LAMC2 promotes cancer progression and gemcitabine resistance through modulation of EMT and ATP-binding cassette transporters in pancreatic ductal adenocarcinoma

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Running title: LAMC2 promotes gemcitabine resistance in PDAC

Funding: The present work was supported by the CA72851, CA187956, CA202797 and CA214254 grants from the National Cancer Institute, National Institute of Health; In addition, this work was also supported by a pilot research award from the City of Hope Ludwig Cancer Research-Hilton Foundation Partnership award.

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Conflict of Interest: None of the authors has any potential conflicts to disclose.

Author contribution: Study concept and design: YO, AG; Specimen providers: NT; Acquisition of clinical data: NT; Analysis and interpretation of data and statistical analysis: YO, NS, AG; Drafting of the manuscript: YO, SN, and AG.

Summary (40 words)

LAMC2 plays a significant role in pancreatic cancer cells through regulation of EMT and ABC transporters associated with cellular migration and invasion, resulting in poor prognosis and gemcitabine sensitivity in patients with pancreatic cancer.

ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease with poor prognosis. Gemcitabine remains an effective option for the majority of PDAC patients. Unfortunately, currently no reliable prognostic and predictive biomarkers of therapeutic response are available for the patients with PDAC. Laminin γ2 (LAMC2) is overexpressed in several cancers, and its high expression facilitates cancer development and chemoresistance. However, its functional role in PDAC remains unclear, and a better understanding of this will likely help improve the prognosis of PDAC patients. This study aimed to elucidate the clinical and biological role of LAMC2 in PDAC. We first analyzed the expression levels of LAMC2 by real-time reverse transcription PCR in a cohort of 114 PDAC patients. Interestingly, higher expression of LAMC2 significantly correlated with poor survival in PDAC cohort. In addition, elevated LAMC2 expression served as a potential prognostic marker for survival. Subsequently, functional characterization for the role of LAMC2 in PDAC was performed by small interfering RNA (siRNA) knockdown in pancreatic cancer (PC) cell lines. Interestingly, inhibition of LAMC2 in PC cells enhanced the gemcitabine sensitivity and induction of apoptosis. Moreover, it inhibited colony formation ability, migration, and invasion potential. Furthermore, LAMC2 regulated the expression of epithelial-mesenchymal transition (EMT) phenotype. In addition, LAMC2 significantly correlated with genes associated with the expression of ATP-binding cassette (ABC) transporters in PC cells and PDAC patients. In conclusion, these results suggest that LAMC2 regulates gemcitabine sensitivity through EMT and ABC transporters in PDAC and may be a novel therapeutic target in PDAC patients.

Keywords: LAMC2, pancreatic ductal adenocarcinoma, gemcitabine, EMT, ABC transporter

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and lethal cancers, which is projected to become the second most common cause of cancer-related deaths in the United States by 2030 (1-3). Although the clinical outcomes from several other cancers have improved considerably in last decades, the 5-year survival rates for PDAC still remain less than 10%. In spite of the recent improvements in chemotherapeutic regimens, most patients experience relapse following surgery, which is one of the reasons for this increased morbidity and mortality (4-7). The median overall survival (OS) of patients with metastatic PDAC does not extend beyond one year (4,6).

Gemcitabine is a pyrimidine nucleoside analog that is commonly used in the treatment of solid cancers such as the breast, lung, and pancreatic cancers. Systemic chemotherapy with gemcitabine has been the standard treatment for advanced PDAC (8). However, median survival in gemcitabine-treated PDAC patients is ~6 months (8). Consequently, other combination regimens that are gemcitabine-based have been developed for improving survival; however, none of these strategies has resulted in a significant improvements in the overall clinical outcomes because of the underlying intrinsic or acquired therapeutic resistance (4,9-11). The molecular mechanisms of gemcitabine resistance can be attributed to cell plasticity, tumor heterogeneity, altered metabolism, epithelial–mesenchymal transition (EMT) and the regulation of drug influx & efflux (12-15).

