TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Silencing of *LAMC2* Reverses Epithelial-Mesenchymal Transition and Inhibits Angiogenesis in Cholangiocarcinoma via Inactivation of the Epidermal Growth Factor Receptor Signaling Pathway

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Cholangiocarcinoma (CCA) is a malignant cancer that is associated with high mortality rates. The relationship between laminin γ 2 chain gene (*LAMC2*) and epidermal growth factor receptor (EGFR) has been previously documented in gastric cancer and oral squamous cell carcinoma. This study investigates the role of *LAMC2* in epithelial-mesenchymal transition (EMT) and angiogenesis in CCA and explores the underlying mechanism(s). Differentially expressed genes related to CCA were initially screened using a microarray analysis, and the interaction between *LAMC2* and the EGFR signaling pathway was identified. To determine the regulatory effects of *LAMC2* on CCA progression, *LAMC2* was silenced or overexpressed and the EGFR signaling pathway was activated or blocked. Subsequently, the regulation effects of *LAMC2* were evaluated on the expression of EMT markers, invasion and migration of CCA cells, as well as microvessel density in nude mice. Microarray analysis demonstrated that highly expressed *LAMC2* is linked to CCA development, which involves the EGFR signaling pathway. When *LAMC2* expression was increased, the EGFR signaling pathway and EMT were activated in CCA tissues. Silencing of *LAMC2* as well as EGFR signaling pathway inhibition led to suppression of EMT, cell invasion, and migration abilities *in vitro*, as well as angiogenesis *in vivo*. This study demonstrates that *LAMC2* silencing suppresses the activity of the EGFR signaling pathway, thus functioning as a tumor suppressor in CCA. (*Am J Pathol* 2019, 189: 1637–1653; <https://doi.org/10.1016/j.ajpath.2019.03.012>)

Cholangiocarcinoma (CCA) is manifested with irregular proliferation of intrahepatic or extrahepatic epithelial cells in the bile ducts.¹ The incidence of CCA substantially has increased in the past several decades.² It has been confirmed that CCA may be induced by liver fluke infestations, hepatolithiasis, thorotrast exposure, as well as other potential etiologies, including hepatitis B virus infection, hepatitis C virus infection, and cirrhosis.^{3,4} The high metastatic potential of CCA is still attributed to the challenges in diagnosis of early-stage CCA.⁵ Epithelial-mesenchymal transition (EMT) is closely related with tumor cell invasion and metastasis.⁶ Several highly expressed genes in CCA tissues have been associated with CCA progression, including *TIAM1* and *HPIA*. The specific down-regulation of these genes can prevent CCA tumorigenesis.^{7,8} Further exploration of the

molecular activities involved in the development of CCA, in particular regarding EMT and tumor metastasis, may provide new targets for therapeutic strategies in the treatment of CCA.

Laminins are a large family of extracellular matrix glycoproteins; they are multidomain trimeric basement membrane proteins that regulate a wide variety of biological processes, including cell adhesion, differentiation, migration, phenotype stability, and resistance to anoikis.⁹ The laminin γ 2 chain

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gene (*LAMC2*) is an epithelium-specific basement membrane protein that is implicated in anaplastic thyroid carcinoma cell migration and tumor invasion.¹⁰ Previously, it has been revealed that *LAMC2* protein is highly expressed in the stomach, tongue, bladder, colorectal cancers, lung, cervix, melanomas, anaplastic thyroid carcinomas, and squamous cell carcinomas of the vulva.¹¹ In addition, the noncontinuous expression pattern of *LAMC2* exerts a predictive value for the prognosis of esophageal squamous cell carcinoma. Moreover, secreted serum *LAMC2* is correlated with aggressive pancreatic cancer.¹² In relation to CCA, the aberrant expression of *LAMC2* has been identified in CCA in recent research.^{13,14} These results suggest that *LAMC2* may play an important role in CCA and might serve as an ideal target for new therapeutic approaches to treat patients with CCA. In anaplastic thyroid carcinoma, immunoprecipitation studies suggest that *LAMC2* could bind to epidermal growth factor receptor (EGFR) and silence *LAMC2*, thereby partially preventing the activation of EGF-mediated activation of EGFR as well as its downstream pathways.¹⁰ The EGFR pathway is the first oncogenic driver found to promote development of malignancy through modulation of angiogenesis, apoptosis, and metastasis in lung cancer.¹⁵ EGFR is an important factor that contributes to cancer progression, the inhibition of which is envisaged as a target for therapy in CCA.¹⁶ However, the interaction of *LAMC2* and the EGFR pathway in CCA is currently not well understood. Therefore, we studied the effects of *LAMC2* in CCA and its role in regulating tumor progression through the EGFR pathway.

Materials and Methods

Ethics Statement

The study was approved by the Ethics Committee of the Institutional Review Board of Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College (Hangzhou, China). All participants signed written informed consents. Animal experimental protocols were conducted in strict adherence to the NIH's *Guide for the Care and Use of Laboratory Animals*.¹⁷

Microarray-Based Gene Expression Analysis

CCA-related chip data were retrieved from the Gene Expression Omnibus database, and three CCA chips (<https://www.ncbi.nlm.nih.gov/geo>; accession numbers GSE45001, GSE77984, and GSE26566) were obtained as a result. The differential gene analysis in CCA and normal samples from GSE45001 and GSE77984 was conducted using the limma package of R language. Log fold change > 2 and $P < 0.05$ were regarded as the threshold for differentially expressed gene (DEG) screening, and the Venn map was plotted for analysis of the top 200 DEGs from the GSE45001 and GSE77984 chips online (<http://bioinformatics.psb.ugent.be/webtools/Venn>, last accessed August 1, 2018). The

GSE26566 chip was used to verify the expression of *LAMC2* in CCA and normal samples.

Gene-Interaction Network Analysis

The DisGeNET database (<http://www.disgenet.org/web/DisGeNET/menu/home>, last accessed August 1, 2018) was used for CCA-related gene retrieval. The top 10 genes with the highest scores were used to construct gene-interaction networks. The Search Tool for the Retrieval of Interacting Genes (STRING) database (<https://string-db.org>, last accessed August 1, 2018) was used to establish a gene-interaction network for the intersection of DEGs from chip data and the known CCA-related genes.

Analysis of *LAMC2* Expression

The UALCAN database (<http://ualcan.path.uab.edu/index.html>, last accessed August 1, 2018) is a user-friendly, interactive, web-based tool for cancer transcriptome data analysis. The CCA-related chip data, obtained from The Cancer Genome Atlas, were analyzed using the UALCAN database, and a box plot of *LAMC2* expression was obtained. The database was used to analyze the expression of *LAMC2* in CCA and normal samples from The Cancer Genome Atlas database.

Study Subjects

A total of 121 CCA and adjacent normal tissues (generally defined as tissues no more than 3 cm from tumor tissues) were obtained from 121 patients with pathologically confirmed CCA. These patients underwent surgical resection at the Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, from May 2009 to May 2015. The 121 patients consisted of 65 males and 56 females with a range in age of 29 to 79 years (mean age, 59 years); three patients had a tumor diameter <5 cm, and 58 patients had tumor diameters >5 cm. According to the eighth edition of the 2017 American Joint Committee on Cancer tumor-node-metastases classification,¹⁸ 51 cases were categorized as stage I/II and 70 cases were categorized as stage III/IV. In terms of histologic grade, 27 cases were graded as highly differentiated, 63 were graded as moderately differentiated, and 31 were graded as poorly differentiated. Assessment of metastasis showed that there were 52 cases with lymph node metastasis (LNM) and 69 cases without LNM. There were 53 cases with portal vein invasion and 68 cases without portal vein invasion. Freshly obtained specimens were frozen in liquid nitrogen and stored at -80°C . None of the patients received chemoradiotherapy or other treatments before specimen collection. Detailed information is shown in Table 1.

