LAMC2 is a potential prognostic biomarker for cholangiocarcinoma

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Abstract. Cholangiocarcinoma is a common malignancy with increasing incidence worldwide. Most patients are diagnosed at the advanced stage with poor survival rate. Laminin subunit γ2 (LAMC2) is a heparin binding-associated gene involved in tumorigenesis and has been implicated in the prognosis of various types of cancers. However, it is unclear whether expression of LAMC2 is associated with the clinical outcome of patients with cholangiocarcinoma. In the present study, the role and prognostic value of LAMC2 expression in patients with cholangiocarcinoma was investigated. Clinical information and pathological characteristics were analyzed and the association between LAMC2 expression and clinical characteristics, pathological findings and patient outcomes, including metastasis-free and disease-specific survival, were investigated. Data from 182 patients with cholangiocarcinoma were evaluated. High LAMC2 expression was associated with higher tumor stage (P<0.001), large duct type (P=0.024) and poor histological grade (P=0.002). Kaplan-Meier analysis showed high LAMC2 expression was associated with lower overall (P=0.003), disease-specific (P=0.0025), local recurrence-free (P<0.0001) and metastasis-free survival (P<0.0001). Moreover, multivariate analysis demonstrated that increased LAMC2 expression was a significant predictive risk factor for overall [hazard ratio (HR) 1.713; P=0.034], disease-specific (HR 2.011; P=0.039), local recurrence-free (HR 2.721; P<0.001) and metastasis-free survival (HR 3.117; P<0.001). Gene enrichment analysis using Gene Ontology showed that terms associated with LAMC2 upregulation were 'regulation of platelet-derived growth factor receptor-βsignaling pathway' and 'platelet-derived growth factor receptor-β signaling pathway'. The present study indicated that LAMC2 was upregulated in cholangiocarcinoma tumor tissue and had an inverse association with overall, disease-specific, local recurrence-free and metastasis-free survival in patients with cholangiocarcinoma. These results suggested that LAMC2 may serve as a potential biomarker for cholangiocarcinoma.

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Introduction

Cholangiocarcinoma is a malignant tumor located in the bile duct epithelium and is the second most common primary hepatobiliary malignancy after hepatocellular carcinoma (1). According to the American Cancer Society, ~12,000 people in the United States are diagnosed with cholangiocarcinoma each year (2). Cholangiocarcinoma is more common in East and Southeast Asia, potentially because eating raw, fermented or undercooked fish leads to parasitic (liver fluke) infection, which in turn triggers chronic bile duct inflammation and increases cancer risk (3,4). Since cholangiocarcinoma lacks diagnostic markers and has limited diagnostic methods, the five-year survival rate of patients with cholangiocarcinoma is <10% (5). Treatment guidelines for cholangiocarcinoma are primarily surgery, radiation therapy and chemotherapy (CT), depending on the disease stage (6). However, most patients with cholangiocarcinoma are asymptomatic at the early stage and are typically only diagnosed when the cholangiocarcinoma has spread to other tissue beyond the bile duct, which limits the treatment options (7). Accordingly, comprehensive identification of potential cholangiocarcinoma diagnostic biomarkers may facilitate design of more effective and targeted therapeutic strategies.

Laminin subunit γ2 (LAMC2) is a member of the extracellular matrix (ECM) glycoprotein family (8). It has been reported that LAMC2 is implicated in various biological processes, including cell adhesion, differentiation, migration, signaling and cancer metastasis (9). For example, previous report have shown that LAMC2 increases cell migration, invasion and metastasis in lung adenocarcinoma by regulating epithelial-mesenchymal transition (EMT) (10). Additionally, expression of LAMC2 enhances cell migration and invasion via directly targeting EMT regulator zinc finger E-box binding homeobox 1 in colorectal cells (11). Conversely, the inhibition of LAMC2 expression promotes gemcitabine sensitivity and decreases cancer progression via EMT signaling and ATP-binding cassette transporters in pancreatic ductal adenocarcinoma (12). Moreover, clinical data have demonstrated that LAMC2 is upregulated in patients with pancreatic (13), bladder (14), lung (10), colorectal (11) and cervical cancer (15). Furthermore, high expression of LAMC2 is associated with worse clinical outcome for different cancer types, such as pancreas, stomach, tongue, bladder, colorectal, lung, squamous cell carcinoma of vulva, cervix andesophagus (squamous) as well as melanoma and anaplasticthyroid carcinom (9). However, the association between LAMC2 expression, clinical significance and survival outcomes in patients with cholangiocarcinoma is unknown.

The present study aimed to investigate the expression of LAMC2 in cholangiocarcinoma and how it can impact prognosis. By uncovering the potential of LAMC2 as a prognostic indicator, the present study aim to provide valuable insights that can improve the care and treatment outcomes for individuals with cholangiocarcinoma.

Materials and methods

Analysis of expression profiles from publicly available cholangiocarcinoma transcriptomic datasets. The cholangiocarcinoma gene expression dataset (accession no. GSE26566) includes information on 59 non-cancerous liver and 104 cholangiocarcinoma tumor tissue samples; data were downloaded from Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) and analyzed using GeneChip™ Human Genome U133 Plus 2.0 Array (Thermo Fisher Scientific). The comparative analysis was conducted to generate the heatmap of significantly differently expressed genes associated with heparin binding (GO:0008201; geneontology.org/). The expression of the genes was then calculated by probes combinations without preselection or filtering. Genes with significant differential expression (log2 ratio >2; P<0.01) were used for further study.

Patients and tumor specimens. Paraffin-embedded tissue blocks were retrieved from 182 patients with intrahepatic cholangiocarcinoma who had no lymph node or distant metastasis and had received curative surgery. Only individuals with T1-3N0M0 disease were included. No patients received adjuvant CT or radiotherapy. The initial diagnosis was made from January 1990 to December 2010 at The Chi Mei Medical Center (Tainan, Taiwan). The present study was conducted in accordance with the Declaration of Helsinki and approved by The Institutional Review Board of Chi-Mei Medical Center (approval no. 09912003). Informed consent was signed and obtained from all subjects.

In addition, histological subtypes were reevaluated by two pathologists. The tumor stage was assessed by the 7th edition of the American Joint Committee on Cancer (AJCC) staging system (16).