The EMT is an important biological process that plays a seminal role in the cancer progression of metastasis. During this process, cancer cells downregulate the expression of cellular adhesion molecules and gain mesenchymal properties through acquisition of mesenchymal features (16). Several studies have now reported that EMT not only enhances the ability of tumor cells to metastasize, but also orchestrates chemoresistance (17-19). However, the precise mechanisms that govern EMT-mediated chemoresistance to gemcitabine-based treatment in PDAC remain poorly understood.

Accumulating evidence in recent years have revealed that ATP-binding cassette (ABC) transporters are major players that contribute to chemoresistance in cancers, by virtue of their ability to increase the intracellular drug efflux and reduce the drug accumulation in cancer cells

(12). However, their role in gemcitabine-based resistance in pancreatic cancer (PC) remains unclear. Any insights into this important, clinically-relevant issue, would have a significant impact in improving therapeutic responses to gemcitabine-based therapies in cancer, including PDAC.

In the present study, we have identified a novel function for Laminin γ2 (LAMC2) in mediating PC prognosis and gemcitabine resistance via EMT, along with the upregulation of a family of ABC transporters. LAMC2 is a subunit of the heterotrimeric glycoprotein laminin-332 (LAM-332, formerly laminin-5), which consists of the α3, β3, and γ2 chains. LAM-332 is an essential component of this multimeric functional complex that regulates cell adhesion, differentiation and migration, as well as the invasion of epithelial cells in normal tissues (20,21). In addition, LAMC2 is overexpressed in various cancers (22-26). Moreover, recent studies have reported that LAMC2 is frequently over-expressed in cancer cells, particularly that have undergone EMT in different cancer types (25,27,28). In addition, the expression levels of LAMC2 are directly regulated by the EMT master regulator ZEB1 and activated β-catenin in invasive colorectal carcinoma cells (29,30). However, the role of LAMC2 in gemcitabine resistance in PDAC has not been systematically characterized. Herein, we for the first-time provide evidence that high expression of LAMC2 is associated with not only poor survival but also gemcitabine resistance in PDAC patients, and that inhibition of LAMC2 in PC cells increased their sensitivity to gemcitabine, preferentially through suppression of EMT and ABC transporter signaling. We further demonstrate that LAMC2 promotes migration, invasion and inhibit apoptosis in human PC cells.

MATERIALS AND METHODS

Patient cohorts

In this study, we enrolled a total of 413 PDAC patients from multiple cohorts, including two public datasets (GSE71729 [n=123] and The Cancer Genome Atlas [TCGA] [n=176]) and an in-house clinical cohort (n=114). The GSE71729 dataset was downloaded from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). Likewise, TCGA dataset was downloaded from the UCSC Xena Browser (https://xenabrowser.net).

In the clinical cohort, all patients underwent surgery for PC at the Saitama Cancer Center, Japan, between April 1997 and May 2013. None of the patients received preoperative cancer treatment, and all tumors were diagnosed as PDAC. 92 patients were treated with the gemcitabine-based adjuvant chemotherapy after surgery. The PDAC tissues were obtained from resected specimens and were immediately frozen and stored at -80°C until use. All patients were followed until death or January 2019. The study was conducted in accordance with the Declaration of Helsinki. A written informed consent was obtained from all patients, and the study was approved by the institutional review boards of City of Hope Comprehensive Cancer Center and Saitama Cancer Center.

Total RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from fresh-frozen tissue specimens using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany). The synthesis of complementary DNA (cDNA) was performed from 500 ng of total RNA using the High Capacity cDNA Reverse-Transcription Kit (Invitrogen, Carlsbad, CA, USA). We performed quantitative real-time reverse transcription PCR analysis (qRT-PCR) using the SensiFAST™ SYBR® Lo-ROX Kit (Bioline, London, UK) and the QuantStudio 6 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) and expression levels were evaluated using the Applied Biosystems QuantStudio 6 Flex Real Time PCR System Software. The relative abundance of target transcripts was evaluated and normalized to the expression levels of β-actin as an internal control by employing the 2-ΔCt method; ΔCt means the difference of Ct values between the mRNAs of interest and the normalizer. Normalized values were further

log2 transformed (31-33). The PCR primers used in the current study were described in **Supplementary Table S1**.