RT-qPCR

Total RNA was extracted from samples using the Trizol kit (15596018; Life Technologies, Carlsbad, CA). According to

Table 1 Relationship between *LAMC2* Expression and Clinicopathologic Features of CCA Patients

Feature	No. of patients	LAMC2		Positive rate, %	P value
		—	+		
Age, years					0.441
<65	69	30	39	56.52	
≥65	52	19	33	63.46	
Sex					0.711
Male	65	25	40	61.54	
Female	56	24	32	57.14	
Portal vein invasion					<0.001
Yes	53	12	41	77.36	
No	68	37	31	45.59	
LNM					<0.001
Yes	52	13	39	75.00	
No	69	36	33	47.83	
Histologic grade					<0.001
Poor differentiation	27	9	18	66.67	
Moderate differentiation	63	19	44	69.84	
High differentiation	31	21	10	32.26	
TNM stage					<0.001
I/II	51	28	23	45.10	
III/IV	70	21	49	70.00	

CCA, cholangiocarcinoma; *LAMC2*, laminin γ 2 chain gene; LNM, lymph node metastasis; TNM, tumor-node-metastases.

the instructions provided by the One Step SYBR PrimeScript RT Kit (RR047A; Takara, Tokyo, Japan), RNA was reverse transcribed into cDNA. The reaction conditions used were as follows: 15 minutes at 37°C, 5 seconds at 85°C, and subsequently stored at 4°C. Quantitative RT-PCR (RT-qPCR) instruments and the Bio-Rad iQ5 software version 2.1.97 (Bio-Rad, Inc., Hercules, CA) were used for amplification and quantitative analysis. The total reaction system consisted of the following mixture: 0.6 μ L of forward and 0.6 μ L of reverse primers (10 μ mol/L), 1 μ L of cDNA solution, 10 μ L of SYBR Green I Master (Roche, Mannheim, Germany), and 7.8 μ L of distilled water. The reaction conditions were as follows: 10 minutes at 95°C; PCR for 15 seconds at 95°C and 1 minute at 60°C, for a total of 40 cycles. The primers of *LAMC2*, *EGFR*, phosphatidylinositol 3-kinase (*PI3K*), *K-Ras*, *CDH1*, *VIM*, *ZEB1*, and *U6* were synthesized by the Beijing Genomics Institute (Shenzhen, China) (Table 2). *U6* was used as the internal reference. The term $2^{-\Delta Ct}$ represented the multiple proportions of target gene expression between the experimental group and the control group. The formula used was as follows: $\Delta Ct = Ct_{miRNA} - Ct_{U6}$.

Ct represents the number of amplification cycles required for the set threshold of the real-time fluorescence intensity, and the amplification was in logarithmic growth. The experiment was repeated three times, and the protocol was used for all subsequent cell experiments.

Western Blot Analysis

Total protein was extracted from tissues and cells using radioimmunoprecipitation assay lysis buffer (R0010; Beijing

Solarbio Science & Technology Co, Ltd, Beijing, China) containing phenylmethylsulfonyl fluoride. According to manufacturer's instructions, the protein concentration was determined by using a bicinchoninic acid protein assay kit (BCA1-1KT; Sigma, St. Louis, MO). Approximately 20 μ g of each sample was separated by polyacrylamide gel electrophoresis (10% separation gel and 5% concentrated gel) for 2 hours and transferring onto a polyvinylidene fluoride membrane. The membrane was then blocked with 5% skim milk in Tris-buffered saline with Tween-20 for 1 hour. After that, the membrane was incubated with the diluted antibodies

Table 2 Primer Sequences of Related Genes for RT-qPCR

Gene	Primer sequence
<i>LAMC2</i>	F: 5'-CCAGAAGGTTTCAGATGCC-3' R: 5'-TGGCTTCCAAGTTCAGACTC-3'
<i>EGFR</i>	F: 5'-CGAATGGGCCTAAGATCCCG-3' R: 5'-CTTCGCATGAAGAGGCCGAT-3'
<i>PI3K</i>	F: 5'-CCACGACCATCATCAGGTGAA-3' R: 5'-ACCACAGTCATCGAGAGGACC-3'
<i>K-Ras</i>	F: 5'-GCTCAGGACTTAGCAAGAAG-3' R: 5'-GTATTTACATAATTACACACTTTG-3'
<i>E-cadherin</i>	F: 5'-AGAACGCATTGCCACATACACTCTC-3' R: 5'-CGGTTACCGTGATCAAAATCTCCA-3'
<i>Vimentin</i>	F: 5'-TGCAGGAGGAGATGCTTCAG-3' R: 5'-ATTCCACTTTGCGTTCAAGG-3'
<i>ZEB1</i>	F: 5'-GCCAATAAGCAAACGATTCTG-3' R: 5'-TTTGGCTGGATCACTTTCAAG-3'
<i>U6</i>	F: 5'-GTGCTCGCTTCGGCAGCATATAC-3' R: 5'-AAAAATATGGAACGCTTCACGAATTTG-3'

F, forward; R, reverse; RT-qPCR, quantitative RT-PCR.

Figure 1 *LAMC2* is highly expressed in cholangiocarcinoma (CCA). **A** and **B**: The heat map of 50 differentially expressed genes (DEGs) of GSE77984 and GSE45001 chips. **C**: Venn analysis of top 200 DEGs reveals that *LAMC2*, *ALDH2*, and *AOX1* are displayed in the intersection of GSE77984 and GSE45001 chips. **D**: Correlation analysis of *LAMC2* and *ALDH2*, with the known CCA-related genes. **E** and **F**: Expression of *LAMC2* in CCA samples by analysis of the GSE26566 chip ($P = 6.99 \times 10^{-9}$) and The Cancer Genome Atlas (TCGA) database.

overnight at 4°C: *LAMC2* (ab210959; dilution 1:1000; Abcam, Inc., Cambridge, MA), EGFR (ab81440; dilution 1:1000; Abcam, Inc.), PI3K (ab189403; dilution 1:1000; Abcam, Inc.), vimentin (ab8978; dilution 1:1000; Abcam, Inc.), v-Ki-ras2-Kirsten rat sarcoma 2 viral oncogene homolog (K-Ras; ab157255; dilution 1:2000; Abcam, Inc.), zinc finger E-box-binding homeobox (ZEB1; NBP2-2384H; dilution 1:2000; Shanghai Xuanling Biotechnology Co, Ltd, Shanghai, China), and E-cadherin (ab1416; dilution 1:50; Abcam, Inc.). Glyceraldehyde-3-phosphate dehydrogenase (ab8245; dilution 1:500; Abcam, Inc.) was used as internal reference. Subsequently, the membrane was incubated with secondary goat anti-mouse monoclonal antibody horseradish peroxidase-labeled IgG (IgG; ab6785; dilution 1:1000; Abcam, Inc.) at room temperature for 1 hour. The membrane was visualized with 1 mL enhanced chemiluminescence solution, prepared using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific Inc., Waltham, MA) on X-ray film. The experiment was

repeated three times, and this procedure was used for the subsequent cell experiments.

Hematoxylin and Eosin Staining

CCA and adjacent normal tissues were fixed in 4% paraformaldehyde (P1110; Beijing Solarbio Science & Technology Co, Ltd), cut into serial frozen sections (5 μ m thick), and stored at -20°C for further use. After being stained with hematoxylin for 7 minutes, sections were taken out (G1140; Beijing Solarbio Science & Technology Co, Ltd), washed under running water for 1 minute, and treated with acidic alcohol for 15 seconds. After another wash under running water for 4 minutes, the sections were stained with eosin solution for 3 minutes and treated with gradient ethanol at the concentrations of 70%, 80%, and 90% for 1 minute, 95% for 2 minutes, and 100% (I and II, respectively) for 5 minutes. Sections were cleared with xylene I and II, respectively, for 10 minutes. Finally, the sections

Table 3 The Known Top 10 Cholangiocarcinoma-Related Genes

Gene	Score	PMIDs
<i>TP53</i>	0.291	24
<i>PTGS2</i>	0.215	19
<i>EGFR</i>	0.213	21
<i>ERBB2</i>	0.211	14
<i>IL6</i>	0.208	18
<i>K-Ras</i>	0.205	13
<i>SLC5A5</i>	0.203	1
<i>GNAS</i>	0.203	2
<i>PTEN</i>	0.201	6
<i>IDH2</i>	0.201	5

Score of the reliability of the gene-disease association, on the basis of the type and number of sources in which it is reported, and the number of PubMed Unique Identifiers (PMIDs) (ie, total number of PMIDs supporting the association).

were cleared with neutral gum and observed under a 200-fold optical light microscope (BX-51; Olympus, Tokyo, Japan).