Immunohistochemistry (IHC) staining. The tissue blocks of cholangiocarcinoma were fixed in 4% paraformaldehyde in PBS (4 ˚C), made transparent, paraffin-embedded, and sliced into 4-μm thick serial sections using a microtome. For antigen retrieval, slides were pressure-cooked in 10 mmol/l citrate buffer at pH 6 for 7 min and washed using TBS buffer with 0.1% Tween-80. The tissues were dewaxed, rehydrated in a graded ethanol submerged in 0.3% H_2O_2 and in 95% ethanol for 5 min and placed in citrate buffer (pH 6). For H&E staining, tissue section was stained in Mayers Hematoxylin for 1 mi followed by staining blue nuclei in 1X PBS for 1 min and counterstaining in Alcoholic-Eosin for 1 min. Then the tissue sections were dehydrated through 100% EtOH. For immunohistochemistry staining, the sections were stained overnight at 4˚C with anti-LAMC2 primary antibody (cat. no. ab125679; Abcam; 1:100) followed by incubation with secondary antibody HRP polymer (car. no. ab214880; Abcam; 1:2,000) for 30 min at room temperature. A total of two pathologists calculated H-score as follows: H-score=π(i +1), where π is the percentage of stained tumor cells and i is the degree of staining (0-3). The i values are indicated as 0 (no evidence of staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). Based on the median H-score, the immunostaining was categorized as low or high expression of LAMC2.

Gene function prediction and classification. To determine the function of LAMC2 in intrahepatic cholangiocarcinoma, the association between the mRNA expression levels of LAMC2

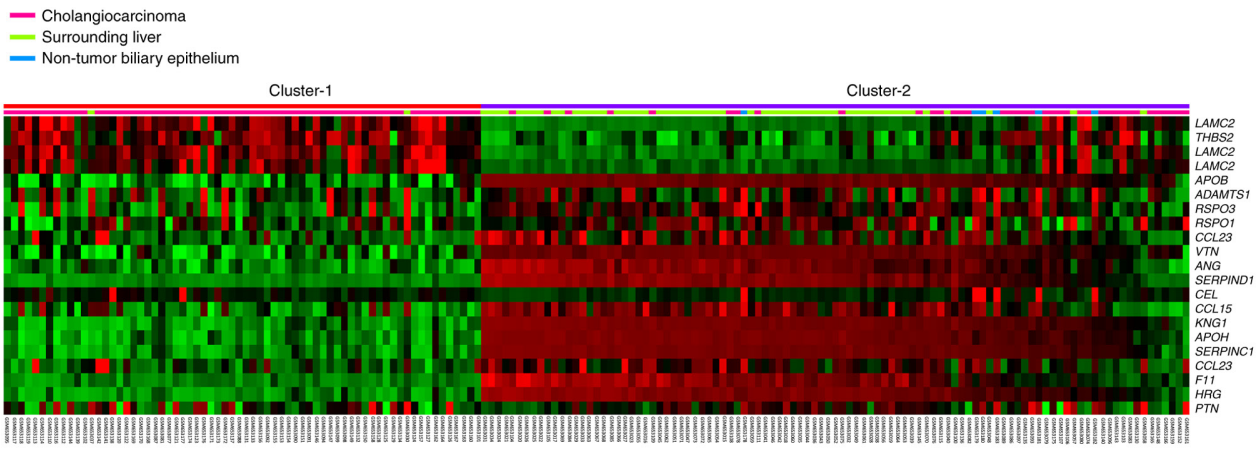

Figure 1. Analysis of gene expression in cholangiocarcinoma using a published trascriptome dataset (GSE26566). Heatmap showing differential expression of genes associated with heparin binding (GO:0008201) in cholangiocarcinoma (cluster 1) and non-cholangiocarcinoma tumors (cluster 2). Black, mean expression; green, downregulation; red, upregulation. A total of three probes were utilized to detect LAMC2.

and its co-expressed genes from the cholangiocarcinoma dataset containing 51 samples in The Cancer Genome Atlas (TCGA) database (dbGaP Study Accession no.phs000178, cancer.gov/ccg/research/genome-sequencing/tcga) were assessed. The top 200 differentially expressed transcripts exhibiting positive or negative associations with LAMC2 were downloaded. These genes were undergoing functional annotation by the GO classification system (geneontology.org/) and rated by fold enrichment. Fisher's exact test was performed to identify GO terms that were over-represented amongst differentially expressed genes. In this test, the P-value denotes the likelihood of observing ≥x genes from the entire set of n genes associated with a specific GO term. Subsequently, to minimize false positives (type I errors), the original P-value was adjusted for multiple hypothesis testing, resulting in false discovery rate (FDR). P-value and FDR <0.05 were considered to indicate a statistically significant difference.

Statistical analysis. All the data were analyzed using SPSS version 17.0 software (SPSS, Inc.). To explore the association between LAMC2 expression and clinicopathological characteristics in patients with cholangiocarcinoma, medical records were collected and overall, disease-specific, local recurrence-free and metastasis-free survival of patients with cholangiocarcinoma from treatment start date to the event occurrence were analyzed. Using uni- and multivariate analysis, LAMC2 expression and clinicopathological variables were discovered as predictors of OS (measured from curative surgery to the time of any cause mortality), DSS (measured from curative surgery to the time of cancer mortality), LRS (measured from curative surgery to the time of first local recurrence) and MFS (measured from curative surgery to the first metastasis). Survival curves were obtained by Kaplan-Meier analysis and log-rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

Heparin binding-associated gene LAMC2 is significantly upregulated in patients with cholangiocarcinoma. To identify a potential target for diagnosis of patients with cholangiocarcinoma, the public cholangiocarcinoma transcriptome dataset (accession no. GSE26566) in the GEO database, which contains 104 cholangiocarcinoma tumor and 59 non-cancerous liver tissue samples. The comparative analysis was conducted to detect significantly differently expressed genes associated with heparin binding (GO:0008201). The heatmap data revealed 19 heparin binding-associated genes with significant differential expression (Fig. 1). In GO Term database, three probes for LAMC2 are used including: ILMN_1701424, ILMN_1653824 and ILMN_1706519. All LAMC2 probes exhibited significant expression fold-change between cholangiocarcinoma tumor tissue and non-cancerous liver tissue. Specifically, ILMN_1701424 probe exhibited the highest expression fold change (log ratio, 2.7229; Table I). Collectively, these findings demonstrated that LAMC2 may play an essential role in cancer progression in cholangiocarcinoma.