Cell lines

The human PC cell lines, PANC-1, MIAPaCa-2, BXPC-3, and CAPAN-2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM) or Dulbecco's Modified Eagle Medium (DMEM) from the Thermo Fisher Scientific, Waltham, MA, USA, and were supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin-streptomycin (Thermo Fisher Scientific). The cells were maintained at 37℃ in a humid atmosphere containing 5% CO2. All cell lines were tested and authenticated using a panel of genetic and epigenetic markers every 4-6 months.

Small interfering RNA (siRNA) induced inhibition of LAMC2

Specific double-stranded LAMC2 siRNAs (Silencer Select ® s534191; Ambion, Austin, TX) were transfected into PANC-1 and BxPC-3 cells (1.0×10^5) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Silencer Negative Control #1 siRNA (Ambion) was used as a negative control. Cells were incubated in culture media for 48 h after transfection prior to harvesting for analyses. All experiments were conducted in triplicates, and at least three independent experiments were performed.

Cell viability assay

Cell viability was assessed by WST-8 assay using Cell Counting Kit-8 (Dojindo, Japan) as described previously (34). After the incubation of siRNA or negative control siRNA for 24 h, cell growth was assayed in 96-well plates after 72 h with the treatment of gemcitabine (Sigma-Aldrich, St. louis, MO). The absorbance at 450 nm was recorded with a 96-well plate reader (TECAN, infinite 200Pro, i-control software).

Colony formation assay

A total of 1000 cells transfected with siLAMC2 or negative control were seeded into 6-well plates

and cultured for 10 days in a humidified CO_2 incubator at 37°C. Cells were incubated for a total of 10 days and thereafter stained with crystal violet as described previously (35). The number of colonies with more than 50 cells were counted using the Image J program, ver. 1.51 (National Institutes of Health, Bethesda, MD).

Cell invasion, migration, and wound healing assays

Two days following transfection with LAMC2 or negative control siRNA, invasion and migration assays were performed with a BD BioCoat Matrigel Invasion Chamber (BD Biosciences, San Jose, CA, USA) that had 8 μm pore size membranes with Matrigel (for the invasion assay) or without Matrigel (for the migration assay) as described previously (36). For wound healing assays, cell monolayers transfected with LAMC2 or control siRNA were scratched with a sterile 200 μl pipette tip, and cell migration was observed for up to 24 h. All experiments were performed in triplicate.

Western blot analysis

Total cellular protein was extracted and western immunoblotting was performed as described previously (35,37). Briefly, proteins were extracted from cells using a RIPA lysis and extraction buffer (Thermo Fisher Scientific) containing Protease Inhibitor Cocktail (Thermo Fisher Scientific). Total protein concentration in the lysates was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc.). Protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking using 5% low-fat dry milk in Tris-buffered saline with 0.1% Tween (TBS-T) for 1 hour at room temperature, the membranes were probed with a mouse anti-LAMC2 (E-6, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-poly ADP-ribose polymerase-1 (PARP-1) (sc-7150, 1:300, Santa Cruz Biotechnology), rabbit anti-caspase 3 (sc-7148, 1:300, Santa Cruz Biotechnology), rabbit anti-E-cadherin (H-108, 1:500, Santa Cruz Biotechnology), rabbit anti-vimentin (H-84, 1:500, Santa Cruz Biotechnology), goat anti-ZEB1 (C-20, 1:1000, Santa Cruz Biotechnology), mouse anti-ZEB2 (E-11, 1:1000, Santa Cruz Biotechnology), and mouse anti-β-actin (A5441, 1:5000, Sigma-Aldrich). Membranes were thereafter incubated with horseradish peroxidase-conjugated IgG (Sigma-Aldrich). All protein bands on the membranes were visualized

using the ChemiDoc™ MP Imaging System and Image Lab™ Software version 5.2.1 (Bio-Rad Laboratories, Inc., California, USA). Band intensity was quantified using the Image J program and expressed as a ratio to β-actin band intensity.