Immunohistochemistry

CCA and adjacent normal tissues extracted from patients and nude mice (tumor formation experiments *in vivo*) were cut into serial sections (4 μ m thick) and left to dry in an oven for 20 minutes at 68°C. The sections were then treated with xylene (GD-RY1215-12; Guduo Biotechnology Co, Ltd, Shanghai, China) for 25 minutes, dehydrated with 100%, 95%, and 75% ethanol, respectively, for 5 minutes for each concentration, and washed three times in distilled water (3 minutes per wash). After adding 3% H₂O₂, the sections were incubated for another 10 minutes at 37°C, and then washed with distilled water three times (3 minutes per wash). The endogenous peroxidases were inactivated using hydrogen peroxide. The sections were washed with phosphate-buffered saline (PBS). High-pressure antigen retrieval was conducted twice after the sections were dried. The sections were then blocked with 10% goat serum for 15 minutes before being incubated with primary monoclonal antibodies: mouse anti-human *LAMC2* (ab210959; dilution 1:500; Abcam, Inc.) and rabbit anti-mouse CD31 (ab28364; dilution 1:50; Abcam, Inc.) at 4°C overnight. On the following day, the sections were warmed in a 37°C incubator for 45 minutes and washed thrice with PBS (5 minutes for each time). Biotin-labeled secondary antibodies goat anti-mouse (dilution 1:1000; ab6785; Abcam, Inc.) and goat anti-rabbit (dilution 1:1000; ab150077; Abcam, Inc.) IgGs were added and incubated at 37°C for 30 minutes, followed by three PBS washes (5 minutes per wash). The sections were stained with a streptavidin-peroxidase staining kit (Phylas, Changzhou, China) for 10 to 15 minutes, according to the manufacturer's instructions. Next, the sections were stained with diaminobenzidine [Chemical Abstracts Service

(CAS): 7411-49-6; Suzhou Yacoo Science Co Ltd, Suzhou, China] for 3 minutes and then washed under running water for 3 minutes. The sections were then counterstained with hematoxylin for 3 minutes, and washed with distilled water thrice, 5 minutes per wash. The sections were dehydrated with gradient ethanol, cleared with xylene, and mounted with neutral balsam. A double-blind method was used to evaluate the final results. Five high-power fields ($\times 400$) were randomly selected for observation under an optical microscope (CX41-12C02; Olympus). Tan particles in the cytoplasm represented a positive expression for *LAMC2*. Protein staining results were calculated according to the percentage of positive cells using the following evaluating criteria: a positive expression indicated that the number of positive cells was $>10\%$ (+), whereas the number of positive cells $<10\%$ indicated a negative expression (−). Immunohistochemistry was also used for detection of microvessel density (MVD) in xenograft tumor in nude mice. The principle of microvessel counting and criteria of CD31 positive were used according to the following: any endothelial cell cluster or single endothelial cell that stained either brown or tan with defined borders with adjacent tumor cells, microvessels, and surrounding connective tissues was regarded as a microvessel.

Cell Culture and Selection

CCA cell lines RBE and QBC939 were obtained from the Department of Liver and Gallbladder Hepatobiliary Surgery, Southwest Hospital, Third Military Medical University (Chongqing, China). CCA cell line HCCC-9810 was obtained from the Central Laboratory of Zhongshan Hospital, Xiamen University (Xiamen, China), whereas cell lines HuCC-T1 and SSP-25 were procured from the ATCC (Manassas, VA) cell bank. Cancer cell lines were cultured at 37°C in a humidified incubator (BB15; Thermo Fisher Scientific Inc., Waltham, MA) with 5% CO₂ containing Dulbecco's modified Eagle's medium (31600-034; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (26140079; Gibco, Grand Island, NY). The medium was replaced every 24 hours, and the cell lines were passaged every 72 hours. The cells were washed three times with PBS without medium and detached with 0.25% trypsin for 3 minutes. The detachment was terminated with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. A single-cell suspension was prepared after trituration using a pipette. The mRNA expression of *LAMC2* in the three cell lines was determined by RT-qPCR. The cell line with the highest expression level of *LAMC2* was selected for subsequent experiments. The above procedure was repeated three times.

Vector Construction

siRNA and shRNA sequence design was in accordance with the mRNA complete sequence of *LAMC2*, published by GenBank

Figure 2 *LAMC2*, epidermal growth factor receptor (EGFR), phosphatidylinositol 3-kinase (PI3K), v-Ki-ras2-Kirsten rat sarcoma 2 viral oncogene homolog (K-Ras), vimentin, and zinc finger E-box-binding homeobox 1 (ZEB1) are highly expressed, and E-cadherin is lowly expressed, in cholangiocarcinoma (CCA) tissues. **A:** The mRNA levels of *LAMC2*, EGFR, PI3K, K-Ras, vimentin, and ZEB1 in CCA tissues and adjacent normal tissues, as determined by quantitative RT-PCR. **B:** The gray value of *LAMC2*, EGFR, phosphorylated EGFR (p-EGFR), PI3K, phosphorylated PI3K (p-PI3K), K-Ras, E-cadherin, vimentin, and ZEB1 protein bands in CCA tissues and adjacent normal tissues, as determined by Western blot analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference. **C:** The protein levels of *LAMC2*, EGFR, PI3K, K-Ras, E-cadherin, vimentin, and ZEB1, as well as the extent of EGFR and PI3K phosphorylation in CCA tissues and adjacent normal tissues, as determined by Western blot analysis. The experiment was repeated three times. $n = 121$ CCA and adjacent normal tissues (**A** and **C**). * $P < 0.05$ versus adjacent normal tissues (unpaired t -test).

(<https://www.ncbi.nlm.nih.gov/genbank>; accession number NM_005562). According to siRNA and shRNA design principles, sequences of siRNA-*LAMC2* and shRNA-*LAMC2* were designed using the online design website of Thermo Fisher Scientific. The forward sequence of siRNA-*LAMC2* is 5'-GCAGAAUACAGUCUCCAUA-3', and the reverse sequence is 5'-UAUGGACACUGUAUUCUGC-3'. The forward sequence of shRNA-*LAMC2* is 5'-CACCGC-CTCAACTGCAATGCAACACGAATGTTGTCATTGCAG-TTGAGGC-3', and the reverse sequence is 5'-AA-AAGCCTCAACTGCAATGACAACATTCGTGTTGTCAT-TGCAGTTGAGGC-3'. All of the oligonucleotide fragments were synthesized by Shanghai Sangon Biotech Co, Ltd (Shanghai, China). After annealing, these sequences were inserted into BamHI and EcoR (ER0052 and ER0271, respectively; Cobioer, Nanjing, China) linearized pSIREN restriction sites (HAB 2-9; Huaaobio, Beijing, China). The constructed recombinant plasmids pSI-REN/S (recombinant siRNA-*LAMC2* vector, S represented siRNA) and pSIREN/CN (recombinant pcDNA-*LAMC2* vector, CN represented cDNA) were transformed into *Escherichia coli* DH5 α . The ampicillin-resistant transformed *E. coli* DH5 α colonies were selected after sequencing. The plasmids were rapidly and quantitatively prepared, followed by nucleic acid sequencing identification. Correct sequenced colonies were selected and cultured for subsequent experiments.

Stable *LAMC2* Silencing in the QBC939 Cell Line

The puromycin-resistant lentiviral vector siRNA-*LAMC* was synthesized with lentiviral vector PLKO.1-puro directed by

U6 promoter. After annealing, the sense and antisense strands of siRNA-*LAMC* were restored to double strands. The lentiviral vector was then ligated to the T4 DNA ligase. Next, 4.5 μ L of ligation products was transformed into DH5 α competent cells (TIANGEN Biotech Co, Ltd, Beijing, China). Resistant strains were selected in the Luria-Bertani plate coated with puromycin (Sigma). The plate was then incubated at a temperature of 37°C. The extracted plasmid was sequenced (Beijing Genomics Institute) to verify whether the inserted sequence was correct. The recombinant lentiviral vector was subsequently produced and packaged with a virus titer of 10^6 transducing units (Sigma). Next, 2×10^5 QBC939, HuCC-T1, and SSP-25 cells were paved to the 6-well plate 2 days before transfection. When cells reached 60% to 70% confluence, 8 μ L/mL polymer gel was added to each well (Sigma), followed by transfection with 50 μ L of recombinant lentiviral vector. After transfection for 48 hours, the medium was replaced with RPMI 1640 medium supplemented with 1 μ g/mL puromycin. The cells were regularly checked, and the culture medium was replaced every 2 to 3 days until the nontransfected cells were completely killed. The colonies were amplified and cultured. The colonies were obtained by the limiting dilution assay, and cell lines with stable *LAMC2* silencing were obtained from resistant clones.

Cell Grouping and Transfection

The wild-type QBC939, HuCC-T1, and SSP-25 cell lines were assigned into blank group (without any transfection of plasmid but only PBS treatment), negative control (NC) group

Figure 3 Protein expression of *LAMC2* is higher in cholangiocarcinoma (CCA) tissue than in adjacent normal tissue. **A:** Histopathology of CCA and adjacent normal tissues, stained with hematoxylin and eosin. **B:** *LAMC2* protein expression in CCA and adjacent normal tissues, as measured by immunohistochemistry. **C:** Positive expression rate of *LAMC2* in CCA and adjacent normal tissues. $n = 121$ CCA and adjacent normal tissues (**C**). * $P < 0.05$ versus adjacent normal tissues (unpaired t -test). Scale bars: 100 μm (**A**); 25 μm (**B**). Original magnification, $\times 400$ (**B**).