LAMC2 expression is associated with poorer clinical pathological parameters of patients with cholangiocarcinoma. The aforementioned data confirmed that high expression of LAMC2 may be associated with cholangiocarcinoma progression. Therefore, the association between LAMC2 expression and the clinicopathological features of patients with cholangiocarcinoma was explored (Table II). A total of 182 patients with cholangiocarcinoma were collected including 108 male patients and 75 patients ≥65 years old. Moreover, the clinicopathological parameters were analyzed; LAMC2 (low vs. high expression) in the tumors of patients with cholangiocarcinoma was significantly associated with the status of primary tumor, histological variant and the histological grade. However, sex, age, hepatitis, intrahepatic lithiasis and surgical margin showed no significant difference between tumor tissue of patients with cholangiocarcinoma with differential LAMC2 expression. LAMC2 protein expression in human cholangiocarcinoma tumor tissue was further confirmed by IHC staining. Low-stage cholangiocarcinoma tissue had lower LAMC2 expression (Fig. 2A-D) than high-stage cholangiocarcinoma tissue (Fig. 2E-H). These data showed that LAMC2 expression was

Table I. Alteration of genes associated with heparin binding (accession no. GO:0008201) in cholangiocarcinoma (accession no.GSE26566).

	Cholangiocarcinoma vs. non-tumor[a]		Cholangiocarcinoma vs. normal intrahepatic bile duct[b]				
Probe	Log ratio	P-value	Log ratio	P-value	Gene	Molecular function	Biological process
ILMN_1701424	2.7229	<0.0001	2.3705	<0.0001	LAMC2	'Heparin binding', 'protein binding'	'Cell adhesion', 'epidermis development'
ILMN_1653824	1.7967	<0.0001	1.7476	<0.0001	LAMC2	'Heparin binding', 'protein binding'	'Cell adhesion', 'epidermis development'
ILMN_1678842	1.2588	0.0008	1.9732	<0.0001	THBS2	'Structural molecule activity', 'heparinbinding', 'calcium ion binding','protein binding'	'Cell adhesion'
ILMN_1706519	0.7668	0.0007	0.626	<0.0001	LAMC2	'Heparin binding', 'protein binding'	'Cell adhesion', 'epidermis development'
ILMN_1813753	-0.3156	0.0065	-0.114	0.0043	PTN	'Cytokine activity', 'protein phosphatase inhibitor activity', 'heparin binding, growth factor activity'	Cell proliferation', 'transmembrane receptor protein tyrosine phosphatase signaling pathway', 'positive regulation of cell proliferation'
ILMN_1682937	-0.3774	0.0003	-0.2562	<0.0001	RSPO1	'Electron carrier activity', 'iron ion binding', 'heparin binding'	'Wnt receptor signaling pathway', 'electron transport'
ILMN_1764030	-0.6538	0.0005	-0.3667	<0.0001	CCL23	'Heparin binding', 'chemokine activity'	Cell-cell signaling', 'negative regulation of cell proliferation', 'chemotaxis', 'calcium ion homeostasis', 'G-protein coupled receptor protein signaling pathway', 'signal transduction', 'inflammatory response'
ILMN_1807101	-1.0034	0.0001	-1.7784	<0.0001	F11	'Coagulation factor XIa activity', 'peptidase activity', 'heparin binding', 'coagulation factor IXa activity'	'Blood coagulation'
ILMN_1681983	-1.0244	<0.0001	-0.3955	<0.0001	RSPO3	'Electron carrier activity', 'iron ion binding', 'heparin binding'	'Wnt receptor signaling pathway', 'electrontransport'

Table I. Continued.

Probe	Cholangiocarcinoma vs. non-tumor[a]		Cholangiocarcinoma vs. normal intrahepatic bile duct[b]		Gene	Molecular function	Biological process
	Log ratio	P-value	Log ratio	P-value			
ILMN_1686109	-1.349	0.0001	-0.7483	<0.0001	CCL23	'Heparin binding', 'chemokine activity'	'Cell-cell signaling', 'negative regulation of cell proliferation', 'chemotaxis', 'calcium ion homeostasis', 'G-protein coupled receptor protein signaling pathway', 'signal transduction, inflammatory response'
ILMN_1696974	-1.9949	0.0006	-2.6032	<0.0001	ANG	'Pancreatic ribonuclease activity', 'hydrolase activity', 'ribonuclease activity', 'DNA binding', 'endonuclease activity', 'receptor binding, copper ion binding', 'rRNA binding', 'heparin binding, actin binding'	'Negative regulation of protein biosynthesis', 'calcium-dependent phospholipase A2 activation', 'positive regulation of endothelial cell proliferation', 'homeostasis', 'response to hypoxia', 'angiogenesis', 'phospholipase C activation', 'ovarian follicle development', 'diacylglycerol biosynthesis', 'ribosome biogenesis', 'rRNA transcription', 'cell differentiation', 'positive regulation of protein secretion', 'negative regulation of smooth muscle cell proliferation', 'cell communication', 'actin filament polymerization'
ILMN_1707975	-2.0503	0.0002	-3.0614	<0.0001	SERPIND1	'Serine-type endopeptidase inhibitor activity', 'heparin binding'	'Blood coagulation', 'chemotaxis'
ILMN_1691127	-2.0625	0.0053	-2.6248	<0.0001	VTN	'Heparin binding', 'protein binding'	'Immune response', 'cell adhesion'

Table I. Continued.

