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RESEARCH PAPER

LOX and ACSL5 as potential relapse markers for pancreatic cancer patients

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ABSTRACT
Pancreatic cancer is one of the most malignant diseases and has a poor prognosis. The screening and validation of biomarkers with predictive value for prognosis and treatment efficacy are important. To identify potential prognostic markers of pancreatic cancer patients, we conducted a study that included 99 pancreatic cancer patients. Three patients with PFS>18 months were enrolled in the treat group, and three patients with PFS<12 months were enrolled in the control group. Differentially expressed genes (DEGs) between these two groups were analyzed by whole-genome expression microarray. A total of 178 DEGs were identified, including 110 up-regulated and 68 down-regulated genes. Next, 24 candidate genes were selected for validation by qPCR based on fold change and previous studies. The results showed that the mRNA levels of four candidate genes, including ACSL5, SLC44A4, LOX, and TOX3, were correlated with PFS. Immunohistochemical staining was performed to validate the protein expression levels of these four markers. The results showed that patients with LOX ^{high}, ACSL5 ^{low} and TOX3 ^{low} expression had a significantly shorter PFS than those with LOX ^{low}, ACSL5 ^{high} and TOX3 ^{high} expression. Multivariable analysis revealed differentiation, tumor stage, LOX expression, and ACSL5 expression were independent prognostic factors for PFS. Then, we use the TCGA database to explore the underlying mechanism of LOX influence pancreatic cancer progression. Protein–protein interaction network of ACSL5 was established by STRING to uncover the potential regulation mechanism. Our findings reveal that LOX and ACSL5 are potential prognostic markers for the prognosis of pancreatic cancer patients.

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Pancreatic cancer; prognostic markers; LOX; ACSL5; microarray; bioinformatic analysis; EMT

Introduction

Pancreatic cancer is a common digestive system cancer with a high incidence rate, and mortality.[1] Although there has been a steady increase in survival for most cancers, the 5-year relative survival for pancreatic cancer is currently 8% in the United States.[2] It is because of diagnosis at a late stage and resistance to chemotherapy and radiotherapy.[2,3] The currently available markers cannot accurately predict survival, and identifying novel markers for use in personalized medicine, that could improve outcomes is difficult. Therefore, it is crucial to find diagnostic and prognostic biomarkers and therapeutic targets.

Recently, considerable progress in bioinformatics and the high-throughput sequencing technique has led to more knowledge regarding whole human genome sequencing.[4] Others have used gene expression microarray analysis to report molecular signatures associated with pancreatic cancer disease progression.[5,6] Hundreds of genes that are differentially expressed in pancreatic cancer tissues compared to normal pancreatic tissues may serve as biomarkers.[7] Although several genes and protein biomarkers have been proposed,[8] their sensitivity and specificity are inadequate. For example, carbohydrate antigen 19–9 (CA19–9) and carcinoembryonic antigen (CEA), which have been commonly used in clinical settings, have thus far not met the initial expectations.[9] Other biomarkers, such as KRAS, CDKN2A, TP53, and SMAD4, have been successful primarily in the study of biological functions, such as apoptosis regulation, cell cycle progression and DNA damage control.[10]

To identify biomarkers that could predict prognosis, we investigated different gene expression profiles with respect to survival. To further evaluate the clinical outcome relevance of these biomarkers, we analyzed survival based on mRNA and protein expression levels and assessed the potential of these genes as novel prognostic markers for evaluating pancreatic cancer.

Results

Clinicopathological characteristics and follow-up of pancreatic cancer patients

The clinicopathological characteristics of the 99 pancreatic cancer patients are summarized in Table 1. In this study, a total of 99 patients (60 males, 39 females) with a median age of 61 years (ranging from 36 to 80) were enrolled. Among them, six patients were diagnosed with well-differentiated tumors (6.1%), 75 were diagnosed with moderately differentiated tumors (75.7%), and 18 patients were diagnosed with poorly differentiated tumors (18.2%). A total of 46 tumors were classified as T1-T2 (46.5%), and 53 tumors were classified as T3-T4 (43.5%). Twenty-five cases (25.3%) of the lymphatic invasion were present, and 74 cases (74.7%) had no lymphatic invasion. Thirty-one cases

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Table 1. Association between clinicopathologic factors and overall survival (OS) and progression-free survival (PFS) times in pancreatic carcinoma patients.

Variable	No. (%)	PFS (M± SD, months)	*P Value*	OS (M± SD, months)	*P Value*
Sex					
Female	39(39.4%)	18.47 ± 13.61	0.124	21.98 ± 14.13	0.105
Male	60(60.6%)	14.48 ± 11.69		17.73 ± 11.58	
Age (years)					
≤60	48(48.5%)	14.95 ± 11.98	0.400	18.64 ± 13.22	0.565
>60	51(51.5%)	17.09 ± 13.13		20.12 ± 12.37	
Degree of differentiation					
High	6(6.1%)	19.86 ± 10.93	**0.002**	29.27 ± 15.11	**<0.001**
Moderate	75(75.7%)	17.62 ± 12.67		20.79 ± 12.25	
Poor	18(18.2%)	8.22 ± 9.77		10.31 ± 9.27	
T stage					
T1-T2	46(46.5%)	20.15 ± 14.33	**0.010**	22.41 ± 14.03	0.054
T3-T4	53(53.5%)	12.49 ± 9.60		16.79 ± 11.00	
Lymphatic invasion					
Absent	74(74.7%)	17.90 ± 13.21	**0.014**	21.34 ± 13.30	**0.014**
Present	25(25.3%)	10.57 ± 8.47		13.66 ± 8.91	
Stage					
I	31(31.3%)	22.86 ± 15.00	**0.010**	24.39 ± 15.00	0.053
IIA	40(40.4%)	14.81 ± 10.63		19.35 ± 11.69	
IIB	24(24.2%)	10.60 ± 8.66		13.81 ± 9.06	
III	4(4.0%)	8.46 ± 7.24		12.82 ± 9.76	
Vascular invasion					
Absent	82(82.8%)	17.22 ± 12.80	**0.007**	20.46 ± 13.04	**0.037**
Present	17(17.2%)	8.54 ± 7.53		13.11 ± 9.30	

(31.3%), 40 cases (40.4%), 24 cases (24.2%) and 4 cases (4%) were diagnosed as stage I, IIA, IIB, and III, respectively, according to AJCC Guidelines Version 7. The median progression-free survival (PFS) was 11 months, while the median overall survival (OS) was 17.03 months. The 2-year survival was 35.35%. Generally, the PFS was significantly associated with differentiation ($P = 0.002$), TNM stage ($P = 0.01$) and vascular invasion ($P = 0.007$). The OS was significantly associated with differentiation ($P < 0.001$), lymphatic invasion ($P = 0.014$) and vascular invasion ($P = 0.037$).

Screening for differentially expressed genes (DEGs) between pancreatic cancer patients with different prognoses by gene expression microarray

To identify potential prognostic markers that are correlated with the survival of pancreatic cancer patients, six patients were divided into two groups according to their prognosis; and the DEGs were then analyzed by gene expression microarray (Figure 1). Three patients with PFS>18 months were enrolled in the treatment group, and three patients with PFS<12 months were enrolled in the control group. According to the results of the microarray analysis, 178 DEGs were significantly different (|FC|>1), of which the expression of 110 genes (65.48%) was up-regulated, and 68 genes (34.52%) was down-regulated.