(transfection with NC), siRNA-*LAMC2* group (transfection with siRNA-*LAMC2* plasmid), siRNA-*LAMC2* + EGF group (treatment with EGFR signaling pathway activator, EGF, 10 $\mu\text{g/L}$, and transfection with siRNA-*LAMC2* plasmid), and AG1478 group (treatment with EGFR signaling pathway inhibitor, AG1478, 10 $\mu\text{g/L}$; Tocris, Minneapolis, MN). The cell lines with stable *LAMC2* silencing were allocated to blank group (without any transfection of plasmid but only PBS treatment), NC group (transfection with negative control), pcDNA-*LAMC2* group (transfection with pcDNA-*LAMC2* plasmid purchased from HanBio Co, Ltd, Shanghai, China), and the pcDNA-*LAMC2* + AG1478 group (transfection with pcDNA-*LAMC2* plasmid and treatment with AG1478 of 10 $\mu\text{g/L}$). Cells were inoculated in 6-well plates with a cell density of 3×10^5 cells per well. Cell transfection was performed using Lipofectamine 2000 reagent (Invitrogen Inc., Carlsbad, CA) when the cell confluence reached 50% to 80%. Subsequently, 4 μg of the target plasmid and 10 μL of Lipofectamine 2000 were separately diluted with 250 μL of Opti-MEM medium (Gibco) and allowed to sit at room temperature for 5 minutes. The two dilutions were mixed and left to incubate at room temperature

for 20 minutes. The mixture was added to the culture wells and cultured in an incubator with 5% CO_2 at 37°C . After the transfected plasmids were incubated for 6 hours, the medium was replaced with complete medium. After another 12 hours of incubation, the cells were treated with EGF and AG1478 for 48 hours and then harvested for the subsequent experiments.

Morphologic Observation

The stable *LAMC2*-silencing cells were cultured at 37°C with 5% CO_2 for 72 hours. Changes in cell phenotype of each group were observed and imaged under an inverted-phase contrast microscope (Olympus).

Immunofluorescence Assay

Cells in the logarithmic growth phase were detached with trypsin and centrifuged. Collected cells were seeded to 24×24 -mm coverslips and cultured for 24 hours to reach 70% cell confluence. Cells were rinsed once in precooled PBS, fixed in 4% paraformaldehyde for 20 minutes, rinsed twice in PBS (5 minutes per wash), and blocked with 10% goat serum for 15 minutes. The primary antibodies rabbit anti-human antibody to E-cadherin (ab40772; dilution 1:500; Abcam, Inc.) and vimentin (ab92547; dilution 1:300; Abcam, Inc.) were added to the cells and left to incubate at 4°C overnight. These samples were warmed for 45 minutes at 37°C and rinsed three times with PBS (5 minutes per wash). The coverslips labeled with rabbit anti-human E-cadherin were added with 40 μL of Alexa Fluor 488 anti-rabbit fluorescence IgG (dilution 1:400; Proteintech, Chicago, IL), and coverslips labeled with rabbit anti-human vimentin were added with 40 μL of Alexa Fluor 594 anti-rabbit fluorescence IgG (dilution 1:400; Proteintech). The cells were incubated at room temperature for 2 hours and washed three times with PBS (5 minutes per wash) in a dark room. The cells were then stained with DAPI (dilution 1:10; Vector Laboratories, Burlingame, CA) in the dark for 5 minutes, followed by three rinses with running water (5 minutes per wash). Next, the specimens were dehydrated with gradient ethanol, cleared with xylene, mounted with neutral balsam, and observed under a light microscope.

Transwell Assay

The Transwell chamber (Corning Incorporated, Corning, NY), sampling tube, and PBS were precooled at 4°C for one night. Matrigel (Becton Dickinson, Franklin Lakes, NJ) was thawed at 4°C , mixed at the ratio of 1:1 with serum-free medium, and then added into the apical chamber of the Transwell chamber (50 μL per well). The plates were incubated at 37°C for 4 hours. Cells in logarithmic growth phase were starved with serum-free RPMI 1640 medium (Gibco) for 12 hours. After removal of the medium, the cells were washed twice with PBS (5 minutes per wash), detached with trypsin, and then centrifuged at $716 \times g$ for 5 minutes. The supernatant was removed,

Figure 4 *LAMC2* expression is highest in the QBC939 cell line. **A:** *LAMC2* expression in cell lines HCCC-9810, RBE, QBC939, HuCC-T1, and SSP-25, detected by quantitative RT-PCR. **B:** Protein bands, produced by Western blot analysis, showing the levels of *LAMC2*, epidermal growth factor receptor (*EGFR*), and v-Ki-ras2-Kirsten rat sarcoma 2 viral oncogene homolog (*K-Ras*) in cell lines HCCC-9810, RBE, QBC939, HuCC-T1, and SSP-25. **C:** Protein-level quantification of *EGFR* and *K-Ras* in cell lines HCCC-9810, RBE, QBC939, HuCC-T1, and SSP-25. The experiment was repeated three times. * $P < 0.05$ versus HCCC-9810 cell line (one-way analysis of variance); † $P < 0.05$ versus RBE cell line (one-way analysis of variance). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

and the cells in the chamber were resuspended with 2 mL of serum-free RPMI 1640 medium. The cell suspension was diluted to 1×10^6 cells/mL. The cell suspension was added into the apical chamber, and 600 μ L of RPMI 1640 medium, containing 10% serum, was added into the basolateral chamber. After incubation at 37°C with 5% CO₂ for 24 hours, the cells were washed twice with PBS (5 minutes per wash). The chamber was then placed in a 24-well culture plate. Each well was stained for 30 minutes with 600 μ L of crystal violet (0.1%), washed three times with PBS (5 minutes per wash), and air dried. According to the five-point sampling method, five visual fields were acquired and observed under an inverted microscope (high-power lens). The mean value was then calculated. The experiment was repeated three times.

Scratch Test

After transfection, cells were seeded in a 6-well plate with 2×10^5 cells per well. Lines were produced by scratching evenly and horizontally on the posterior side of the plate using a marker pen. When cells reached 80% to 90% confluence, a thin wound was generated lightly along the center of each well with a 200 μ L pipette tip (the width of each scratch was the same). The wells were washed twice gently with PBS (5 minutes per wash) to remove floating cells. Serum-free RPMI 1640 medium was added into the wells for subculturing the

cells for 0.5 to 1 hour. After cell recovery, images were acquired at the initial time, and 24 hours later. To evaluate the cell migration ability, 15 straight lines were evenly drawn in each field. The width of the 15 lines across the scratch was measured, and the average width of the scratch was calculated. Cell migration distance was calculated by the following: cell distance = width of initial scratch – width of scratch, whereby distance was measured using Image-Pro Plus Analysis software version 6.0 (Media Cybernetics, Bethesda, MD).

Tumor Formation in Nude Mice

A total of 30 male BALB/c nude mice (aged 10 to 12 weeks; weighing 20 to 25 g) were fed in the laboratory animal center of Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College ($n = 5$ mice in each cage) at 25°C with a light and dark cycle of illumination. The mice were randomly divided into the blank, NC, shRNA-*LAMC2*, EGF, and shRNA-*LAMC2* + EGF groups. The cells in the logarithmic growth phase were resuspended in serum-free RPMI 1640 medium to generate a cell suspension of 4×10^6 cells/mL. Next, cell suspensions were subcutaneously injected into both axillae of mice (200 μ L per injection). Tumor morphology and nodular growth of mice were observed and recorded every 3 days since the seventh day after initial cell suspension injection. Three

Figure 5 The expression of epidermal growth factor receptor (EGFR) pathway-related genes is down-regulated by *LAMC2* gene silencing. **A:** mRNA expression of *LAMC2*, *EGFR*, phosphatidylinositol 3-kinase (*PI3K*), and v-Ki-ras2-Kirsten rat sarcoma 2 viral oncogene homolog (*K-Ras*) in the wild-type QBC939 cell line, as detected by quantitative RT-PCR (RT-qPCR). **B:** Protein bands of *LAMC2*, *EGFR*, phosphorylated *EGFR* (p-*EGFR*), *PI3K*, phosphorylated *PI3K* (p-*PI3K*), and *K-Ras* in the wild-type QBC939 cell line, as determined by Western blot analysis. **C:** Protein expression of *LAMC2*, *EGFR*, *PI3K*, and *K-Ras*, and the extent of *EGFR* and *PI3K* phosphorylation in the wild-type QBC939 cell line, as determined by Western blot analysis. **D:** mRNA expression of *LAMC2*, *EGFR*, *PI3K*, and *K-Ras* in the *LAMC2*-silencing QBC939 cell line, as determined by RT-qPCR. **E:** Protein bands of *LAMC2*, *EGFR*, p-*EGFR*, *PI3K*, p-*PI3K*, and *K-Ras* in the *LAMC2*-silencing QBC939 cell line, as determined by Western blot analysis. **F:** Protein expression of *LAMC2*, *EGFR*, *PI3K*, and *K-Ras*, and the extent of *EGFR* and *PI3K* phosphorylation, in the *LAMC2*-silencing QBC939 cell line, as determined by Western blot analysis. The experiment was repeated three times. * $P < 0.05$ versus the blank and negative control (NC) groups (one-way analysis of variance). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

weeks later, the mice were euthanized and the xenograft tumors were excised from nude mice in the blank, NC, shRNA-*LAMC2*, EGF, and shRNA-*LAMC2* + EGF groups and sliced into sections. The MVD was also detected using immunohistochemistry.