Probe	Cholangiocarcinoma vs. non-tumor[a]		Cholangiocarcinoma vs. normal intrahepatic bile duct[b]		Gene	Molecular function	Biological process
	Log ratio	P-value	Log ratio	P-value			
ILMN_1740609	-2.2868	<0.0001	-1.5285	<0.0001	CCL15	'Chemokine activity', 'chemoattractant activity', 'signal transducer activity', 'heparin binding'	'Signal transduction', 'immune response', 'antimicrobial humoral response (sensu Vertebrata)', 'cell-cell signaling', 'chemotaxis', 'calcium ion homeostasis'
ILMN_1664024	-2.4224	0.0010	-3.0416	<0.0001	APOB	'Receptor binding', 'lipid transporteractivity', 'heparin binding'	'Circulation, cholesterol metabolism', 'lipid trans port', 'lipid metabolism', 'steroid metabolism', 'signal transduction'
ILMN_1807339	-2.6598	0.0005	-3.8929	<0.0001	HRG	'Heparin binding', 'cysteine protease inhibitor activity'	
ILMN_1761511	-2.7374	0.0001	-3.4698	<0.0001	APOH	'Lipid transporter activity', 'heparin binding'	'Defense response'
ILMN_1673566	-2.8885	<0.0001	-0.6183	<0.0001	ADAMTS1	'Zinc ion binding, metal ion binding', 'integrin binding, heparin binding', 'metalloendopeptidase activity'	'Negative regulation of cell proliferation', 'integrin-mediated signaling pathway'
ILMN_1753729	-3.1226	0.0014	-3.8105	<0.0001	KNG1	'Receptor binding, cysteine proteaseinhibitor activity', 'zinc ion binding', 'heparin binding'	'Diuresis', 'negative regulation of cell adhesion', 'vasodilation', 'positive regulation of apoptosis', 'blood coagulation', 'smooth muscle contraction', 'natriuresis','negative regulation of blood coagulation', 'inflammatory response'
ILMN_1762605	-3.6489	<0.0001	-4.0868	<0.0001	SERPINC1	'Serine-type endopeptidase inhibitor activity', 'heparin binding', 'protein binding'	'Blood coagulation'
ILMN_1723418	-6.3865	<0.0001	-0.3866	0.0084	CEL	'Hydrolase activity', 'serine esterase activity', 'triacylglycerol lipase activity', 'sterol esterase activity', 'heparin binding'	'Pancreatic juice secretion', 'protein amino acid esterification', 'cholesterol absorption', 'cholesterol catabolism', 'triacylglycerol metabolism', 'fatty acid catabolism', 'lipid metabolism', 'lipid catabolism'

Cholangiocarcinoma (n=104) vs. [a]surrounding liver (n=59) and normal intrahepatic bile duct tissue (n=6) and [b]normal intrahepatic bile duct tissue (n=6).

Table II. Association between LAMC2 expression and clinicopathological parameters in primary localized cholangiocarcinoma.

Parameter	n	LAMC2 expression		P-value
		Low	High	
Sex				
Male	108	57	51	0.365
Female	74	34	40	
Age, years				
<65	107	49	58	0.175
≥65	75	42	33	
Hepatitis				
B	72	38	34	0.353
C	29	17	12	
Non-B, non-C	81	36	45	
Intrahepatic lithiasis				
Absent	102	53	49	0.550
Present	80	38	42	
Surgical margin				
R0	163	83	80	0.467
R1	19	8	11	
Primary tumor stage				
T1	87	56	31	<0.001[a]
T2	61	27	34	
T3	34	8	26	
Histological type				
Large duct	105	45	60	0.024[a]
Small duct	77	46	31	
Histological grade				
Well differentiated	61	38	23	0.002[a]
Moderately differentiated	66	36	30	
Poorly differentiated	55	17	38	

[a]P<0.05. LAMC2, laminin subunit γ2.

markedly associated with clinicopathological characteristics and cancer progression in patients with cholangiocarcinoma.

LAMC2 expression is associated with survival of patients with cholangiocarcinoma. Whether differential expression of the LAMC2 gene affects the survival outcomes of patients with cholangiocarcinoma was explored. Kaplan-Meier survival analysis was performed to confirm that LAMC2 expression was associated with clinicopathological characteristics and prognosis in patients with cholangiocarcinoma. High LAMC2 expression was significantly associated with lower overall (Fig. 3A), disease-specific (Fig. 3B), local recurrence-free (Fig. 3C) and metastasis-free survival (Fig. 3D). Univariate and multivariate analyses revealed the association between prognostic factors of LAMC2 expression and clinicopathological factors in patients with cholangiocarcinoma. Sex, surgical margin (R0 and R1), primary tumor stage (T1, T2 and T3) and LAMC2 expression (high or low) were significantly associated with overall and disease-specific survival (Table III). However, age, hepatitis, intrahepatic lithiasis and histological type (large and small duct) and grade (well, moderately or poorly differentiated) did not differ significantly in overall and disease-specific survival (Table III). The association between local recurrence-free and metastasis-free survival with clinical characteristics was also evaluated by univariate and multivariate analyses. Local recurrence-free and metastasis-free survival were markedly associated with surgical margins, primary tumor stage and LAMC2 expression. Local recurrence-free survival was significantly associated with histological type and grade by univariate, but not multivariate, analysis (Table IV). These results demonstrated that LAMC2 may be a potential indicator of prognosis in patients with cholangiocarcinoma.

LAMC2 gene function prediction. To determine the functions of LAMC2 in cholangiocarcinoma, the top 200 differentially expressed transcripts exhibiting positive (Table SI) or negative association (Table SII) with LAMC2 were downloaded from TCGA cholangiocarcinoma dataset (n=51). GO enrichment showed that the most significant biological processes associated with LAMC2 upregulation were the 'regulation of platelet-derived growth factor receptor-β signaling pathway' (GO: 2000586; fold-enrichment, 38.22) and 'platelet-derived growth factor receptor-β signaling pathway' (GO: 0035791; fold-enrichment, 38.22; Fig. 4A). Lysyl oxidase (LOX) gene was involved in both aforementioned biological processes. The most significant molecular function associated with LAMC2 upregulation was 'laminin binding' (GO: 0043236; fold-enrichment, 25.48; Fig 4B). Moreover, the most significant cellular component associated with LAMC2 upregulation was 'integrin alpha3-beta1 complex' (GO: 0034667; fold-enrichment, >100; Fig. 4C). The integrin subunit β1 (ITGB1) and ITGA3 genes, which are implicated in both laminin binding and integrin α3-β1 complex, were identified.