Statistical analysis

All statistical analyses were performed using Medcalc statistical software V.19.1.0 (Medcalc Software bvba, Ostend, Belgium). Differences between groups were estimated by the Student's *t* test, the Chi square test, Mann-Whitney U test, as appropriate. Spearman's correlation was used to evaluate the linear relationship between two variables. Patients divided into the high or low expression group of LAMC2 were classified by Youden's index derived cutoff thresholds. Categorical variables were compared using $\chi 2$ or Fisher exact test. All P values were calculated using a two-sided test. For time-to-event analyses, survival estimates were calculated using the Kaplan–Meier analysis, and the survival differences between groups were compared using the log-rank test. Associations between OS and clinicopathologic features were evaluated by univariate Cox proportional hazards regression analysis. Parameters determined to be significant by univariate analysis were included in multivariate Cox proportional hazards regression analysis. Error bars denote standard deviation for the columns in the figures. All p-values less than 0.05 were considered statistically significant.

RESULTS

High LAMC2 expression is associated with poor survival outcomes in PDAC patients

To assess the impact of LAMC2 on prognosis and survival, we initially performed the survival analysis in two public datasets (GSE71729 and TCGA). Interestingly, we observed a significant association of high-LAMC2 levels with poor OS (**Supplementary Fig.S1A and B**). To determine the cut-off thresholds of LAMC2 expression for dichotomizing the high and low-risk groups, we evaluated the LAMC2 expression by qRT-PCR in the in-house clinical cohort (**Table 1**). In this cohort of 114 patients, 81 patients exhibited low LAMC2 expression, and 33 had high LAMC2 expression, as determined by qRT-PCR. Next, Kaplan–Meier analysis for OS and relapse-free survival (RFS) was performed in order to evaluate the prognostic potential of LAMC2 expression. Interestingly, OS and RFS was significantly reduced in the group with high LAMC2 expression (OS: 23.0 vs 14.2 months, Hazard ratio [HR]=1.61; 95% confidence interval [CI], 1.09-2.79, $P < 0.01$, RFS: 9.0 vs 6.2 months, HR=1.61, 95%CI, 0.96-2.71, $P < 0.05$, **Fig. 1A and B**). The CA19-9 levels are the most commonly used biomarker for the management of patients with PDAC (38). Next, we performed the Kaplan-Meier analysis based on CA19-9 values in this cohort. As expected, high CA19-9 group was associated with poor OS (HR=1.65; 95% CI, 1.10-2.48, $P < 0.05$, **Supplementary Fig.S2A**), while no significant differences were observed for RFS in this group (HR=1.45; 95% CI, 0.90-2.35, $P = 0.17$, **Supplementary Fig.S2B**).

To evaluate the clinical significance of LAMC2 levels, we performed univariate and multivariate analysis using the Cox's proportional hazard model by considering other clinicopathological factors into the equation. Of interest, the multivariate analysis revealed that the patients with high LAMC2 expression were associated with poor OS (HR=1.71; 95% CI, 1.09-2.68; $P = 0.02$, **Fig. 1C**). These data suggest that LAMC2 expression was a potential prognostic marker in our in-house clinical cohort. To examine whether the expression of LAMC2 correlates with gemcitabine response, we next investigated LAMC2 mRNA expression of patients who were treated with gemcitabine therapy in adjuvant settings. Intriguingly, the patients with high LAMC2 demonstrated a significantly worse prognosis (OS: $P < 0.05$; RFS: $P < 0.05$, **Supplementary Fig. S2C and D**), highlighting that LAMC2 is not only a potential prognostic but also a potential predictive biomarker of therapeutic response to gemcitabine in patients with PDAC.