Analysis of mRNA expression level and prognostic value for survival of differentially expressed genes (DEGs)

To validate the prognostic value of the differentially expressed genes, the expression levels of 24 candidate genes were assayed by qRT-PCR (Table 2). The correlation between the mRNA expression levels of these genes and the survival of the patients was analyzed. ROC curve for the PFS showed that the expression levels of six mRNAs, including ACSL5 (AUC = 0.281, 95%CI: 0.116–0.447, $P = 0.023$), SLC44A4 (AUC = 0.295, 95% CI: 0.126–0.463, $P = 0.033$); LOX (AUC = 0.695, 95%CI: 0.551–0.840, $P = 0.042$); TOX3 (AUC = 0.306, 95%CI: 0.160–0.453, $P = 0.044$); SHISA3 (AUC = 0.310, 95%CI: 0.159–0.461, $P = 0.048$); APOBEC1 (AUC = 0.310, 95%CI: 0.141–0.479, $P = 0.048$), were significantly correlated with the PFS of the patients. However, we did not find any mRNA to be correlated with the OS (Figure 2).

To investigate whether individual gene expression level could predict the survival of pancreatic cancer patients, the survival of the high and low expression groups was analyzed. The expression levels of four candidate genes, including LOX, ACSL5, TOX3, and SLC44A4, were correlated with the PFS of the patients. High expression of LOX was correlated with a poor PFS, while patients with high expression levels of ACSL5, TOX3 or SLC44A4 had significantly longer survival time than the low expressers. (Figure 3).

Evaluation of protein expression levels and association of clinicopathologic parameters with four candidate biomarkers

To further evaluate the prognostic value of the four candidate biomarkers whose mRNA expression was correlated with the PFS, the protein expression of these markers was examined by IHC assay (Figure 4). The association of the expression level of these four biomarkers with the clinicopathologic parameters was analyzed. High expression of LOX was significantly correlated with the presence of lymphatic invasion, recurrence at 18 months and death at 18 months. In addition, low expression of ACSL5 and TOX3 was related to the tumor stage, a higher ratio of recurrence at 18 months and a higher death rate at 18 months. However, the expression of SLC44A4 had no significant correlation with the clinicopathological features of pancreatic cancer patients (Table 3).

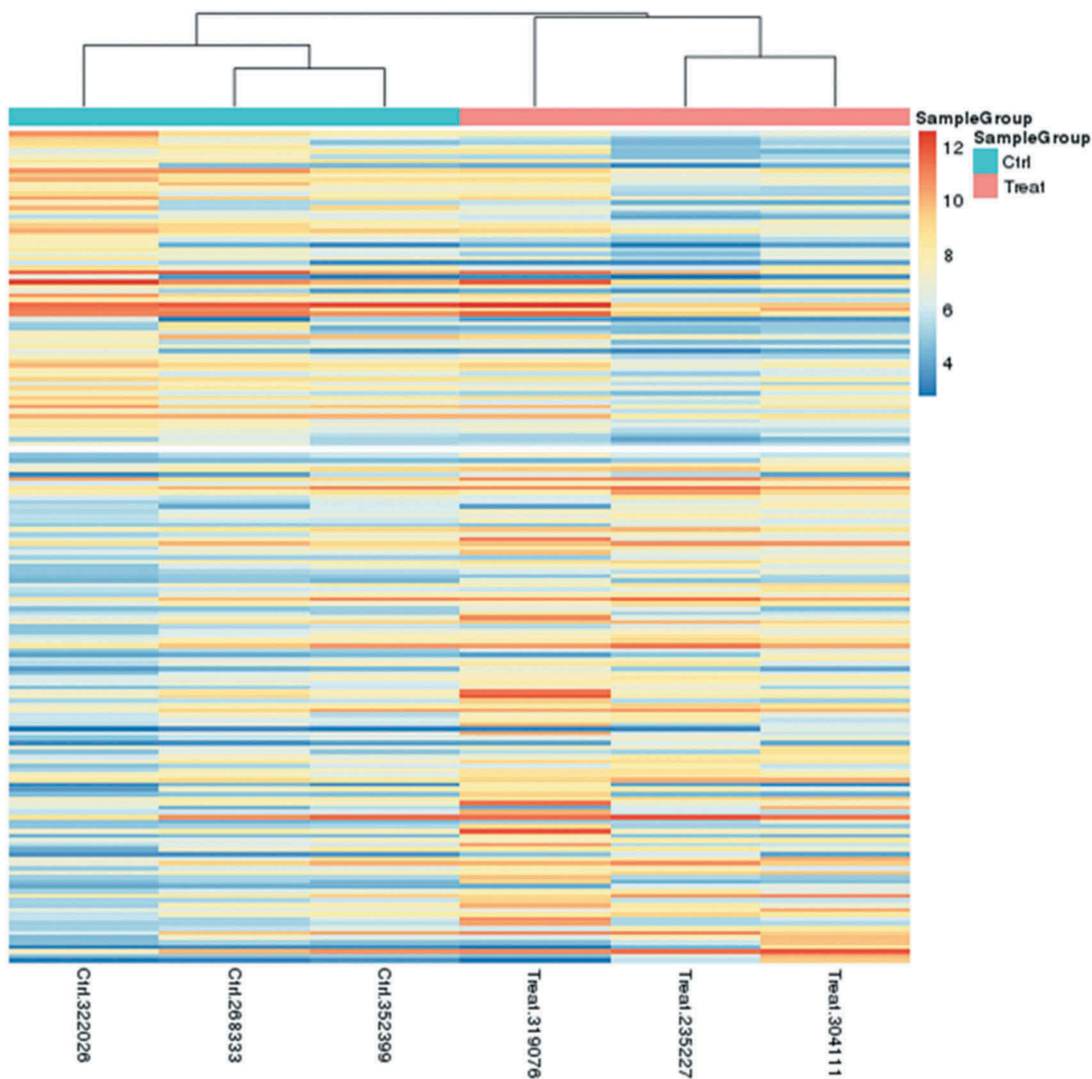

Figure 1. Hierarchical clustering analysis of gene expression levels between the treat group and control group.
To identify differentially expressed genes in the treat group (OS>24 months) and control group (OS<12 months), tumor tissues from patients were collected and assayed by gene expression microarray. A total of 110 up-regulated genes and 68 down-regulated genes were identified in the treat group. "Red" and "Blue" indicate up-regulated and down-regulated transcripts, respectively.