Statistical Analysis

Statistical analyses were conducted using SPSS 21.0 (IBM Corp., Armonk, NY). Measurement data were expressed as means $\pm$ SD. Comparisons between two groups were conducted by a *t*-test, and multiple comparisons among multiple

groups were conducted using one-way analysis of variance. $P < 0.05$ was considered to be significant.

Results

LAMC2 Was Expressed at a High Level in CCA

The data belonging to GSE77984 and GSE45001 CCA chips were obtained from the Gene Expression Omnibus database, and differential analysis was performed on GSE77984 and GSE45001 CCA chips. In the GSE77984 chip, 396 DEGs were found in CCA samples, of which 137 genes were

prominently up-regulated in CCA and 259 genes were notably down-regulated in CCA. In the GSE45001 chip, 1083 DEGs were obtained, among which 404 genes were up-regulated in CCA samples and 679 genes were down-regulated in CCA samples. The heat map of 50 DEGs of GSE79984 and GSE45001 chips was constructed (Figure 1, A and B). To further screen CCA-related genes, the Venn analysis of the top 200 DEGs was conducted to evaluate the gene intersection (Figure 1C). Finally, *LAMC2*, *ALDH2*, and *AOX* were found in the intersection of the two chips among the top 200 DEGs. This result implied that *LAMC2*, *ALDH2*, and *AOX* were likely to be associated with CCA. To further screen CCA-related genes associated with *LAMC2*, *ALDH2*, and *AOX*, gene-interaction analysis was performed on above-mentioned three DEGs and the known CCA-related genes. The known CCA-related genes were retrieved from the DisGeNET database, and the top 10 genes with the highest scores were selected for subsequent screening of CCA-related genes, which were used to construct a gene interaction network (Table 3 and Figure 1D). It was revealed that there were direct or indirect interactions of *LAMC2* and *ALDH2*, with known CCA-related genes, such as *TP53* and *EGFR*. Further literature retrieval on the relationship between *LAMC2/ALDH2* and CCA indicated that there was almost no report of aldehyde dehydrogenase 2 (*ALDH2*) associated with CCA, whereas several studies revealed that the expression of *LAMC2* was directly related to CCA.^{19,20} Although the mechanism of *LAMC2* in CCA was still poorly understood, studies suggest that *LAMC2* could regulate the progression of tumor through the EGFR signaling pathway.^{10,11,21} Moreover, previous research has demonstrated that EGFR was closely related to the development of CCA.^{22–24} Taken together, these studies suggest that *LAMC2* was highly expressed in CCA and might be associated with CCA development through the EGFR signaling pathway. To further confirm the differential expression of *LAMC2* in CCA, another independent GSE26566 CCA chip was retrieved from the Gene Expression Omnibus database for further analysis. Meanwhile, the expression of *LAMC2* in CCA samples retrieved from The Cancer Genome Atlas database was analyzed (Figure 1, E and F). It was demonstrated that *LAMC2* from both GSE26566 chip and The Cancer Genome Atlas database exhibited high expression in CCA. Thus, *LAMC2* might affect the progression of CCA through the EGFR signaling pathway.

EGFR Signaling Pathway and EMT Are Induced in CCA

To further verify *LAMC2* expression in CCA and EGFR signaling pathway in relation to CCA, the expression of *LAMC2* and the EGFR signaling pathway-related genes *EGFR*, *PI3K*, and *KRAS* was determined in CCA tissues and adjacent normal tissues by RT-qPCR (Figure 2C) and Western blot analysis (Figure 2, A and B). EMT markers E-cadherin, vimentin, and ZEB1 were also determined to show the progression of CCA. Results showed that mRNA and protein levels of *LAMC2*, *EGFR*, *PI3K*, K-Ras, vimentin, and ZEB1, as well as the extent of EGFR and *PI3K* phosphorylation, were significantly higher in CCA tissues compared with those detected in adjacent normal tissues. E-cadherin expression was remarkably lower in CCA tissues than that in adjacent normal tissues (all $P < 0.05$). These results suggest that *LAMC2* is highly expressed in CCA, thereby activating the EGFR signaling pathway and inducing EMT.

High *LAMC2* Expression Is Correlated with CCA Progression

The expression of *LAMC2* in CCA samples was analyzed in response to different clinicopathological features. Hematoxylin and eosin staining demonstrated that CCA tissues presented with diffused distribution and obscure entrance of bile duct. Immunohistochemistry was conducted to determine *LAMC2* protein expression in CCA cells and tissues. Under the microscope, *LAMC2* protein was mainly expressed in the cytoplasm, and presented as brown particles (Figure 3A). The positive rate of *LAMC2* expression was 59.50% (72/121) in CCA tissues, significantly higher compared with that in adjacent normal tissues [37.38% (42/121)] ($P < 0.05$) (Figure 3, B and C). The *LAMC2* level was closely related to the differentiation degree, LNM, invasion, and tumor-node-metastases ($P < 0.05$), but showed no relation to sex and age of patients ($P > 0.05$) (Table 1). Compared with patients without portal vein invasion, the positive expression rate of *LAMC2* was prominently higher in patients with portal vein invasion. There was a higher expression rate of *LAMC2* in patients with LNM compared with patients without LNM. Moreover, the positive expression rate of *LAMC2* was remarkably higher in patients with poor differentiation in comparison with moderately and highly differentiated

Figure 6 Epithelial-mesenchymal transition (EMT) is inhibited by silencing *LAMC2*. **A:** mRNA expression of vimentin, E-cadherin, and zinc finger E-box-binding homeobox 1 (*ZEB1*) in the wild-type QBC939 cell line, as determined by quantitative RT-PCR (RT-qPCR). **B:** Protein bands of vimentin, E-cadherin, ZEB1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the wild-type QBC939 cell line, as determined by Western blot analysis. **C:** Protein expression of vimentin, E-cadherin, ZEB1, and GAPDH in the wild-type QBC939 cell line, as determined by Western blot analysis. **D:** mRNA expression of vimentin, E-cadherin, and ZEB1 in the *LAMC2*-silencing QBC939 cell line, as determined by RT-qPCR. **E:** Protein bands of vimentin, E-cadherin, ZEB1, and GAPDH in the *LAMC2*-silencing QBC939 cell line, as determined by Western blot analysis. **F:** Protein expression of vimentin, E-cadherin, ZEB1, and GAPDH in the *LAMC2*-silencing QBC939 cell line, as determined by Western blot analysis. **G:** EMT cellular morphology comparison in the wild-type QBC939 cell line. **H:** EMT cellular morphology comparison in the *LAMC2*-silencing QBC939 cell line. **I:** The location of E-cadherin and vimentin in the wild-type QBC939 cell line, as observed by immunofluorescence. **J:** The location of E-cadherin and vimentin in the *LAMC2*-silencing QBC939 cell line, as observed by immunofluorescence. The experiment was repeated three times. * $P < 0.05$ versus the blank and negative control (NC) groups (one-way analysis of variance). Scale bars: 100 μm (**G** and **H**); 25 μm (**I** and **J**). Original magnifications: $\times 100$ (**G** and **H**); $\times 400$ (**I** and **J**). EGF, epidermal growth factor.

Figure 7 The number of invasive cells is reduced by silencing *LAMC2*. **A:** Transwell assay showing cell invasion in the wild-type QBC939 cell line. **B:** The number of invasive cells in the wild-type QBC939 cell line, as tested by Transwell assay. **C:** Cell invasion in the *LAMC2*-silencing QBC939 cell line, as measured by Transwell assay. **D:** The number of invasive cells in the *LAMC2*-silencing QBC939 cell line, as measured by Transwell assay. The experiment was repeated three times. * $P < 0.05$ versus the blank and negative control (NC) groups (one-way analysis of variance). Scale bars = 100 μm (A and C). Original magnification, $\times 100$ (A and C). EGF, epidermal growth factor.

patients. Compared with stage I/II patients, the positive expression rate of *LAMC2* in stage III/IV patients was significantly higher. All these data indicate that high protein expression of *LAMC2* is detected in CCA tissues, which is relevant to the progression of CC.

QBC939 Cell Line Demonstrates Highest *LAMC2* Expression

To select the cell line with the highest *LAMC2* expression for further experiments, RT-qPCR and Western blot analysis were conducted to detect the expression of *LAMC2* in cell lines HCCC-9810, REB, QBC939, HuCC-T1, and SSP-25 (Figure 4). The expression of *LAMC2* in cell lines RBE, QBC939, HuCC-T1, and SSP-25 was significantly higher compared with cell line HCCC-9810 ($P < 0.05$). The expression of *LAMC2* was markedly increased in cell line QBC939 in comparison with that in cell lines RBE, HuCC-T1, and SSP-25. In addition, the expression of EGFR and K-Ras was also detected in cell lines HCCC-9810, REB, QBC939, HuCC-T1, and SSP-25. No differences were found

in the K-Ras expression, whereas the highest EGFR expression was detected in cell line QBC939. Therefore, the cell line QBC939 was selected for the subsequent experiments.