LAMC2 promotes gemcitabine resistance in pancreatic cancer cells

To determine the biological impact of LAMC2 in PDAC, we first examined the expression of LAMC2 in several PC cell lines to identify those with high endogenous LAMC2 expression (**Supplementary Fig. S3**). PANC-1 and BxPC-3 cell lines were subsequently chosen for siRNA transfection experiments since these cells demonstrated the highest levels of LAMC2 expression. Following siRNA based experiments, suppression of LAMC2 was confirmed at both mRNA and protein expression levels (**Supplementary Fig. S4A and B**). Considering that overexpression of LAMC2 is associated with gemcitabine resistance in our clinical cohort, we were curious to investigate the role of LAMC2 on the regulation of gemcitabine resistance in PC cells. Therefore, we examined the gemcitabine sensitivity of PC cells with or without LAMC2 siRNA. The IC50 values for gemcitabine in PANC-1 with or without LAMC2 siRNA were 105.42 ± 15.31 and 50.63 ± 8.85 μM, respectively; i.e., the IC50 of PANC-1 with LAMC2 siRNA was significantly lower compared to that of PANC-1 with negative control ($p < 0.05$; **Fig. 2A**). Similarly, the IC50 value of gemcitabine in BxPC-3 cells with LAMC2 siRNA (16.78 ± 1.82 nM) was significantly lower than that of the negative control (35.29 ± 4.54 nM, $p < 0.05$). Furthermore, to investigate the underlying mechanism of the LAMC2 effect on PC cells, the degree of apoptosis was examined by performing western blotting assays for PARP and procaspase-3 in PC cells treated with gemcitabine. Interestingly, these assays revealed that the expression of PARP-1 and procaspase-3 was significantly decreased in both cell lines transfected with LAMC2 siRNA at 72 h after treatment with gemcitabine in comparison with the control cells (**Fig. 2B**). Taken together, these results suggest that the downregulation of LAMC2 may enhance the gemcitabine sensitivity in addition to apoptosis.

LAMC2 downregulation inhibits colony formation, as well as invasion and migration potential in pancreatic cancer cells

To further evaluate the functional significance of LAMC2 in PDAC, we next examined colony formation assays. Both cells treated with LAMC2 siRNA resulted in significantly reduced number of colonies compared to the negative control siRNA cells (**Fig. 3A**). In view of the previous evidence that LAMC2 might influence the in invasive and migratory potential (25,39), we next

performed migration and invasion assays. As expected, a wound-healing assay confirmed that LAMC2 depletion significantly inhibited cellular motility (**Fig. 3B**). Moreover, transwell chamber assays demonstrated that LAMC2 inhibited cell invasion and migration ability of both PC cells (**Fig. 3C and D**); indicating that LAMC2 might be intimately involved in the malignant potential of PC by enhancing colonogenic survival as well as the invasive and migratory potential of PC cells.

LAMC2 regulates chemoresistance through EMT in pancreatic cancer cells

Previous studies have shown that EMT plays an important role in mediating chemoresistance in various cancers (18,19). Moreover, EMT is known to be closely related to cancer invasion and migration (40). Therefore, we hypothesized that LAMC2 may mediate chemoresistance through regulation of EMT. To investigate this hypothesis further, we next examined the expression of EMT related genes, E-cadherin, vimentin, ZEB1, and ZEB2 in PC cell lines treated with LAMC2 siRNA or negative controls. As expected, knockdown of LAMC2 in PC cell lines resulted in reduced vimentin, ZEB1, and ZEB2 expression, while E-cadherin expression was significantly upregulated compared to negative control transfected cells, at both mRNA (**Fig. 4A**) and protein expression levels (**Fig. 4B**). Thus, these results strongly suggest that LAMC2 may enhance the gemcitabine resistance through the induction of EMT.

LAMC2 enhances gemcitabine sensitivity by regulating ABC transporters in pancreatic cancer cells

Emerging evidence indicates that ABC transporters are implicated in inducing chemoresistance in tumor cells (12,41). Therefore, we next evaluated whether the expression of ABC transporters mediate chemoresistance through the suppression of LAMC2. Interestingly, mRNA levels of ABCA1, ABCD3, ABCB4, and CFTR were significantly decreased in PANC-1 and BxPC-3 cell lines transfected with LAMC2 siRNA compared to negative control (**Fig. 5A and Supplementary Fig. S5**). To assess the clinical relevance of this finding, we investigated the association between LAMC2 and these four ABC transporter genes in cancer tissues from PDAC patients who received the gemcitabine therapy. While the expression of ABCA1 and CFTR was not correlated with LAMC expression, the expression of ABCD3 and ABCB4 positively correlated with LAMC2 expression ($r = 0.344$, $P < 0.001$ and $r = 0.472$, $P < 0.0001$ respectively; **Fig. 5B**). Collectively, these data

indicate that LAMC2 promote gemcitabine resistance through enhancement of ABCD3 and ABCB4 as well as EMT activation.