Prognostic value of LOX, ACSL5, SLC44A4, and TOX3 for the evaluation of pancreatic cancer patients

To investigate whether the expression levels of these four biomarkers correlated with the survival probability of pancreatic cancer patients, a survival analysis was performed. As shown in Figure 5, LOX ^{high}, ACSL5 ^{low} and TOX3 ^{low} patients had a significantly poorer PFS than LOX ^{low}, ACSL5 ^{high} and TOX3 ^{high} patients (P = 0.037; P = 0.031 and P = 0.019). However, there was no significant difference between the expression of SLC44A4 and the survival of pancreatic cancer patients. These results indicated that LOX, ACSL5, and TOX3 have prognostic value for evaluating pancreatic cancer patients. Multivariable analysis was also performed to determine the independent prognostic value of LOX, ACSL5 and TOX3 expression (Table 4). Differentiation and tumor stage were also included based on their significance in the univariable analysis. For PFS, differentiation (P = 0.005), tumor stage (P = 0.033), LOX expression (P = 0.017) and ACSL5 expression (P = 0.026) were independent prognostic factors.

The underlying regulation mechanism of LOX and ACSL5 in cancer progression

As LOX and ACSL5 were independent prognostic factors for pancreatic cancer, the underlying mechanism of LOX and ACSL5 was valuable to explore. Pancreatic cancer progression is closely related to the lack of oxygen and epithelial-mesenchymal transition (EMT).[11,12] To deep mine the potential mechanism of LOX in pancreatic cancer, we analyze the relationship between LOX and HIF-1A, CDH1, CDH2, CTNNBIP1, VIM, TWIST1, SNAI1, and SNAI2. In Figure 6, LOX and HIF-1A were positively correlated ($P < 0.001$, r = 0.579). Also, CDH2, VIM, TWIST1, SNAI1 and SNAI2 expression were positively correlated with the expression of LOX, which were signals of EMT (CDH2, $P < 0.001$, r = 0.395; VIM, $P < 0.001$, r = 0.658;

Table 2. Primers designed for the qRT-PCR validation of candidate gene mRNA levels and β-actin.

Symbol	Forward primer (5 'to 3')	Reverse primer (5 'to 3')
ACSL5	AAGGCATTGGTGCTGATAGG	TCAGGTCTTCTGGGCTAGGA
APOBEC1	CAAAAACACCACCAATCACG	TAATAGCCTGGGAGCATTCC
BCL2L15	AACCATTAAGGGACAGACAGGA	GCCACCTGTCCCACTACTTC
DEFB1	TCACTCCCAGCTCACTTGCAGC	ATGGCCTCAGGTGGTAACTTTCTCA
EGR1	GTACAGTGTCTGTGCCATGGATTTC	GAGGATCACCATTGGTTTGCTTG
GABRP	CAGCCAACAGTACCAAAAGTGATT	TGGCGAGATTGTCTAAAATAACTGA
GPX2	GCCTCCTTAAAGTTGCCATA	GCCCAGAGCTTACCCA
INHBA	GCAGTCTGAAGACCACCCTC	ATGATCCAGTCATTCCAGCC
LOX	GCCGACCAAGATATTCCTGGG	GCAGGTCATAGTGGCTAAACTC
MT2A	CCGACTCTAGCCGCCTCTT	GTGGAAGTCGCGTTCTTTACA
OCLN	CAGGGAATATCCACCTATCACTTCAG	CATCAGCAGCAGCCATGTACTCTTCAC
PCDH17	GCACGGTGATCGGGAACAT	GCGCTGCTTGGTGTAGAGG
PLS1	CAAGAGGGAAGGGATTACTGC	TCATTCTCCAGGGCTTTGTT
SFRP2	AACCTACATCAACCGAGATACCA	ACAGCACCGATTTCTTCAGG
SFRP4	GTGGCGCTCAAGGATGATG	CTCTTCCCACTGTATGGATC
SH3BGRL2	CGTGTTCATCGCCTCTTCCT	CCACCTCCTCAAACTCTATCT
SHISA3	GTCTACGTCCCCTTTCTCATCG	AGGTGCAACAATAAATAGCCACT
SLC12A2	CAGCCCTCCAGAATGGTACT	CAACTTTCCTGTGTGCTTTCA
SLC44A4	GGGAAGCCAGTCAAATACGA	CCCCACCACGATGTAACCTA
SLIT2	CGTTTGGAAAATGTGCAGCATAA	TTCGATTGCTTCTCAACATCAAAGT
TFPI2	CCAGATGAAGCTACTTGTATG	GCACATGCACGTTTGCAATC
TIMP3	TCTGCAACTCCGACATCGT	TTGGTGAAGCCTCGGTACAT
TOX3	ATTCCACCAATCACGCCTCC	GGATCGCTGAGGGCTTGAAA
TSPAN8	TCTATGGTCCTGTATTGCCAGATC	GACAGCTGCTCCTGACTTATATA
β-actin	GATGACCCAGATCATGTTTGAG	AGGGCATACCCCTCGTAGAT

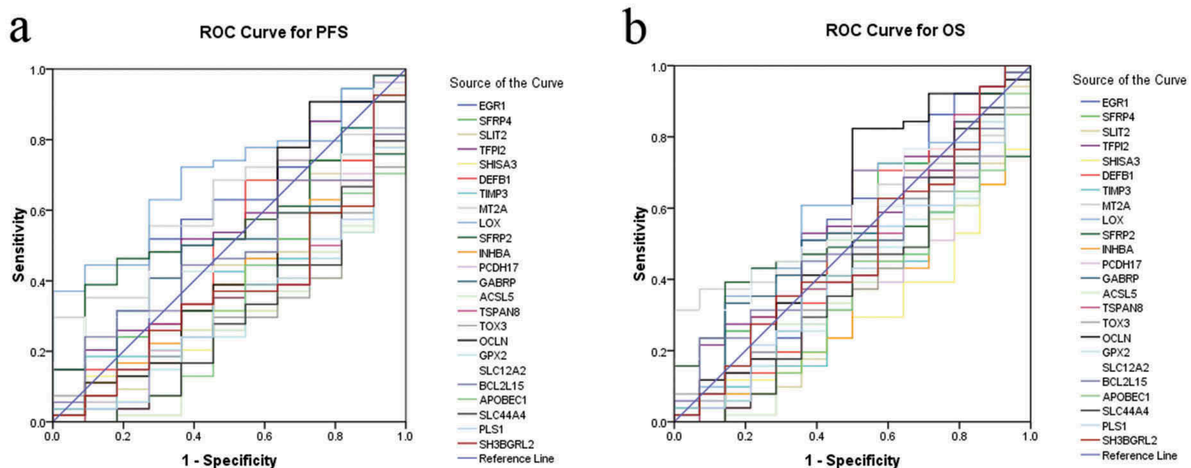

Figure 2. ROC (receiver operating characteristic) curves of 24 candidate genes for prognosis (n = 68).
ROC curves for the PFS showed that the mRNA level of six candidate genes including ACSL5 (AUC = 0.281, 95% CI:0.116–0.447, P = 0.023), SLC44A4 (AUC = 0.295, 95% CI:0.126–0.463, P = 0.033), LOX (AUC = 0.695, 95% CI:0.551–0.840, P = 0.042), TOX3 (AUC = 0.306, 95% CI:0.160–0.453, P = 0.044), SHISA3 (AUC = 0.310, 95% CI:0.159–0.461, P = 0.048) and APOBEC1 (AUC = 0.310, 95% CI:0.141–0.479, P = 0.048), were correlated with PFS (a). ROC curves for the OS indicated that the mRNA levels of all of the 24 candidate genes were not significantly correlated with the overall survival (b).