LAMC2 Stimulates the EGFR Signaling Pathway in CCA

To explore the possible function of *LAMC2* in regulating the EGFR signaling pathway, RT-qPCR and Western blot analysis were used to measure the expression levels of *LAMC2* and EGFR signaling pathway-related genes *EGFR*, *PI3K*, and *KRAS*. The expression of EGFR signaling pathway-related genes did not significantly differ between the blank and NC groups in both the wild-type and the *LAMC2*-silencing QBC939 cells (all $P > 0.05$). In the wild-type QBC939 cells, *LAMC2*, *EGFR*, *PI3K*, and *KRAS* expression, and EGFR and PI3K phosphorylation, were suppressed after transfection with siRNA-*LAMC2* (all $P < 0.05$) in contrast to cells without transfection or transfected with scramble sequences. EGFR, PI3K, and K-Ras expression, and the extent of EGFR and PI3K phosphorylation, were found to be significantly elevated after

Figure 8 Cell migration is reduced by *LAMC2* silencing. **A:** Cell migration in the wild-type QBC939 cell line, as tested by scratch test. **B:** The migration distance in the wild-type QBC939 cell line, as tested by scratch test. **C:** Cell migration in the *LAMC2*-silencing QBC939 cell line, as measured by scratch test. **D:** The migration distance in the *LAMC2*-silencing QBC939 cell line, as measured by scratch test. The experiment was repeated three times. * $P < 0.05$ versus the blank and negative control (NC) groups (one-way analysis of variance). Original magnification, $\times 40$ (**A** and **C**). EGF, epidermal growth factor.

treatment by EGF (all $P < 0.05$) compared with cells without transfection or transfected with scramble sequence. No significant difference was found in the expression of *LAMC2* after EGF treatment when compared with cells without transfection or those transfected with the scramble sequence ($P > 0.05$). Decreased expression levels of *LAMC2* were found after transfection of siRNA-*LAMC2* and treatment with EGF ($P < 0.05$). However, no significant changes were found in EGFR, PI3K, and K-Ras levels, as well as in the extent of EGFR and PI3K phosphorylation (all $P > 0.05$) in comparison to cells without transfection or those transfected with the scramble sequence. The expression of EGFR, PI3K, and K-Ras and the extent of EGFR and PI3K phosphorylation were lower (all $P < 0.05$), whereas *LAMC2* levels did not differ prominently when cells were treated with AG1478 ($P > 0.05$) in contrast to cells without transfection and those transfected with the scramble sequence (Figure 5, A–C). In the *LAMC2*-silencing QBC939 cells, mRNA and protein expression of *LAMC2*, EGFR, PI3K, and K-Ras and the extent of EGFR and PI3K phosphorylation were increased in the pcDNA-*LAMC2* group in comparison with the blank and NC groups (all $P < 0.05$). *LAMC2* levels were also up-regulated in the pcDNA-*LAMC2* + AG1478 group, whereas no obvious difference was observed in the expression of EGFR, PI3K, and K-Ras, as well as the extent of EGFR and PI3K phosphorylation when compared with the blank and NC groups (all $P > 0.05$). All these findings suggest that *LAMC2* is a positive regulator of EGFR in CCA (Figure 5, D–F).

Silencing *LAMC2* Inhibits EMT of CCA Cells

To further investigate the mechanism of *LAMC2* function in CCA, RT-qPCR and Western blot analysis were used to detect the expression of EMT markers, such as vimentin, E-cadherin, and ZEB1, in cell line QBC939. Immunofluorescence staining was used to quantitate the expression of vimentin and E-cadherin and evaluate their location in the cells. In addition, an optical light microscope was used to observe the phenotypic switching during EMT. The results showed that vimentin was expressed in the cytoplasm and E-cadherin was expressed in the cell membrane. The expression of vimentin, E-cadherin, and ZEB1 and phenotypic switching did not differ significantly between the blank and NC groups in both wild-type and *LAMC2*-silencing QBC939 cell lines (all $P > 0.05$). Vimentin, E-cadherin, and ZEB1 levels also did not differ significantly among the blank, NC, and siRNA-*LAMC2* + EGF groups in the wild-type QBC939 cells (all $P > 0.05$) (Figure 6, A, E, G, and I). There was no obvious change from the cobblestone-shaped epithelial cells to spindle-shaped mesenchymal cells. After transfection of siRNA-*LAMC2* and AG1478, E-cadherin expression was prominently increased, whereas vimentin and ZEB1 expression decreased in the wild-type QBC939 cells (all $P < 0.05$). An abundant amount of cobblestone-shaped epithelial cells that transformed into spindle mesenchymal cells were observed (Figure 6B). In the *LAMC2*-silencing QBC939 cell line, the expression of E-cadherin was reduced, but expression of

Figure 9 The microvessel density (MVD) of xenograft tumors is decreased by silencing of *LAMC2*. **A:** The microvessels in the tumor tissues obtained from *in vivo* experiment. **B:** Statistical analysis of the MVD value of xenograft tumors *in vivo*. **C:** mRNA expression of vascular endothelial growth factor (VEGF), as detected by quantitative RT-PCR. The experiment was repeated three times. * $P < 0.05$ versus the blank group (one-way analysis of variance). Scale bars = 25 μ m (**A**). Original magnification, $\times 400$ (**A**). NC, negative control.

vimentin and ZEB1 was increased markedly, after transfection of pcDNA-*LAMC2* when compared with cells without transfection or transfected with scramble sequences (all $P < 0.05$) (Figure 6, C, D, F, and H). An obvious change was seen in the cobblestone phenotype epithelial cells that switched to spindle phenotype mesenchymal cells. There were no significant differences in the expression of vimentin, E-cadherin, and ZEB1 in the *LAMC2*-silencing QBC939 cells treated with pcDNA-*LAMC2* + AG1478 compared with cells without transfection or transfected with scramble sequence (all $P > 0.05$). In addition, there was phenotypic switching from cobblestone epithelial cells to spindle mesenchymal cells (Figure 6J). Meanwhile, results obtained from the above experiment repeated in cell lines HuCC-T1 and SSP-25 revealed no significant changes compared with the QBC939 cell line (Supplemental Figures S1 and S2). The aforementioned data concluded that the silencing of *LAMC2* inhibits EMT of CCA cells.

Silencing *LAMC2* Restrains CCA Cell Invasion

To study the effect of *LAMC2* on CCA cell invasion, a Transwell assay was conducted to evaluate the cell invasion ability when *LAMC2* was silenced or overexpressed in CCA cells. The extent of cell invasion did not differ dramatically between the blank and NC groups in either wild-type or *LAMC2*-silencing QBC939 cells ($P > 0.05$). In the wild-type QBC939 cells, the number of cells invading through the chamber in the siRNA-*LAMC2* and AG1478 groups was

significantly lower ($P < 0.05$), whereas that in the EGF group was significantly higher ($P < 0.05$), compared with the blank and NC groups (Figure 7, A and B). Cell invasion did not differ prominently among the blank, NC, and siRNA-*LAMC2* + EGF groups. On the other hand, the number of cells invading through the chamber was increased by transfecting with pcDNA-*LAMC2* into *LAMC2*-silencing QBC939 cells (all $P < 0.05$) compared with that in the blank and NC groups. No remarkable difference was observed among the blank, NC, and pcDNA-*LAMC2* + AG1478 groups ($P > 0.05$) (Figure 7, C and D). These findings suggested that suppression of *LAMC2* led to declined expression of EGFR and depressed invasion of CCA cells.

Silencing *LAMC2* Suppresses CCA Cell Migration

The scratch test was used to assess the cell migration ability when *LAMC2* was silenced or overexpressed in CCA cells. In both wild-type and *LAMC2*-silencing QBC939 cells, cell migration did not differ remarkably between the blank and NC groups ($P > 0.05$). In the wild-type QBC939 cells, cell migration was significantly lower in the siRNA-*LAMC2* and AG1478 groups in comparison with the blank or NC group ($P < 0.05$), whereas cell migration was more prominent in the EGF group ($P < 0.05$). Cell migration capability did not change significantly in the siRNA-*LAMC2* + EGF group when compared with the blank and NC groups ($P > 0.05$) (Figure 8, A and B). In the *LAMC2*-silencing QBC939 cells,

Figure 10 Silencing of *LAMC2* inhibits epithelial-mesenchymal transition (EMT) of cholangiocarcinoma (CCA) cells through inactivation of the epidermal growth factor receptor (EGFR) pathway. *LAMC2* is highly expressed in CCA, which activates the EGFR pathway. This increases vimentin and zinc finger E-box-binding homeobox 1 (ZEB1) expression while decreasing E-cadherin, suggesting a promotive effect on EMT. K-Ras, v-Ki-ras2-Kirsten rat sarcoma 2 viral oncogene homolog; PI3K, phosphatidylinositol 3-kinase.

enhancement of cell migration in the pcDNA-*LAMC2* group was observed in contrast to the blank and NC groups ($P < 0.05$). Cell migration was declined in the pcDNA-*LAMC2* group when compared with the blank and NC groups ($P > 0.05$) (Figure 8, C and D). All these data suggest that the depressed expression of *LAMC2* suppresses CCA cell migration.