DISCUSSION

The extracellular matrix (ECM) is an essential component of tissues that constitute multicellular organisms (42). Accumulating evidence indicates that the ECM not only triggers cancer progression, but also plays a central role in mediating drug resistance in PDAC (43,44). Laminins are a family of large molecular weight glycoproteins that accumulate mainly in the ECM (20). In the present study, we have shown that high expression of LAMC2, which is one of the subunits of the protein laminin-332 (laminin-5), was associated with poor OS and RFS in PDAC patients in our clinical cohort. Moreover, we successfully identified that high expression of LAMC2 was predictive of therapeutic response to gemcitabine-based therapy. Furthermore, we demonstrated that down-regulation of LAMC2 enhanced the sensitivity of gemcitabine in PC cells. We also showed that the inhibition of LAMC2 significantly induced apoptosis, as well as impaired invasion and migration capability. In addition, we identified that LAMC2 regulates EMT activation, and the expression of several ABC transporters, resulting in gemcitabine resistance in PC cells.

We first identified that PDAC patients with high expression of LAMC2 had significantly poorer prognosis. Moreover, the multivariate analysis for OS in our cohort revealed that LAMC2 expression is a prognostic biomarker for OS. Additionally, our data revealed that LAMC2 is an excellent marker for gemcitabine therapy in patients with PDAC. In this study, we performed the LAMC2 expression analysis by analyzing only one patient cohort. To overcome this limitation, future studies are required to confirm and support the validity of our findings.

To further understand the biological function of LAMC2 in PDAC prognosis, we investigated the ability of LAMC2 in PC cells through siRNA knockdown experiments. This result showed that silencing LAMC2 not only inhibited gemcitabine resistance through the induction of apoptosis but also the ability for colony formation, cell invasion, and migration in PC cells. Consistent with our findings, other studies have suggested that LAMC2 might play a role in PDAC development (45,46). Although further investigations are required to fully understand the effects of LAMC2 inhibition, our results suggest that LAMC2 may serve as a promising prognostic biomarker and could play a pivotal role in malignant potential and gemcitabine resistance in PDAC.

Several previous studies have indicated that EMT is associated with gemcitabine drug resistance in PDAC. Moreover, the up-regulation of LAMC2 facilitates EMT in several cancers

(25,47). Herein, we hypothesized that LAMC2 may induce gemcitabine resistance through the induction of EMT in PDAC. As expected, our hypothesis was consistent with our results that silencing of LAMC2 significantly inhibited the EMT phenotype of PC cells, thereby resulting in an increased sensitivity to gemcitabine.

Furthermore, we showed that overexpression of LAMC2 significantly increased the mRNA levels of ABCA1, ABCD3, ABCB4, and CFTR in PC cell lines. Interestingly, ABCD3 and ABCB4 are significantly correlated with LAMC2 expression in PDAC patients. ABCD3 and ABCB4 function as an efflux pumps to limit the intracellular accumulation of cytotoxic drugs, including taxanes, anthracyclines and vinca alkaloids (48). However, to the best of our knowledge, no studies have interrogated whether LAMC2 regulates ABC transporter expression in PC cells. We first carried out the downstream regulation of ABC transporters through knockdown of LAMC2 in PC.

Considering our findings, LAMC2 may be a potential therapeutic target in PDAC. A characteristic feature of PDAC is the presence of an abundant desmoplastic stroma (49). Moreover, PC cells induce a desmoplastic response within the tumor stroma including ECM (50). One limitation of our study is that we assessed LAMC2 expression only in PC cells. Therefore, co-culture with cancer associated with fibroblast (CAF) or in vivo experiments will be needed for a more detailed analysis.

In conclusion, we have shown that LAMC2 promotes PC development and resistance to gemcitabine therapy by up-regulating EMT related markers and down-regulating ABC transporters, ABCD3 and ABCB4. In addition, our clinical data supports that high expression levels of LAMC2 correlated with poor prognosis and gemcitabine resistance in PDAC patients. Collectively, this is the first demonstration for the biological and clinical significance of LAMC2, as a result, it may be an effective therapeutic strategy for PC.