TWIST1, $P < 0.001$, r = 0.708; SNAI1, $P < 0.001$, r = 0.604; SNAI2, $P < 0.001$, r = 0.794). The expression of CTNNBIP1 was negatively correlated with the expression of LOX (P = 0.021, r = −0.171), but there is no statistical correlation between CDH1 and LOX (P = 0.360, r = −0.068). Functional protein association network of ACSL5 was analyzed by an online biological tool, String. As the results are shown in Figure 7, ACSL5 interacted with ACADL, ACOX1, ACOX2, CPT1A, CPT1B, CPT1C, ELOVL1, ELOVL6, FADS2 and FASN.

Discussion

Pancreatic cancer is a malignant disease with a very poor prognosis. Identification of prognostic biomarkers for personalized treatment might improve the outcomes. In this study, we identified biomarkers that could predict the prognosis of pancreatic cancer patients. The different gene expression profiles between the two groups of patients with different survival times, one group with PFS>18 months and one group with PFS<12 months, were determined. Then, the mRNA and protein expression of candidate genes was validated by qRT-PCR and IHC assays. The results indicated that LOX and ACSL5 had prognostic value for evaluating pancreatic cancer patients.

We found that LOX ^{high} patients had a higher ratio of lymphatic invasion, higher recurrence/death rates at 18 months and significantly poorer PFS/OS times. LOX is one of the most important regulators of the extracellular matrix (ECM). LOX family members, including LOX, LOXL1, LOXL2, LOXL3, and LOXL4, mediate collagen cross-linking and thus promote ECM stiffening.[13–15] It has

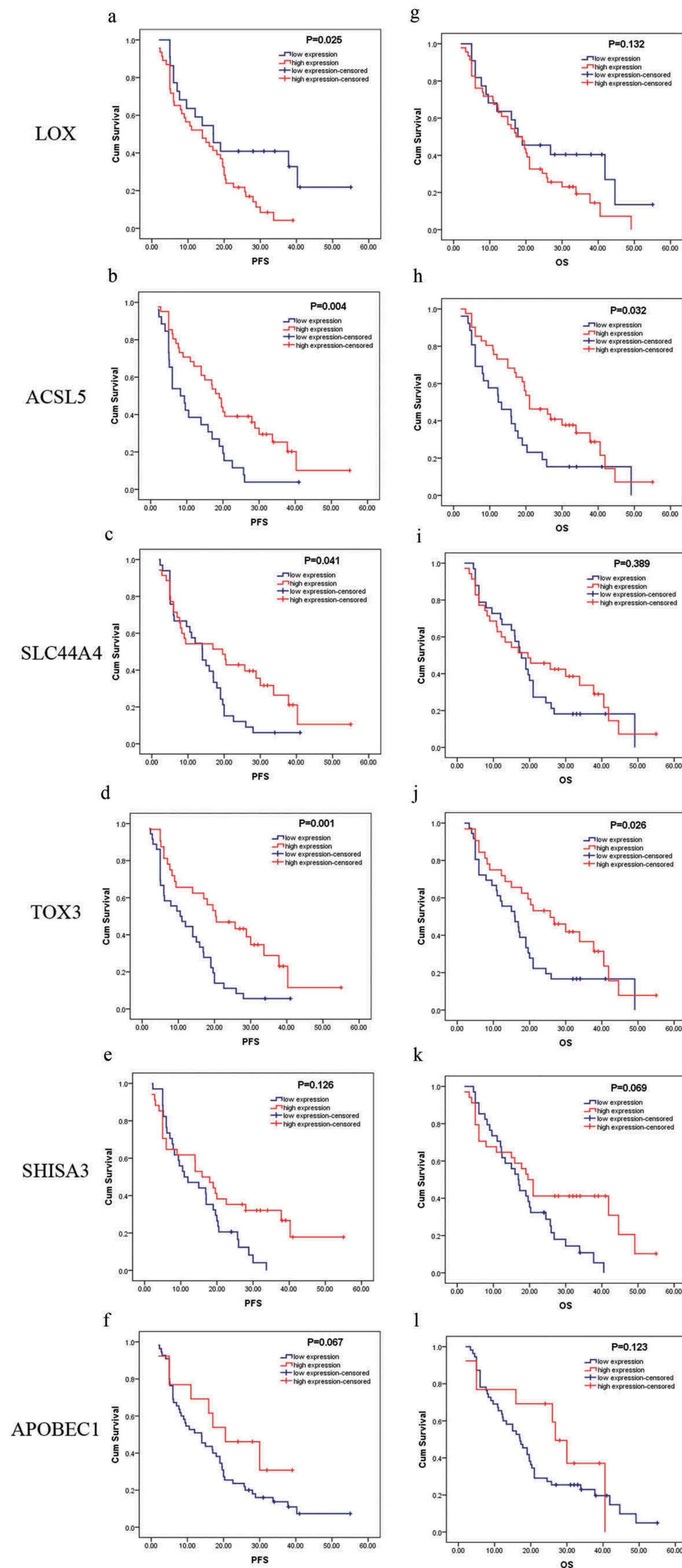

Figure 3. The relationship between the mRNA expression levels of six candidate genes and the survival of pancreatic cancer patients (n = 68).

Pancreatic cancer patients were divided into high expression and low expression group according to the mRNA levels of each candidate gene. The PFS and OS of these two groups were analyzed. The results showed that the PFS was correlated with the expression levels of LOX, ACSL5, SLC44A4 and TOX3 ($P = 0.025$, $P = 0.004$, $P = 0.041$, $P = 0.001$) but not with the mRNA levels of SHISA3 and APOBEC1 (a-f). The OS survival was significantly different in the ACSL5 and TOX3high and low expression groups ($P = 0.032$, $P = 0.026$) (g-l).

