPS (Japan Society for the Promotion of Science) KAKENHI Grant numbers 15K10191 and 16K19939.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Umezaki N, Nakagawa S, Yamashita Y-I, et al. Lysyl oxidase induces epithelial-mesenchymal transition and predicts intrahepatic metastasis of hepatocellular carcinoma. *Cancer Sci*. 2019;110:2033-2043. https://doi.org/10.1111/cas.14010