ACKNOWLEDGEMENTS

We thank Satoshi Nishiwada, Geeta Sharma, Tatsuhiko Kakisaka, Yuma Wada, In Seob Lee, Divya Sahu, Xiaoe Zhang, Yinghui Zhao, and Yuetong Chen for helping with performing the experiments and analysis.

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FIGURE LEGENDS

Figure 1. Prognostic potential of LAMC2 for PDAC patients in the clinical cohort. (A, B) Kaplan-Meier curves for (A) OS and (B) RFS in PDAC patients with high (blue) or low (red) LAMC2 expression in the clinical cohort. (C) Forest plot with hazard ratio of clinicopathological variables and LAMC2 expression in univariate and multivariate analysis by Cox regression model. The P-values were obtained by the log-rank test for the Kaplan-Meier survival analysis.

Figure 2. LAMC2 facilitates gemcitabine resistance in pancreatic cancer cells. (A) The sensitivities of PANC-1 and BxPC-3 cells transfected with LAMC2 siRNA or negative control to gemcitabine were assessed by the WST-8 assay. The line graphs (left) are presented as the treated to control cell ratios, and the bar graphs (right) illustrate IC50 values. (B) Expression of PARP and procaspase-3 in pancreatic cancer cells transfected with LAMC2 siRNA or negative control. The siRNA transfected cells were treated with gemcitabine (PANC-1: 100μM, BxPC-3: 30nM) for 3 days, and Western blotting for PARP and procaspase-3 were performed. β-Actin was used as a loading control. Error bars mean ± SD. GEM, gemcitabine. NC, negative control. *P < 0.05, **P < 0.01.

Figure 3. LAMC2 downregulation inhibit the colony forming ability, migration, and invasion in vitro. (A) Colony forming ability of pancreatic cancer (PC) cell lines transfected with LAMC2 siRNA or negative control. Colony formation assay was performed after 10 days incubation. (B) Wound healing assay in PC cell lines transfected with LAMC2 siRNA or negative control. The distance of wound healing was measured and calculated as a percentage of the distance at 0 h. Original magnification, ×100. (C, D) Invasion and migration assays for PC cell lines transfected with LAMC2 siRNA or negative control. All experiments were carried out three times. Error bars mean ± SD. NC, negative control. *P < 0.05, **P < 0.01.

Figure 4. LAMC2 knockdown inhibits the EMT process in pancreatic cancer cells. (A, B) EMT marker (E-cadherin, vimentin, ZEB1, and ZEB2) expression of PC cell lines transfected with LAMC2 siRNA or negative control by (A) quantitative RT-PCR and (B) Western blotting. Western blot data were scanned by densitometry and analyzed using ImageJ software. Error bars mean ± SD. NC, negative control. *P < 0.05, **P < 0.01.

Figure 5. LAMC2 regulates the expression of ABC transporter genes in pancreatic cancer cells. (A) Expression of ABC transporters (ABCA1, ABCD3, ABCB4, and CFTR) in pancreatic cancer cells transfected with LAMC2 siRNA or negative control. (B) Correlation between LAMC2 and ABC transporter mRNA expression in PDAC cancer specimens. Error bars mean ± SD. NC, negative control. *P < 0.05, **P < 0.01.

Supplementary Figure S1. LAMC2 expression is associated with prognosis in PDAC patients in the publicly available datasets. (A, B) Kaplan-Meier curves for OS in PDAC patients with high (blue) or low (red) LAMC2 expression in (A) GSE71729 and (B) TCGA datasets. The P-values were obtained by the log-rank test for the Kaplan-Meier survival analysis.

Supplementary Figure S2. Survival analysis in the in-house clinical cohort. (A, B) Kaplan-Meier curves for (A) OS and (B) RFS in PDAC patients with high (blue) or low (red) CA19-9 values in the clinical cohort. (C, D) Kaplan-Meier curves for (C) OS and (D) RFS in PDAC patients who received gemcitabine-based adjuvant treatment with high (blue) or low (red) LAMC2 expression in the clinical cohort. The P-values were obtained by the log-rank test for the Kaplan-Meier survival analysis.

Supplementary Figure S3. Expression status of LAMC2 in PC cell lines. mRNA levels of LAMC2 were measured by quantitative RT-PCR. Error bars mean ± SD.