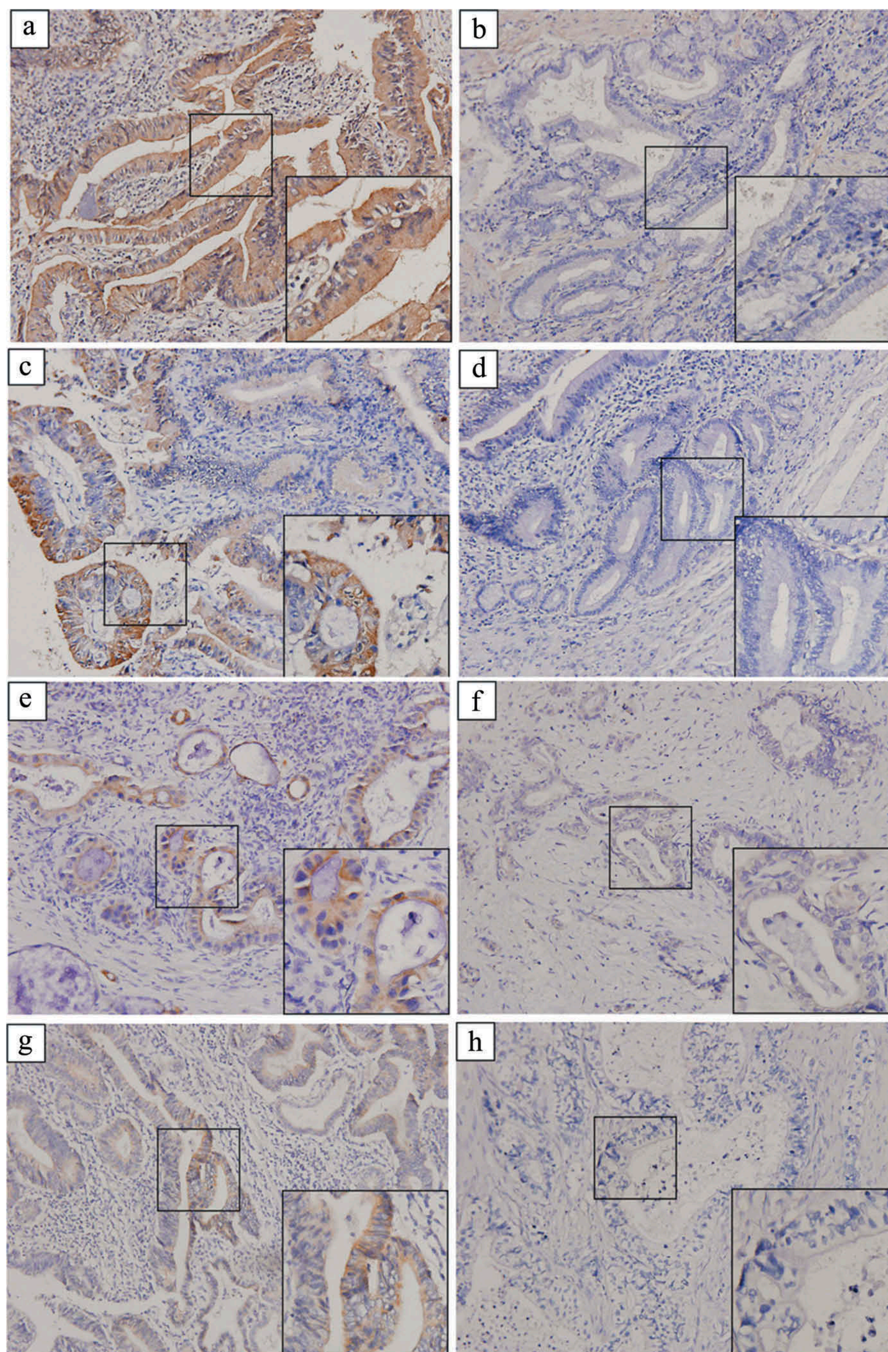

Figure 4. IHC staining of LOX, ACSL5, TOX3 and SLC44A4 in pancreatic cancer tissues (n = 64) (200×).
Expression of LOX (a, b), ACSL5 (c, d), SLC44A4 (e, f) and TOX3 (g, h) in pancreatic cancer tissues by IHC staining. The figures show positive (a, c, e, g) and negative cases (b, d, f, h). Images are at 20 × 10 magnification, and inserts are at 40 × 10 magnification.

been reported that LOX could increase migration, invasion, and metastasis dissemination and participate in causing chemotherapy resistance by limiting the gemcitabine intratumoral distribution.[16–18] We also analyzed the relationship between LOX and HIF-1a or EMT markers using TCGA datasets. The results showed that LOX was correlated with HIF-1a, CDH2, VIM, TWIST1, SNAI1 and SNAI2, and CTNNBIP1. It has been reported that activation of LOX is sufficient for hypoxic repression of E-cadherin.[19] Up-regulation of HIF-1A could transcriptional induce the expression of LOX and subsequently repressed E-cadherin expression in ovarian carcinoma cells, leading to an enhance in cell motility and invasiveness.[20] Moreover, LOX could transcriptionally regulate SNAI2, which result in the down-regulation of TIMP4 and weaken the ability of cancer cellular invasion and migration.[21] LOX also could alter the invasiveness of epithelial cells in gastric cancer.[22] Our data showed that the increased LOX expression in pancreatic cancer patients was correlated with EMT and poor survival time. In consideration of the junction role of LOX in cancer progression, targeting LOX might improve outcome in pancreatic cancer.

Table 3. The relationship between clinicopathological characteristics and LOX/ACSL5/SLC44A4/TOX3 protein expression levels.

Variable	No.(%)	LOX No. (%)		R value	*P Value*	ACSL5 No. (%)		R value	*P Value*	SLC44A4 No. (%)		R value	*P Value*	TOX3 No. (%)		R value	*P Value*
		Low	High			Low	High			Low	High			Low	High		
Sex																	
Female	24(37. 5%)	12(50.0%)	12(50.0%)	−0.024	0.846	13(54.2%)	11(45.8%)	0.033	0.799	16(66.7%)	8(33.3%)	−0.186	0.14	9(37.5%)	15(62.5%)	0.194	0.125
Male	40(62.5%)	19(47.5%)	21(52.5%)			23(57.5%)	17(42.5%)			19(47.5%)	21(52.5%)			23(57.5%)	17(42.5%)		
Age (years)																	
≤60	30(46.9%)	14(46.7%)	16(53.3%)	−0.033	0.790	18(60.0%)	12(40.0%)	0.071	0.577	19(63.3%)	11(36.7%)	0.163	0.198	16(53.3%)	14(46.7%)	0.063	0.623
>60	34(53.1%)	17(50.0%)	17(50.0%)			18(52.9%)	16(47.1%)			16(47.1%)	18(52.9%)			16(47.1%)	18(52.9%)		
Degree of differentiation																	
Moderate-High	50(78.1%)	27(54.0%)	23(46.0%)	0.210	0.092	29(58.0%)	21(42.0%)	0.067	0.601	26(52.0%)	24(48.0%)	−0.102	0.422	22(44.0%)	28(56.0%)	−0.227	0.072
Poor	14(21.9%)	4(28.6%)	10(71.4%)			7(50.0%)	7(50.0%)			9(64.3%)	5(35.7%)			10(71.4%)	4(28.6%)		
T stage																	
T1-T2	31(48.4%)	15(48.4%)	16(51.6%)	0.000	0.994	13(41.9%)	18(58.1%)	−0.28	**0.025**	17(54.8%)	14(45.2%)	0.003	0.982	13(41.9%)	18(58.1%)	−0.156	0.217
T3-T4	33(51.6%)	16(48.5%)	17(51.5%)			23(69.7%)	10(30.3%)			18(54.5%)	15(45.5%)			19(57.6%)	14(42.4%)		
Lymphatic invasion																	
Absent	48(73.53%)	27(56.2%)	21(43.8%)	0.271	**0.030**	28(58.3%)	20(41.7%)	0.073	0.568	25(52.1%)	23(47.9%)	−0.091	0.476	22(45.8%)	26(54.2%)	−0.144	0.255
Present	16(26.47%)	5(31.2%)	11(68.8%)			8(50.0%)	8(50.0%)			10(62.5%)	6(37.5%)			10(62.5%)	6(37.5%)		
Stage																	
I	18(28.1%)	11(83.3%)	7(16.7%)	0.159	0.204	6(33.3%)	12(66.7%)	−0.289	**0.021**	9(50.0%)	9(50.0%)	−0.059	0.644	5(27.8%)	13(72.2%)	−0.278	**0.026**
II-III	46(71.9%)	20(43.5%)	26(56.5%)			30(65.2%)	16(34.3%)			26(56.5%)	20(43.5%)			27(58.7%)	19(41.3%)		
Vascular invasion																	
Absent	56(87.5%)	27(48.2%)	29(51.8%)	−0.012	0.925	30(53.6%)	26(46.4%)	−0.143	0.26	31(55.4%)	25(44.5%)	0.036	0.78	25(44.6%)	31(55.4%)	−0.283	**0.023**
Present	8(12.5%)	4(50.0%)	4(50.0%)			6(75.0%)	2(25.0%)			4(50.0%)	4(50.0%)			7(87.5%)	1(12.5%)		
Recurrence at 18 months																	
Absent	28(43.7%)	18(64.3%)	10(35.7%)	0.280	**0.025**	12(42.9%)	16(57.1%)	−0.238	0.058	14(50%)	14(50%)	−0.083	0.514	9(32.1%)	19(67.9%)	−0.315	**0.011**
Present	36(56.3%)	13(36.4%)	23(63.6%)			24(66.7%)	12(33.3%)			21(58.3%)	15(41.7%)			23(63.9%)	13(36.1%)		
Death at 18 months																	
Absent	34(53.1%)	23(67.6%)	11(32.4%)	0.409	**0.001**	15(44.1%)	19(55.9%)	−0.26	**0.038**	18(52.9%)	16(47.1%)	−0.037	0.77	12(35.3%)	22(64.7%)	−0.313	**0.012**
Present	30(46.9%)	8(26.7%)	22(73.3%)			21(70.0%)	9 (30.0%)			17(56.7%)	13(43.3%)			20(66.7%)	10(33.3%)		