Discussion

Cholangiocarcinoma is a rare malignant tumor located in the bile duct. However, its incidence is increasing globally and it is a global public health problem that needs attention (1,17). To the best of our knowledge, there is no literature identifying the cause of cholangiocarcinoma. Certain studies have investigated risk factors that may serve essential roles in increasing the risk of cholangiocarcinoma, including primary sclerosing cholangitis, chronic liver disease, smoking, diabetes and liver parasites (liver fluke infection) (18,19). Cholangiocarcinoma is divided into three types based on where it occurs in the bile ducts: Intrahepatic, extrahepatic and distal cholangiocarcinoma (20). Cholangiocarcinoma is asymptomatic in the early stages and is often diagnosed when the disease is already at an advanced stage, which decreases affects treatment options and leads to poor prognosis (21). The 5-year survival rate for intrahepatic cholangiocarcinoma is 9%. However, if the cancer is diagnosed at an early stage, the 5-year survival rate is 25%. If the tumor has spread to the regional lymph nodes,

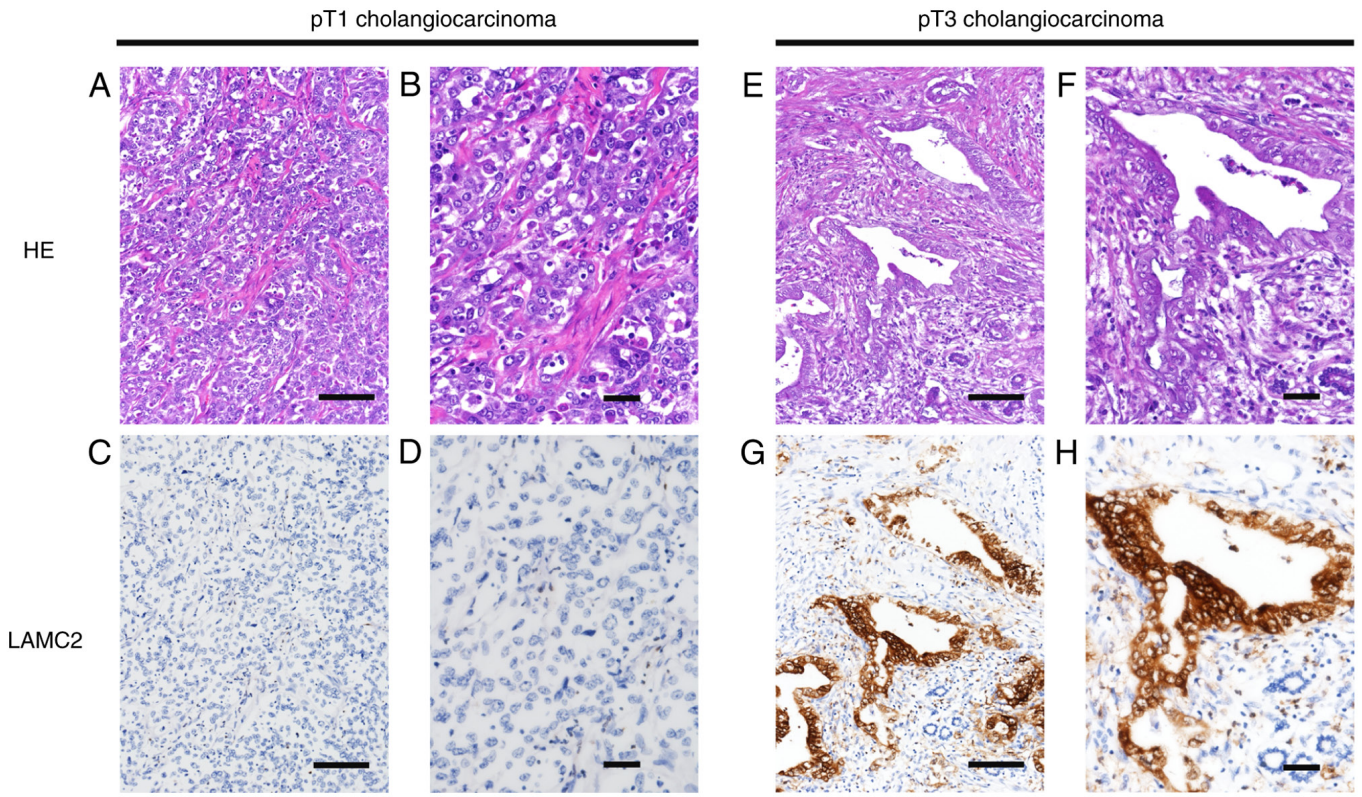

Figure 2. Representative sections of LAMC2 immunostaning. Immunohistochemistry staining showed lower LAMC2 expression in pT1 stage cholangiocarcinoma HE staining at (A) magnification, x200; scale bar, 500 μm and (B) magnification, x400; scale bar 200 μm. LAMC2 staining at (C) magnification, x200; scale bar, 500 μm and (D) magnification, x400; scale bar 200 μm compared with pT3 stage cholangiocarcinoma HE staining at (E) magnification, x200; scale bar, 500 μm and (F) magnification, x400; scale bar 200 μm. LAMC2 staining at (G) magnification, x200; scale bar, 500 μm and (H) magnification, x400; scale bar 200 μm. HE, hematoxylin and eosin; LAMC2, laminin subunit γ2; pT, pathological T.

5-year survival rate is 8%. However, if the tumor has spread to a distant part of the body, 5-year survival rate is 2% (22,23). Thus, identifying potential novel biomarkers is a promising approach to enhancing strategies to treat cholangiocarcinoma.