Silencing *LAMC2* Inhibits CCA Angiogenesis

The *in vivo* experiment was used to further analyze the effect of *LAMC2* on angiogenesis. Immunohistochemistry was performed to measure the MVD of xenograft tumors (Figure 9, A and B). The neovascular endothelial cells treated with anti-CD34 antibody were stained brown, and the microvessels in the tumor tissues appeared irregular in shape. Some vessels appeared as stripes with or without obvious lumens. Compared with the blank group, there was no evident difference in the MVD value belonging to the NC and shRNA-*LAMC2* + EGF groups (both $P > 0.05$). In contrast, the shRNA-*LAMC2* group exhibited a significantly lower MVD value, whereas the EGF group had a markedly higher MVD value, when compared with the blank group (both $P < 0.05$). In addition, RT-qPCR was conducted to detect vascular endothelial growth factor (VEGF) expression in CCA tissues (Figure 9C). The result showed that compared with the blank group, no differences were found in VEGF expression in the NC and siRNA-*LAMC2* + EGF groups, whereas VEGF expression was lower in the siRNA-*LAMC2* group and elevated in the EGF group ($P < 0.05$). These results demonstrate that angiogenesis and LNM of CCA tissues

could be inhibited by silencing *LAMC2* expression and inactivation of EGFR.

Discussion

Gene expression is implicated in cell proliferation and is a key factor in tumor development resulting from dysregulation in expression. Therefore, targeting specific genes that are associated with CCA may serve as an effective therapeutic approach in treatment for CCA.²⁵ A series of studies have provided evidence that the expression of *LAMC2* is related to a variety of cancers by promoting tumorigenesis.^{26–28} The present study investigated the regulatory function of *LAMC2* in the processes of EMT, cell invasion, and cell migration in CCA. Our findings demonstrated that silencing *LAMC2* expression can inactivate the EGFR pathway, thereby inhibiting the EMT of CCA cells and preventing CCA cell invasion and migration.

Initially, this study revealed that *LAMC2* was highly expressed in CCA, and promoted its EMT, angiogenesis, and metastasis. Consistent with our finding, several studies have documented that *LAMC2* was correlated with CCA development.^{19,20} For instance, high expression of *LAMC2* was found in alcoholic hepatitis, a kind of liver disease.²⁹ Govaere et al³⁰ have also suggested that *LAMC2* is implicated in the invasion and metastasis in hepatocellular carcinomas. Similar to our findings, Moon et al¹² have suggested that the up-regulation of *LAMC2* facilitates the EMT in lung adenocarcinoma cells, as well as enhances cell traction force, cell migration, and invasion.

Herein, we hypothesized that *LAMC2* may activate the EGFR signaling pathway in CCA. This hypothesis is consistent with the recent finding that high expression of *LAMC2* significantly accelerates gastric cancer cell invasion and stimulates the activation of the EGFR signaling pathway.²¹ Zanoaruddin et al³¹ report that *LAMC2* and EGFR are closely correlated to the extent of lymph node metastasis in oral squamous cell carcinoma. Collectively, this literature supports our results that establish a connection between *LAMC2* and the EGFR pathway in CCA, whereby *LAMC2* gene silencing inhibits the EGFR signaling pathway. To determine the regulatory effects and mechanism of *LAMC2* in CCA, loss-of-function and gain-of-function approaches were applied in the QBC939 cell line. Our results demonstrated that *LAMC2* gene silencing inhibited the activation of the EGFR signaling pathway, thus reversing EMT and metastasis in CCA. EMT markers, such as vimentin, E-cadherin, and ZEB1, were all monitored during our study. Vimentin is a chief component of the intermediate filament family of proteins, which is specifically expressed in normal mesenchymal cells, but highly expressed in various types of carcinomas.³² E-cadherin is a major constituent of the adherent junctions, essential for cell adhesion and maintenance of the epithelial phenotype.³³ Strikingly, ZEB1 is a critical transcription factor that drives EMT, whereby its

overexpression may induce EMT and lead to a metastatic phenotype in carcinoma cells.^{34,35} By silencing *LAMC2*, a decrease was found in vimentin and ZEB1 expression, whereas the E-cadherin level was elevated in CCA cells, suggesting that EMT was blocked. Before this study, established evidence supported the idea that EGFR, which functions as a receptor tyrosine kinase, contributes to the progression of CCA and EMT in CCA cells.²² Amonyingcharoen et al³⁶ also found that the cell proliferation in CCA was mediated by activation of the EGFR signaling pathway. Another study has also demonstrated that the stimulation of the EGFR/extracellular signal-regulated kinase signaling pathway could facilitate the proliferation, invasion, and metastasis in colorectal cancer.³⁷ A recent study indicated that the down-regulation of EGFR could lead to antiangiogenesis of glioma cells.³⁸ We, therefore, drew a conclusion that the EGFR signaling pathway plays a significant role in the progression of CCA. We propose that the suppression of CCA progression can be achieved through inactivation of the EGFR signaling pathway by silencing *LAMC2*.

Conclusion

In concordance with the earlier findings, the *LAMC2* gene was found to contribute to EMT and metastasis of CCA cells (Figure 10). Silencing *LAMC2* effectively inhibited the EGFR signaling pathway activation to inhibit EMT and LNM of CCA cells. Silencing of *LAMC2* may help manage CCA in the near future. However, its clinical prognostic value will be evaluated in the future.

Acknowledgments

Y.-F.P. designed the study; J.L. collated the data and designed and developed the database; J.C. analyzed data and wrote the manuscript; W.-D.W. and X.-Q.L. wrote the manuscript; all authors have read and approved the final submitted manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.03.012>.