Table 4. Multivariable analyses of factors associated with OS and PFS.

Variable	PFS HR(95.0% CI)	PFS *P value*	OS HR(95.0% CI)	OS *P value*
Poor/Moderate-High degree of differentiation	0.366(0.182–0.738)	**0.005**	0.364(0.171–0.777)	**0.009**
I/II-III	0.419(0.188–0.931)	**0.033**	0.686(0.300–1.571)	0.373
LOX^{High}/LOX^{Low}	0.449(0.233–0.865)	**0.017**	0.367(0.174–0.773)	**0.008**
$ACSL5^{High}/ACSL5^{Low}$	2.071(1.089–3.936)	**0.026**	1.891(0.955–3.745)	0.068
$TOX3^{High}/TOX3^{Low}$	1.136(0.598–2.159)	0.697	1.226(0.599–2.509)	0.577

We also found that the expression level of ACSL5 was related to PFS. Low expression of ACSL5 was correlated with tumor stage and the recurrence/death rates at 18 months. ACSL5 (ACSL isoform 5) is involved in enterocytic differentiation and maturation by regulating both pro-apoptotic and anti-proliferative effects.[23,24] Lower expression of ACSL5 has been reported as a prognostic marker for early recurrence in colorectal adenocarcinoma.[25] ACSL5 mediates antiproliferative activities via Wnt2B palmitoylation, which renders Wnt2B unable to leave the mitochondrial membranes; thus, it can-not translocates into the nucleus to promote the intranuclear Wnt signaling.[19] High activity of ACSL5 enhanced caspase-3 and caspase-7 activity in promoting hepatocellular apoptosis, but death receptor-like DR4, DR5 and TNF-R1 were not accompanied upregulation.[26] We analyzed functional protein association network of ACSL5 by an online biological tool, String. The results showed that ACSL5 interacted with lipid metabolism-related proteins ACADL, ACOX1, ACOX2, CPT1A, CPT1B, CPT1C, ELOVL1, ELOVL6, FADS2, and FASN, which indicated ACSL5 might take the role in cancer progression through affecting lipid metabolism.

In conclusion, high expression of LOX indicated lymphatic invasion, poor PFS and OS in pancreatic cancer patients. Furthermore, LOX was expected to affect epithelial-mesenchymal transition through HIF-1a or other oncogenic transcription factors, including SNAI1, SNAI2, and TWIST1. Low expression of ACSL5 indicated higher T stage, advanced AJCC stage, and poor PFS. LOX and ACSL5 might act as prognostic markers for evaluating pancreatic cancer.

Materials and methods

Patient clinical data

A total of 99 pancreatic cancer patients who underwent surgical resection at Tianjin Medical University Cancer Institute & Hospital (TMUCIH) in China between 2008 and 2015 were enrolled (Table 1). Clinical data were collected from patients' records, and all pancreatic cancer was diagnosed by a pathologist at the Department of Pathology, TMUCIH, China. TNM stages were defined based on the AJCC Guidelines Version 7.

The ages of patients ranged from 36 to 80 years old, with a median age of 61. A total of 60 (60.6%) male and 39 (39.4%) female patients participated in this study. The median follow-up time was 18 months (ranging from 1 to 60 months).

Gene expression microarray analysis

Six out of 99 patients were selected to undergo gene screening. The six patients were divided into two groups, the treat and control groups. The treat group included three patients with a long progression-free survival (PFS) (PFS>18 months) and the other three patients with a short PFS (PFS<12 months) were included in the control group.

Total RNA was extracted by using Trizol/Chloroform and then purified with Agencourt Ampure magnetic beads (APN 000132, Beckman Coulter). Sample preparation for microarray processing was carried out according to the GeneChip® WT PLUS Reagent Kit. Briefly, a total of 500 ng RNA was used for a double-round of cDNA synthesis. After fragmentation of the 2nd-cycle single-stranded cDNA (ss-cDNA), the sample was labeled with biotin by terminal deoxynucleotidyl transferase (TdT). Then, the sample was hybridized to the Affymetrix Human Gene 1.0ST Array for 16–18 h at 45° C. Following hybridization, the microarrays were washed and stained with streptavidin-phycoerythrin on the Affymetrix Fluidics Station 450. Microarrays were scanned by using the Affymetrix® GeneChip Command Console (AGCC), which was installed in GeneChip® Scanner 3000 7G. The data were analyzed with the Robust Multichip Analysis (RMA) algorithm in the Partek® Genomics Suite 6.6, the default analysis settings were used, and global scaling was used as the normalization method. The values presented are the log2 RMA signal intensity.

RNA samples and real-time quantitative PCR (qPCR)

Sixty-eight out of 99 patients' genomic RNA samples were deposited in the TMUCIH tumor tissue bank. cDNA was synthesized from 500 ng of total RNA using the PrimeScript™RT Master Mix Kit (TaKaRa, cat # RR037A) according to the manufacturer's instructions. Candidate genes and β-actin were amplified by real-time PCR in a fluorescence reader ABI Step-one system (Applied Biosystems® 7500 Real-Time PCR Systems). Each sample was run in duplicate using the SYBR® Premix Ex Taq™ II (Tli RNase H Plus) Kit (TaKaRa, cat # RR820A). The cycling conditions were as follows: initial enzyme activation at 95°C for 30 s, followed by 40 cycles of denaturing at 95°C for 5 s, and then annealing/extension at 60°C for 34 s. Ratio = $2^{-\Delta Ct}$ was used to calculate the relative expression of the mRNA, in which ΔCt = (Ct target gene − Ct β-actin). Primer sequences used in this study are displayed in Table 2.