Here, the tumorigenesis-associated genes in the transcriptome of cholangiocarcinoma (GSE26566) were compared with heparin binding in GO (GO:0008201). Heparin-binding associated gene LAMC2 showed upregulated expression in the cholangiocarcinoma compared with non-tumor tissue. LAMC2 is a key laminin in the ECM glycoprotein family and regulates numerous biological processes, including cell adhesion, differentiation, migration, signaling and metastasis (24). Moreover, accumulating evidence indicates that LAMC2 is also involved in regulating progression in multiple types of cancer (25-27). For example, inhibition of LAMC2 expression decreases cell proliferation, migration and invasion in non-small-cell lung cancer (28). In pancreatic cancer, upregulation of LAMC2 enhances cell migration and invasion through the activation of Akt/sodium-hydrogen antiporter 1) signaling (26). Furthermore, overexpression of LAMC2 increases cell proliferation and decreases cell apoptosis via p38/MAPK signaling activation in ovarian cancer (29). Zhou *et al* (27) demonstrated that silencing LAMC2 expression suppresses cell migration, invasion and cancer stemness by inhibiting the PI3K/Akt signaling pathway in oral squamous cell carcinoma. Clinical results have shown that LAMC2 is highly expressed and associated with worse survival outcomes in pancreatic, bladder, colorectal, oral and ovarian cancer (9,30,31). To the best of our knowledge, no studies have investigated the association between LAMC2 expression and prognostic outcomes and survival in patients with cholangiocarcinoma. In the present study, IHC showed that LAMC2 protein was upregulated in advanced cholangiocarcinoma tumor tissues compared with early cholangiocarcinoma tumor tissue. Patients with cholangiocarcinoma with a high LAMC2 expression had worse overall, disease-specific, local recurrence-free and metastasis-free survival than patients with cholangiocarcinoma with low LAMC2 expression. Collectively, these results indicated that LAMC2 may serve as a novel predictive marker for patients with cholangiocarcinoma.

The association between LAMC2 and clinicopathological parameters of patients with cholangiocarcinoma was investigated. It was found that LAMC2 expression was markedly associated with primary tumor stage and histological type and grade. Moreover, univariate log-rank and multivariate analyses were performed for overall, disease-specific, local recurrence-free and metastasis-free survival in primary localized IHCC. Univariate and multivariate analysis indicated that sex, surgical margin, primary tumor stage and LAMC2 expression were markedly associated with overall, disease-specific, local recurrence-free and metastasis-free survival. Additionally, univariate, but not multivariate, analysis showed that histological type and grade were significantly associated with local recurrence-free survival in patients with cholangiocarcinoma. These analyses suggested that LAMC2 may be a potential biomarker in patients with cholangiocarcinoma.

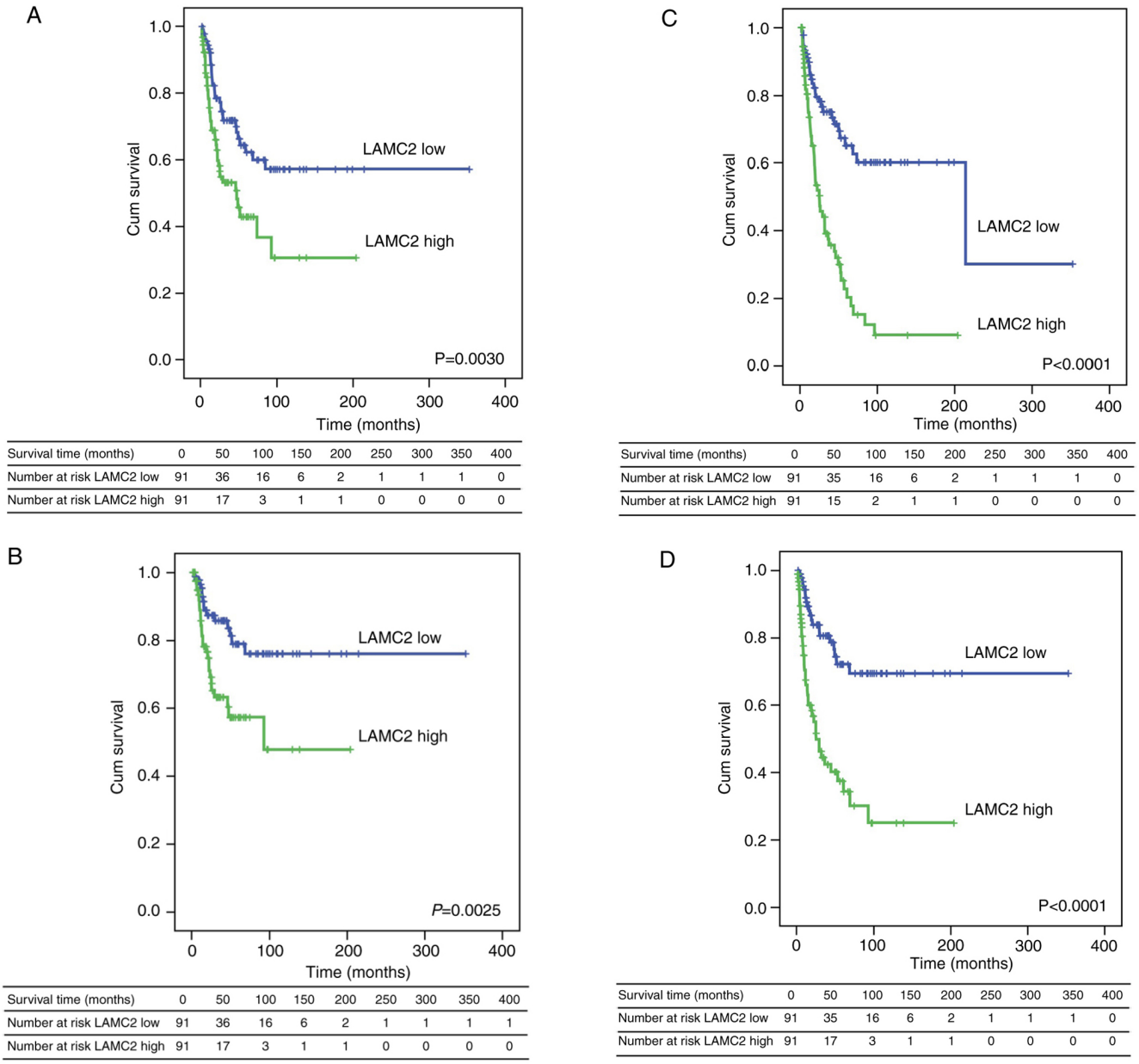

Figure 3. Kaplan-Meier survival analysis of the prognostic significance of LAMC2 expression. Kaplan-Meier survival rates in patients with cholangiocarcinoma showed that high LAMC2 expression associated with worse (A) overall, (B) disease-specific, (C) local recurrence-free and (D) metastasis-free survival. LAMC2, laminin subunit γ2.