References

- Walden D, Kunnimalaiyaan S, Sokolowski K, Clark TG, Kunnimalaiyaan M: Antiproliferative and apoptotic effects of xanthohumol in cholangiocarcinoma. *Oncotarget* 2017, 8:88069–88078
- Bergquist A, von Seth E: Epidemiology of cholangiocarcinoma. *Best Pract Res Clin Gastroenterol* 2015, 29:221–232
- Rizvi S, Gores GJ: Pathogenesis, diagnosis, and management of cholangiocarcinoma. *Gastroenterology* 2013, 145:1215–1229
- Shin HR, Oh JK, Masuyer E, Curado MP, Bouvard V, Fang YY, Wiangnon S, Sripa B, Hong ST: Epidemiology of cholangiocarcinoma: an update focusing on risk factors. *Cancer Sci* 2010, 101:579–585
- Ota Y, Takahashi K, Otake S, Tamaki Y, Okada M, Aso K, Makino Y, Fujii S, Ota T, Haneda M: Extracellular vesicle-encapsulated miR-30e suppresses cholangiocarcinoma cell invasion and migration via inhibiting epithelial-mesenchymal transition. *Oncotarget* 2018, 9:16400–16417
- Qiu YH, Wei YP, Shen NJ, Wang ZC, Kan T, Yu WL, Yi B, Zhang YJ: miR-204 inhibits epithelial to mesenchymal transition by targeting slug in intrahepatic cholangiocarcinoma cells. *Cell Physiol Biochem* 2013, 32:1331–1341
- Cheng W, Liu Y, Zuo Z, Yin X, Jiang B, Chen D, Peng C, Yang J: Biological effects of RNAi targeted inhibiting Tiam1 gene expression on cholangiocarcinoma cells. *Int J Clin Exp Pathol* 2015, 8:15511–15526
- Cheng W, Tian L, Wang B, Qi Y, Huang W, Li H, Chen YJ: Downregulation of HPIalpha suppresses proliferation of cholangiocarcinoma by restoring SFRP1 expression. *Oncotarget* 2016, 7:48107–48119
- Domogatskaya A, Rodin S, Tryggvason K: Functional diversity of laminins. *Annu Rev Cell Dev Biol* 2012, 28:523–553
- Garg M, Kanojia D, Okamoto R, Jain S, Madan V, Chien W, Sampath A, Ding LW, Xuan M, Said JW, Doan NB, Liu LZ, Yang H, Gery S, Braunstein GD, Koeffler HP: Laminin-5gamma-2 (*LAMC2*) is highly expressed in anaplastic thyroid carcinoma and is associated with tumor progression, migration, and invasion by modulating signaling of EGFR. *J Clin Endocrinol Metab* 2014, 99:E62–E72
- Garg M, Braunstein G, Koeffler HP: *LAMC2* as a therapeutic target for cancers. *Expert Opin Ther Targets* 2014, 18:979–982
- Moon YW, Rao G, Kim JJ, Shim HS, Park KS, An SS, Kim B, Steeg PS, Sarfaraz S, Changwoo Lee L, Voeller D, Choi EY, Luo J, Palmieri D, Chung HC, Kim JH, Wang Y, Giaccone G: *LAMC2* enhances the metastatic potential of lung adenocarcinoma. *Cell Death Differ* 2015, 22:1341–1352
- Li Y, Li DJ, Chen J, Liu W, Li JW, Jiang P, Zhao X, Guo F, Li XW, Wang SG: Expression of Laminin gamma2 in extrahepatic cholangiocarcinoma tissues and its influence on tumor invasion and metastasis. *Asian Pac J Cancer Prev* 2015, 16:2099–2102
- Liu W, Tian F, Jiang P, Zhao X, Guo F, Li X, Wang S: Aberrant expression of laminin gamma2 correlates with poor prognosis and promotes invasion in extrahepatic cholangiocarcinoma. *J Surg Res* 2014, 186:150–156
- Carceney E, Moran T, Capdevila L, Cros S, Vila L, de Los Llanos Gil M, Remon J, Rosell R: The epidermal growth factor receptor (EGFR) in lung cancer. *Transl Respir Med* 2015, 3:1
- Vaquero J, Lobe C, Tahraoui S, Claperon A, Mergey M, Merabtene F, Wendum D, Coulouarn C, Housset C, Desbois-Mouthon C, Praz F, Fouassier L: IGF2/IR/IGF1R pathway in tumor cells and myofibroblasts mediates resistance to EGFR inhibition in cholangiocarcinoma. *Clin Cancer Res* 2018, 24:4282–4296
- Committee for the Update of the Guide for the Care and Use of Laboratory Animals National Research Council: Guide for the Care and Use of Laboratory Animals: Eighth Edition. Washington, DC, National Academies Press, 2011
- Pollaers K, Hinton-Bayre A, Friedland PL, Farah CS: AJCC 8th edition oral cavity squamous cell carcinoma staging: is it an improvement on the AJCC 7th edition? *Oral Oncol* 2018, 82:23–28
- Govaere O, Wouters J, Petz M, Vandewynckel YP, Van den Eynde K, Van den Broeck A, Verhulst S, Dolle L, Gremaux L, Ceulemans A, Nevens F, van Grunsven LA, Topal B, Vankelecom H, Giannelli G, Van Vlierberghe H, Mikulits W, Komuta M, Roskams T: Laminin-332 sustains chemoresistance and quiescence as part of the human hepatic cancer stem cell niche. *J Hepatol* 2016, 64:609–617
- Sulpice L, Rayar M, Desille M, Turlin B, Fautrel A, Boucher E, Llamas-Gutierrez F, Meunier B, Boudjema K, Clement B, Coulouarn C: Molecular profiling of stroma identifies osteopontin as

- an independent predictor of poor prognosis in intrahepatic cholangiocarcinoma. *Hepatology* 2013, 58:1992–2000
21. Xu L, Hou Y, Tu G, Chen Y, Du YE, Zhang H, Wen S, Tang X, Yin J, Lang L, Sun K, Yang G, Tang X, Liu M: Nuclear Drosha enhances cell invasion via an EGFR-ERK1/2-MMP7 signaling pathway induced by dysregulated miRNA-622/197 and their targets LAMC2 and CD82 in gastric cancer. *Cell Death Dis* 2017, 8:e2642
 22. Claperon A, Mergey M, Nguyen Ho-Bouloires TH, Vignjevic D, Wendum D, Chretien Y, Merabtene F, Frazao A, Paradis V, Housset C, Guedj N, Fouassier L: EGF/EGFR axis contributes to the progression of cholangiocarcinoma through the induction of an epithelial-mesenchymal transition. *J Hepatol* 2014, 61:325–332
 23. Hoffmann AC, Goekkurt E, Danenberg PV, Lehmann S, Ehninger G, Aust DE, Stoecklacher-Williams J: EGFR, FLT1 and heparanase as markers identifying patients at risk of short survival in cholangiocarcinoma. *PLoS One* 2013, 8:e64186
 24. Yang X, Wang W, Wang C, Wang L, Yang M, Qi M, Su H, Sun X, Liu Z, Zhang J, Qin X, Han B: Characterization of EGFR family gene aberrations in cholangiocarcinoma. *Oncol Rep* 2014, 32:700–708
 25. Li YY, Hanna GJ, Laga AC, Haddad RI, Lorch JH, Hammerman PS: Genomic analysis of metastatic cutaneous squamous cell carcinoma. *Clin Cancer Res* 2015, 21:1447–1456
 26. Kosanam H, Prassas I, Chrystoja CC, Soleas I, Chan A, Dimitromanolakis A, Blasutig IM, Ruckert F, Gruetzmanner R, Pilarsky C, Maekawa M, Brand R, Diamandis EP: Laminin, gamma 2 (LAMC2): a promising new putative pancreatic cancer biomarker identified by proteomic analysis of pancreatic adenocarcinoma tissues. *Mol Cell Proteomics* 2013, 12:2820–2832
 27. Kwon OH, Park JL, Kim M, Kim JH, Lee HC, Kim HJ, Noh SM, Song KS, Yoo HS, Paik SG, Kim SY, Kim YS: Aberrant up-regulation of LAMB3 and LAMC2 by promoter demethylation in gastric cancer. *Biochem Biophys Res Commun* 2011, 406:539–545
 28. Nguyen CT, Okamura T, Morita KI, Yamaguchi S, Harada H, Miki Y, Izumo T, Kayamori K, Yamaguchi A, Sakamoto K: LAMC2 is a predictive marker for the malignant progression of leukoplakia. *J Oral Pathol Med* 2017, 46:223–231
 29. Liu M, Dou Y, Sun R, Zhang Y, Liu Y: Molecular mechanisms for alcoholic hepatitis based on analysis of gene expression profile. *Hepat Mon* 2015, 15:e27336
 30. Govaere O, Komuta M, Berkens J, Spee B, Janssen C, de Luca F, Katoonizadeh A, Wouters J, van Kempen LC, Durnez A, Verslype C, De Kock J, Rogiers V, van Grunsven LA, Topal B, Pirenne J, Vankelecom H, Nevens F, van den Oord J, Pinzani M, Roskams T: Keratin 19: a key role player in the invasion of human hepatocellular carcinomas. *Gut* 2014, 63:674–685
 31. Zannaruddin SN, Saleh A, Yang YH, Hamid S, Mustafa WM, Khairul Bariah AA, Zain RB, Lau SH, Cheong SC: Four-protein signature accurately predicts lymph node metastasis and survival in oral squamous cell carcinoma. *Hum Pathol* 2013, 44:417–426
 32. Satelli A, Li S: Vimentin in cancer and its potential as a molecular target for cancer therapy. *Cell Mol Life Sci* 2011, 68:3033–3046
 33. Mendonsa AM, Na TY, Gumbiner BM: E-cadherin in contact inhibition and cancer. *Oncogene* 2018, 37:4769–4780
 34. Imani S, Hosseini Fard H, Cheng J, Wei C, Fu J: Prognostic value of EMT-inducing transcription factors (EMT-TFs) in metastatic breast cancer: a systematic review and meta-analysis. *Sci Rep* 2016, 6:28587
 35. Imani S, Wei C, Cheng J, Khan MA, Fu S, Yang L, Tania M, Zhang X, Xiao X, Zhang X, Fu J: MicroRNA-34a targets epithelial to mesenchymal transition-inducing transcription factors (EMT-TFs) and inhibits breast cancer cell migration and invasion. *Oncotarget* 2017, 8:21362–21379
 36. Amonyingcharoen S, Suriyo T, Thiantanawat A, Watcharasit P, Satayavivad J: Taurolithocholic acid promotes intrahepatic cholangiocarcinoma cell growth via muscarinic acetylcholine receptor and EGFR/ERK1/2 signaling pathway. *Int J Oncol* 2015, 46:2317–2326
 37. Wei Z, Ma W, Qi X, Zhu X, Wang Y, Xu Z, Luo J, Wang D, Guo W, Li X, Xin S, Yu J, Li G: Pinin facilitated proliferation and metastasis of colorectal cancer through activating EGFR/ERK signaling pathway. *Oncotarget* 2016, 7:29429–29439
 38. Yang HK, Chen H, Mao F, Xiao QG, Xie RF, Lei T: Downregulation of LRIG2 expression inhibits angiogenesis of glioma via EGFR/VEGF-A pathway. *Oncol Lett* 2017, 14:4021–4028