Immunohistochemical (IHC) staining and scoring analysis

Serial sections (4μm) were processed for immunohistochemical staining as followings: the sections were heated in a microwave oven at 65°C for 2 h, then deparaffinized with xylene and dehydrated by gradient ethanol. After washing, the sections were soaked in sodium citrate buffer (pH = 6.0) for

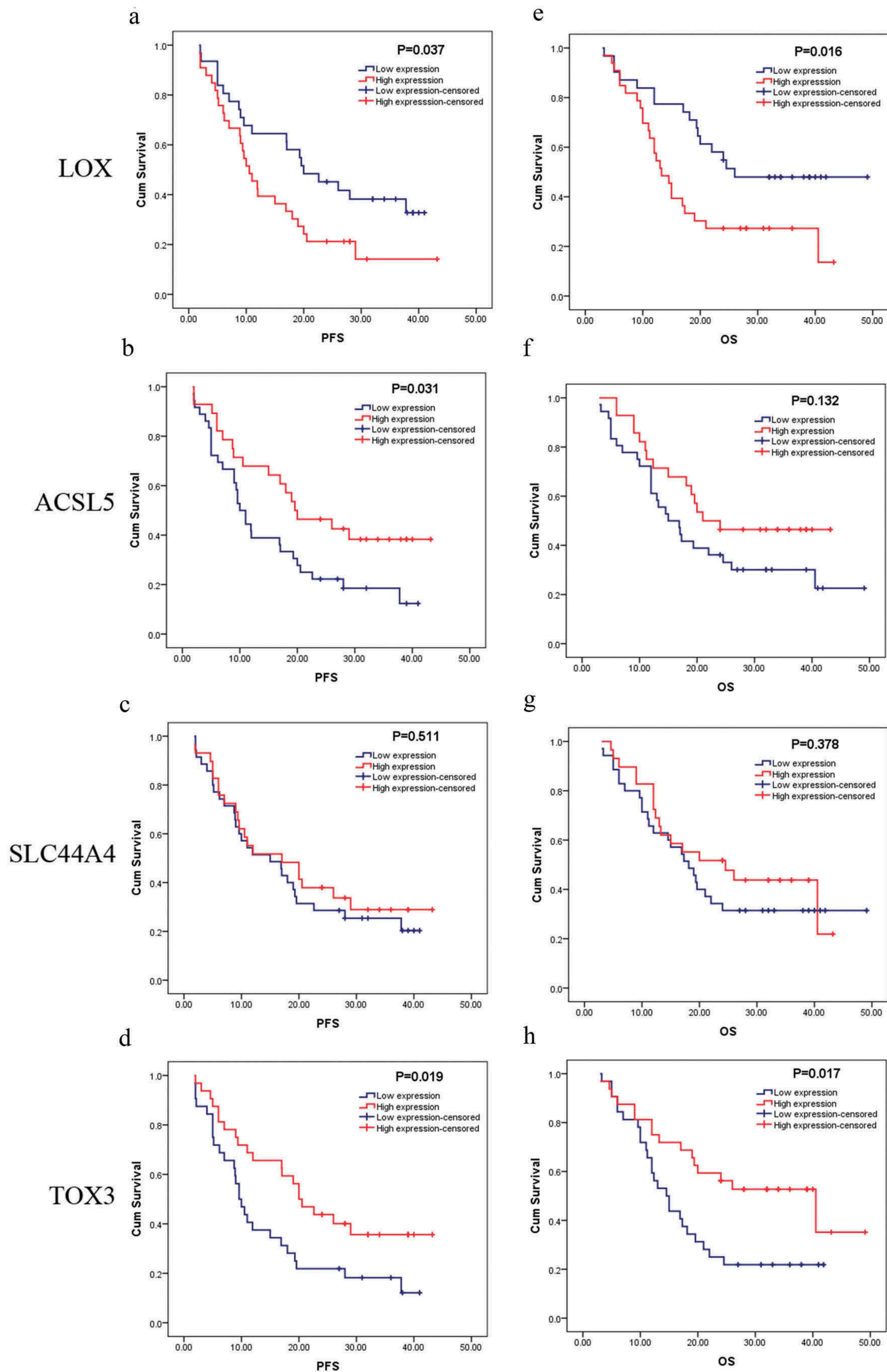

Figure 5. The relationship between the protein expression levels of the four candidate markers and the survival of pancreatic cancer patients (n = 64). Pancreatic cancer patients were divided into high expression and low expression group according to their protein levels of LOX, ACSL5, SLC44A4, and TOX3. The PFS (a-d) and OS (e-h) of these two groups were analyzed. The results showed that the PFS of the patients was correlated with the expression levels of LOX, ACSL5, and TOX3 (P = 0.037; P = 0.031 and P = 0.019). The OS was related to the protein expression of only LOX (P = 0.016) and TOX3 (P = 0.017).