A characteristic of cholangiocarcinoma is dense ECM featuring highly desmoplastic stroma comprising collagen, which increases tumor stiffness and decreases drug penetration (32). The LOX family, composed of LOX and LOX-like 1-4, is characterized by catalytic activity leading to collagen crosslinking and ECM remodeling (33). Notably, LOX was a significant gene that was positively associated with LAMC2 in the context of biological processes. LOX also plays a crucial role in EMT and its elevated expression is associated with poor prognosis in hepatocellular carcinoma (34). Nevertheless, whether LAMC2 promotes cholangiocarcioma progression via LOX needs further exploration. ITGB1 and ITGA3 genes were positively associated with LAMC2 in terms of molecular functions and cellular components. Integrin α3β1, formed of ITGA3 and ITGB1, is a receptor for ECM components including laminin, collagen and fibronectin (35,36). Integrin α3β1 is suggested to play an important role in tumor cell invasion of the basement membrane (37). Additionally, the role of laminin in cholangiocarcinoma cell migration (38) and upregulated ITGA3 and ITGB1 levels in cholangiocarcinoma (39) have been documented. Accordingly, the involvement of ITGA3 and ITGB1 in cholangiocarcinoma development mediated by LAMC2 (a laminin component) deserves further investigation.

The present study research has certain limitations. Firstly, it was a retrospective study conducted at a single institution and lacked experimental validation. Secondly, the exact molecular mechanism underlying disease progression and adverse outcomes in LAMC2-overexpressing cholangiocarcinoma remains unclear. Thirdly, there is currently no standardized immunostaining and scoring scheme for

Table III. Univariate log-rank and multivariate analysis for overall and disease-specific survival in primary localized cholangiocarcinoma.

		Overall survival					Disease-specific survival				
		Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
Parameter	n	n	P-value	HR	95% CI	P-value	n	P-value	HR	95% CI	P-value
Sex											
Male	108	50	0.0254[a]	1	-	0.048[a]	9	0.0072[a]	1	-	0.023[a]
Female	74	21		1.681	1.004-2.814	-	32		2.377	1.123-5.025	-
Age, years											
<65	107	37	0.2626	-	-	-	28	0.2125	-	-	-
≥65	75	34		-	-	-	13		-	-	-
Hepatitis											
B	72	32	0.2379	-	-	-	16	0.4561	-	-	-
C	29	8		-	-	-	19		-	-	-
Non-B, non-C	81	31		-	-	-	6		-	-	-
Intrahepatic lithiasis											
Absent	102	36	0.2831	-	-	-	19	0.1613	-	-	-
Present	80	35		-	-	-	22		-	-	-
Surgical margin											
R0	163	59	<0.0001[a]	1	-	0.002[a]	31	<0.0001[a]	1	-	<0.001[a]
R1	19	12		2.978	1.513-5.862		10		4.446	2.012-9.827	
Primary tumor stage											
T1	87	25	0.0001[a]	1	-	0.012[a]	9	<0.0001[a]	1	-	0.003[a]
T2	61	27		1.579	0.900-2.770	-	19		2.886	1.279-6.510	-
T3	34	19		2.270	1.185-4.347	-	13		3.815	1.544-9.426	-
Histological type											
Large duct	105	43	0.4281	-	-	-	27	0.1984	-	-	-
Small duct	77	28		-	-	-	14		-	-	-
Differentiation											
Well	61	20	0.1663	-	-	-	12	0.3881	-	-	-
Moderately	66	28		-	-	-	16		-	-	-
Poorly	55	23		-	-	-	13		-	-	-
LAMC2 expression											
Low	91	30	0.0030[a]	1	-	0.034[a]	15	0.0025[a]	1	-	0.039[a]
High	91	41		1.713	1.042-2.818	-	26		2.011	1.037-3.901	-

[a]P<0.05. LAMC2, laminin subunit γ2; -, not applicable.

Table IV. Univariate log-rank and multivariate analysis for local recurrence-free and metastasis-free survival in primary localized cholangiocarcinoma.

		Local recurrence-free survival					Metastasis-free survival				
		Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
Parameter	n	n	P-value	HR	95% CI	P-value	n	P-value	HR	95% CI	P-value
Sex											
Male	108	54	0.2170	-	-	-	21	0.1008	-	-	-
Female	74	31		-	-	-	44		-	-	-
Age, years											
<65	107	55	0.2993	-	-	-	42	0.2936	-	-	-
≥65	75	30		-	-	-	23		-	-	-
Hepatitis											
B	72	33	0.7333	-	-	-	26	0.8762	-	-	-
C	29	13		-	-	-	11		-	-	-
Non-B, non-C	81	39		-	-	-	28		-	-	-
Intrahepatic lithiasis											
Absent	102	41	0.0551	-	-	-	31	0.1000	-	-	-
Present	80	44		-	-	-	34		-	-	-
Surgical margin											
R0	163	71	<0.0001[a]	1	-	<0.001[a]	54	<0.0001[a]	1		0.001[a]
R1	19	14		4.120	2.145-7.913		11		3.250	1.607-6.577	
Primary tumor stage											
T1	87	28	<0.0001[a]	1	-	0.004[a]	21	<0.0001[a]	1	-	0.018[a]
T2	61	32		1.445	0.827-2.524		26		1.826	1.011-3.298	
T3	34	25		2.232	1.230-4.048		18		2.166	1.110-4.227	
Histological type											
Large duct	105	58	0.0085[a]	1	-	0.373	43	0.0759	-	-	-
Small duct	77	27		0.803	0.495-1.301		22		-	-	-
Differentiation											
Well	61	28	0.0299[a]	1	-	0.794	22	0.1794	-	-	-
Moderately	66	27		0.869	0.498-1.516		22		-	-	-
Poorly	55	30		1.083	0.616-1.903		21		-	-	-
LAMC2 expression											
Low	91	28	<0.0001[a]	1	-	<0.001[a]	20	<0.0001[a]	1	-	<0.001[a]
High	91	57		2.721	1.656-4.470		45		3.117	1.799-5.403	

[a]P<0.05. LAMC2, laminin subunit γ2; -, not applicable.