Supplementary Figure S4. Knockdown of LAMC2 in PC cell lines. (A, B) LAMC2 expression of PC cell lines transfected with LAMC2 siRNA or negative control by (A) quantitative RT-PCR and (B) Western blotting. Error bars mean ± SD. NC, negative control. *P < 0.05, ***P < 0.001.

Supplementary Figure S5. The expression of ABC transporter genes which are not significant in pancreatic cancer cells lines transfected with LAMC2 siRNA or negative control. (A, B) Expression of ABC transporters in pancreatic cancer cells transfected with LAMC2 siRNA or negative control (A: PANC-1, B: BxPC-3). Error bars mean ± SD.

Table 1: Clinicopathological characteristics in pancreatic cancer patients

Characteristics	Total	LAMC2 expression		*P*-value [a]
	n = 114	Low (n = 81)	High (n = 33)	
Age, years				0.745
< 65, n (%)	56	39	17	
≥ 65, n (%)	58	42	16	
Gender				0.576
Male, n (%)	75	52	23	
Female, n (%)	39	29	10	
Tumor status				0.277[b]
T1-2	10	9	1	
T3-4	104	72	32	
Nodal status				0.349
N0	31	20	11	
N1	83	61	22	
UICC stage (ver. 7)				0.212
IA, IB	4	4	0	
IIA	24	14	10	
IIB	73	55	18	
III, IV	13	8	5	
CA19-9 (U/mL)				0.192
< 37, n (%)	27	21	6	
≥ 37, n (%)	85	59	26	
N/A	2	1	1	
Tumor size (mm)				**0.037**
< 40, n (%)	59	47	12	
≥ 40, n (%)	55	34	21	
Adjuvant therapy				0.848
present	92	65	27	
absent	22	16	6	

[a] Chi-square test

[b] Fisher's exact test

UICC, International Union Against Cancer

Supplementary Table S1 Primer for RT-PCR

No.	Gene	Forward primer	Reverse primer
1	LAMC2	GGACATTCTGAGAGATGCCCA	TTGGCCAACTGGAGACCAAG
2	E-cadherin	TGGAGGAATTCTTGCTTTGC	CGCTCTCCTCCGAAGAAAC
3	Vimentin	GACGCCATCAACACCGAGTT	CTTTGTCGTTGGTTAGCTGGT
4	ZEB1	GATGATGAATGCGAGTCAGATGC	ACAGCAGTGTCTTGTTGTTGT
5	ZEB2	GGAGACGAGTCCAGCTAGTGT	CCACTCCACCCTCCCTTATTTC
6	MDR1	GAGTATCTTCTTCCAAGATTTCACG	TCCCCTTCAAGATCCATCC
7	MRP2	AGGCATTGACCCTATCCAACT	CATCCACAGACATCAGGTTCA
8	ABCA5	TCAAGCAACATTGGAACAGG	TCCCACCAAAGTGTGCTGT
9	ABCA1	GGGAACCCTGGAACTCTTAAAC	GGGATTGGGTTTCCTTCCATAC
10	ABCA2	GAGGTGGCGCATGATAAGAT	GTACTTAGAGCCCAGACCAAAG
11	ABCD3	AATGTCCAGTTGGGTCATATCC	CACTGAGTACGTCCATCCAATC
12	ABCB4	TGTCTCAGGAGCCTATCCTATT	GCTGCACTCACAATTTCATCC
13	ABCB6	GGTGACTGTGGGCAACTAC	GTGAAGAAGAAGCAGGGACTC
14	CFTR	GCTTCCTATGACCCGGATAAC	GGAGCAGTGTCCTCACAATAA
15	TAP1	ATGTTTCCGGCACACCAAAC	AAAAGAGGAGACACCCGCAG
16	TAP2	GACAGAACTGGGCACAAGTAATA	CAGTCCCTTCTCCTACCATACA
17	ABCC3	TGCCTGCTTCAAGCTTATCC	CATGGGGTTGGAGATAAACC
18	β-actin	CCTTTGCCGATCCGCCG	GATATCATCATCCATGGTGAGCTGG