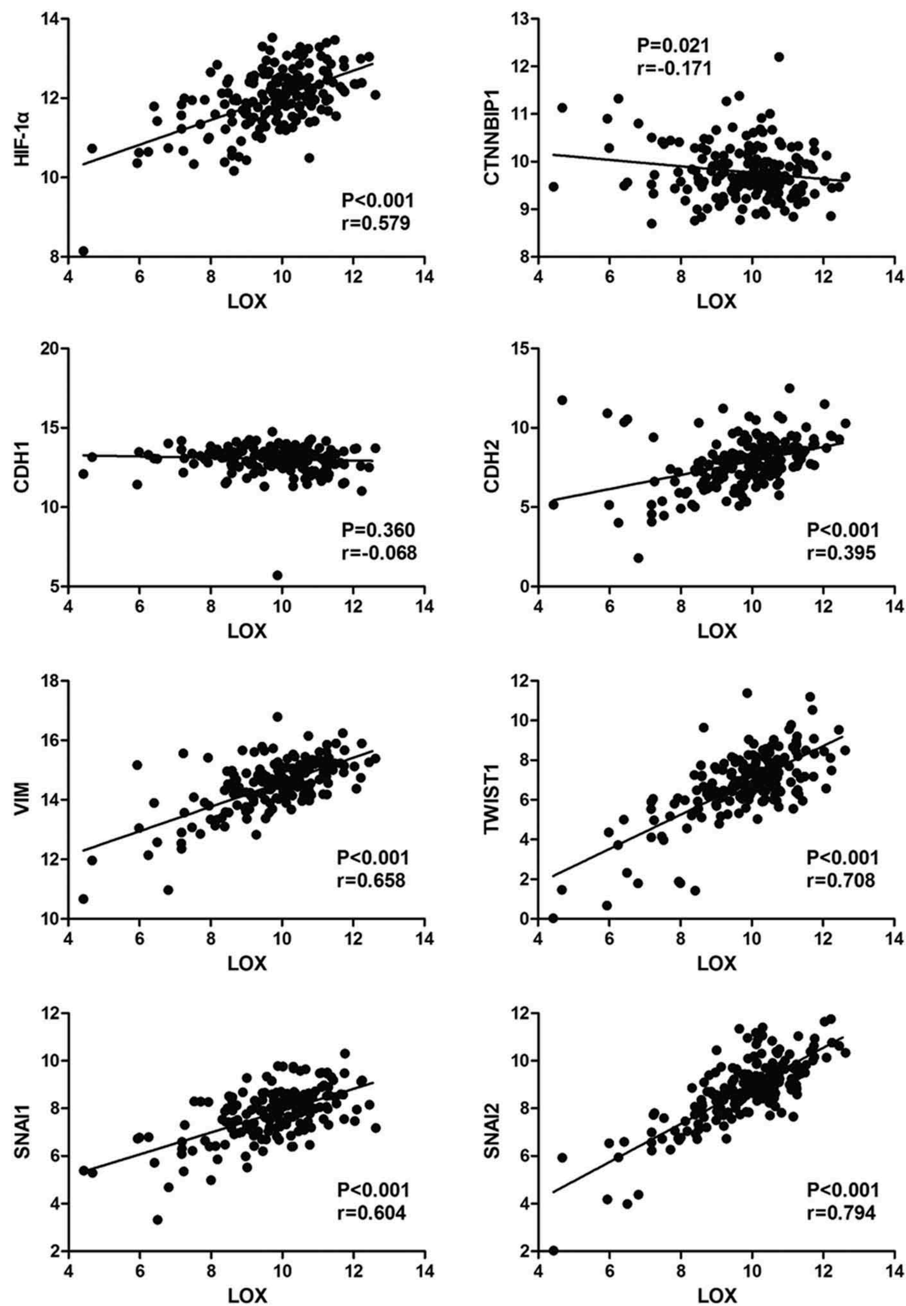

Figure 6. The relationship between LOX and HIF-1A, CDH2, VIM, TWIST1, SNAI1, SNAI2, CDH1, and CTNNBIP1.

A total of 185 pancreatic cancer tissues were analyzed. The results showed LOX was positively correlated with HIF-1A, CDH2, VIM, TWIST1, SNAI1 and SNAI2 (HIF-1A, $P < 0.001$, r = 0.579; CDH2, $P < 0.001$, r = 0.395; VIM, $P < 0.001$, r = 0.658; TWIST1, $P < 0.001$, r = 0.708; SNAI1, $P < 0.001$, r = 0.604; SNAI2, $P < 0.001$, r = 0.794). LOX was negatively correlated with CTNNBIP1 ($P = 0.021$, r = −0.171), but there is no statistical correlation between CDH1 and LOX ($P = 0.360$, r = −0.068).

3 min in an autoclave at 120°C and then cooled to room temperature (RT) for antigen retrieval for at least 30 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min at RT. After washing with PBS 3 times, non-specific binding sites were blocked with normal goat serum for 10 min at RT. The sections were then incubated overnight at 4°C with primary antibodies [rabbit polyclonal anti-LOX (1:200, Novus Biological, NB100–2527); mouse monoclonal anti-ACSL5 (1:50, ab104892); rabbit polyclonal anti-SLC44A4 (1:200, Novus Biological, NBP2-33707); rabbit polyclonal anti-TOX3 (1:50, Abgent, Cat# AH11330-100)]. After washing with PBS 3 times, the sections were incubated with secondary antibodies (Zhongshan Goldenbridge Biological Technology Co., SPN-9001) for 60 min at 37°C, except for TOX3. TOX3 staining was performed by using an UltraSensitiveTM SP IHC Kit (Fuzhou Maixin Biotech, KIT-9710) according to the manufacturer's instructions.[27] The sections were then washed 3 times with PBS, and the sections were visualized with diaminobenzidine tetrahydrochloride (DAB kit, Zhongshan Goldenbridge Biological Technology Co., KIT-9710). Finally, the sections were counterstained with hematoxylin and dehydrated.

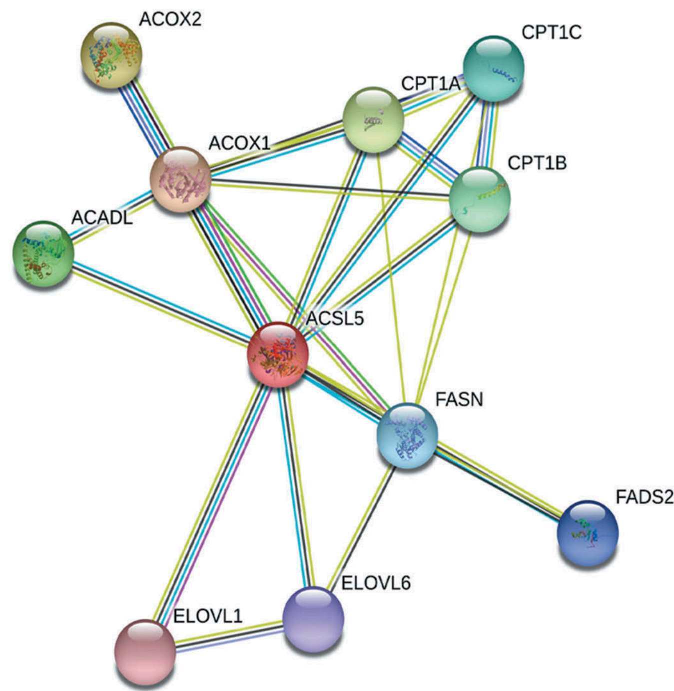

Figure 7. Protein–protein interaction network of ACSL5.

The two-way scoring system was used for staining quantification.[28] The staining intensity was scored as four categories: negative, 0; weak, 1; moderate, 2; and strong, 3. The proportion of positively stained cells of interest was determined as the following: 0–25%, 1; 26–50%, 2; 51–75%, 3; and 76–100%, 4. The final expression level of each protein in each sample was obtained by multiplying the proportion and the intensity. The median score of the biomarkers was used as a cut-off to divide the cases into two groups, the low expression group, and the high expression group, according to the published standard scoring method.[29–31]

TCGA data procession

A total of 185 pancreatic cancer patients' datasets were downloaded from The Cancer Genome Atlas (TCGA) data portal. A total of 144 cases left after removing six cases with uncompleted clinical information, eight cases with neuroendocrine neoplasm, seven African-Americans, 11 Asian-Americans, five cases with unknown race and five cases with AJCC stage IV. The expression values of LOX, HIF-1A, CDH1, CDH2, CTNNBIP1, VIM, TWIST1, SNAI1 and SNAI2 were extracted to analyze the correlation.

STRING online tool

The string was an online biological tool to establish functional protein association network.[33] Input "ACSL5" in single protein by name, then choose homo sapiens in organism box to search the network of ACSL5.

Statistical analysis

Statistical analysis was performed using SPSS software 16.0 for Windows.[34] Receiver operating curve (ROC) and area under the curve (AUC) analyses were estimated by survival time for candidate genes. Difference/correlations between groups were assessed by student's *t* test, one-way ANOVA and Pearson's correlation test. The survival curve was determined by the Kaplan-Meier method, and the statistical significance was assessed using the log-rank test. Multivariable analysis of the independent factors associated with survival was performed using the Cox proportional hazard model. Differences were considered significant when the associated P value < 0.05.

Disclosure of Potential Conflicts of Interest

The authors report no conflict of interest.

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