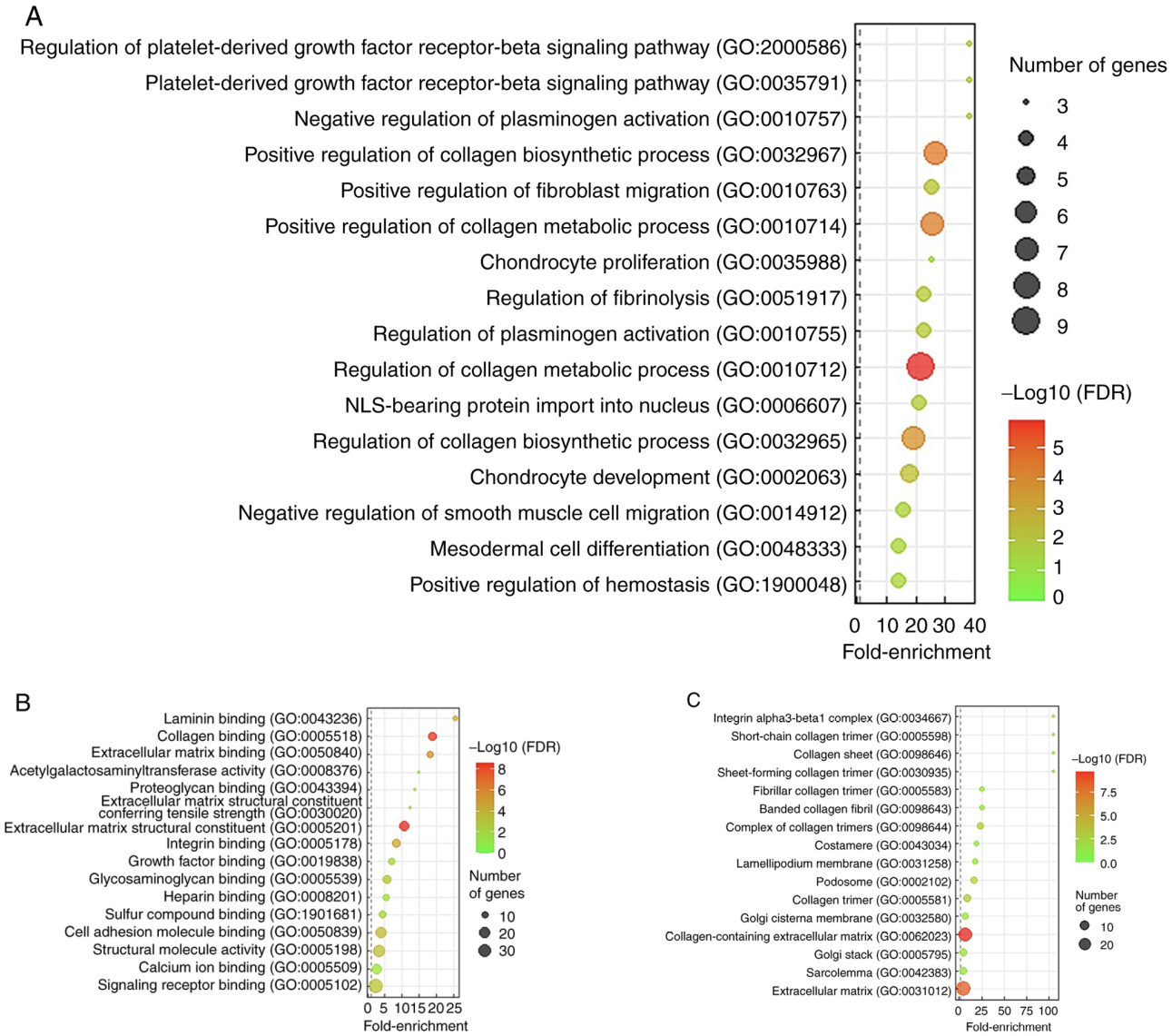

Figure 4. GO terms enriched in LAMC2 upregulation. GO classification of (A) biological process, (B) molecular function and (C) cellular component exhibiting positive association with LAMC2. GO, Gene Ontology; LAMC2, laminin subunit γ2.

assessing LAMC2 expression. Due to the lack of agreed staining standards, it is difficult to reach a consensus. Lastly, to validate the findings, prospective multicenter studies are required.

In conclusion, to the best of our knowledge, the present study is the first to indicate that LAMC2 may serve as a novel biomarker for prognosis of patients with cholangiocarcinoma. Public transcriptome datasets were analyzed with clinical cohorts and LAMC2 was notably upregulated in cholangiocarcinoma tumor tissues. IHC staining was consistent with this result. The expression of LAMC2 in patients with advanced cholangiocarcinoma was higher than in patients with early cholangiocarcinoma. Furthermore, the present study demonstrated that high expression of LAMC2 was associated with poorer overall, disease-specific, local recurrence-free and metastasis-free survival in patients with cholangiocarcinoma. Notably, differential expression of LAMC2 was significantly associated with the primary tumor stage and histological type and histological grade. Therefore, LAMC2 may be a novel biomarker to detect cholangiocarcinoma.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in the Gene Expression Omnibus database (National Center for Biotechnology Information, ncbi.nlm.nih.gov/geo/) and in The Cancer Genome Atlas database (National Cancer Institute and National Human Genome Research Institute, cancer.gov/ccg/research/genome-sequencing/tcga).

Authors' contributions

YLS, CFL, KHO, YYH, HYL and YHK conceptualized the study. SKHH and YFT performed the experiments. HCW, TCC, TJC, DPS, CLC and HHT performed the data analysis. KHO, YYH and HYL wrote the manuscript. CLC, CFL and YHK wrote, reviewed and edited the manuscript. YLS, CFL, YHK, SKHH, YFT, TJC, DPS, HCW, TCC, HHT, KHO, YYH, HYL and CLC confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the Declaration of Helsinki and approved by The Institutional Review Board of Chi-Mei Medical Center (Tainan, Taiwan; approval no. 09912003). Informed consent was signed and obtained from all subjects.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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