Upregulation of KLK8 Predicts Poor Prognosis in Pancreatic Cancer

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Research

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Abstract

Background: Pancreatic ductal adenocarcinoma (PDAC) is a growing cause of cancer-related mortality worldwide. Kallikrein-related peptidase 8 (KLK8) has potential clinical values in many cancers. However, the clinicopathological significances of KLK8 in PDAC remain unknown.

Methods: The relationship of KLK8 to clinicopathological features of PDAC was investigated based on public databases. KLK8 expression was examined in human PDAC tissues. Cell proliferation and apoptosis were evaluated in KLK8-overexpressed human pancreatic cancer cell lines Mia-paca-2 and Panc-1. The related signaling pathways of KLK8 involved in pancreatic cancer progression were analyzed by gene set enrichment analysis (GSEA) and further verified in *in vitro* studies.

Results: KLK8 was up-regulated in tumor tissues in the TCGA-PAAD cohort, and was an independent prognostic factor for both overall survival and disease-free survival of PDAC. KLK8 mRNA and protein expressions were increased in PDAC tissues compared with para-cancerous pancreas. KLK8 overexpression exerted pro-proliferation and anti-apoptotic functions in Mia-paca-2 and Panc-1 cells. GSEA analysis showed that KLK8 was positively associated with PI3K-Akt-mTOR and Notch pathways. KLK8-induced pro-proliferation and anti-apoptotic effects in Mia-paca-2 and Panc-1 cells were attenuated by inhibitors for PI3K, Akt, and mTOR, but not by inhibitor for Notch.

Conclusion: KLK8 overexpression exerts pro-proliferation and anti-apoptotic functions in pancreatic cancer cells via PI3K/Akt/mTOR pathway. Upregulated KLK8 in PDAC predicts poor prognosis and may be a potential therapeutic target for PDAC.

Background

Pancreatic cancer is one of the most leading causes of cancer death in both males and females because of its poor prognosis, with almost as many deaths (n = 432,000) as cases (n = 459,000)[1]. According to 2020 cancer statistics, approximately 57,600 new cases of pancreatic cancer will be diagnosed, killing almost 47,050 people in the United States in 2020, making it the fourth leading cause of cancer-associated death worldwide[2]. Despite the advanced therapeutic approaches, the 5-year relative survival rate of pancreatic cancer remains poor, estimated at 9%[3–5]. To further improve the survival rates, it is critical to identify a more sensitive and effective biomarker associated with the tumorigenesis and progression for early detection, which will improve the prognosis for pancreatic cancer.

Tissue kallikrein-related peptidases (KLKs) are a group of serine proteases encoded by 15 highly conserved trypsin- or chymotrypsin-like serine proteases displaying diverse biological processes, such as embryogenesis and pregnancy, blood coagulation, complement activation, general protein turnover and wound healing. [6–9]. KLK8, an important member of the KLKs family, is a synaptic, plasticity-modulating extracellular serine protease and has been found in many tissues and biological fluids, involved in a variety of biological activities, for instance, epidermal proliferation and differentiation, terminal differentiation of keratinocytes and so on [10, 11]. Abnormal KLK8 expression has been found in several

malignancies, including ovarian, cervical, gland and lung cancers[12–15]. Meanwhile, accumulating evidence support the clinical utility of KLK8 as a biomarker for cancer survival and prognosis. However, the expression pattern and role of KLK8 in pancreatic cancer remains unknown.

In this study, we explored the expression of KLK8 in the pancreatic cancer at both the mRNA and protein levels and investigated the correlation between KLK8 expression and prognosis of pancreatic cancer patients. We also investigated whether and how KLK8 affected the proliferation and apoptosis of pancreatic cancer cells. Our findings demonstrated that upregulation of KLK8 was related to a poor prognosis in pancreatic cancer. Overexpression of KLK8 might promote proliferation and inhibit apoptosis via activating PI3K-AKT-mTOR signaling in pancreatic cancer cells.

Methods

Patients and specimens

Thirty pancreatic cancer tissue samples and their matched para-cancerous pancreas from pancreatic cancer patients who underwent surgery in Fudan University Shanghai Cancer Center (Fudan Center) between June 2016 and April 2018 were obtained during operations. All diagnoses were confirmed by two pathologists. All specimens were acquired after written informed consent following procedures approved by the Ethics Committee of Fudan University Shanghai Cancer Center.

The Cancer Genome Atlas Analysis and GSEA

The GEPIA database provided differential gene expression analysis of 31 kinds of cancers based on integrated analysis of the TCGA and GTEx databases. TCGA Pancreatic Cancer (PAAD) cohort consisted of 178 primary pancreatic cancer and 4 normal samples. And gene expression of 167 normal pancreatic tissue was also downloaded from GTEx (http://commonfund.nih.gov/GTEx/) to explore the differential expressed genes between tumors and normal tissues. All data on expression and clinical features were obtained from the USUC Xena Cancer Genomics Browser(https://xenabrowser.net/datapages/?dataset=TCGA). The differentially expressed genes with $|\log2foldchange| \geq 1$ and $P < 0.05$ were selected based on 178 pancreatic cancer samples and 171 normal pancreas samples. X-tile program (www.tissuearray.org/rimmlab/) was used to determine the optimum cutoff value of KLK8 with minimum p value defined by Kaplan–Meier survival analysis and log-rank test. To elucidate the mechanisms behind KLK8 in pancreatic cancer, Gene set enrichment analysis (GSEA) was performed on the Broad Institute Platform, and statistical significance (false discovery rate, FDR) was set at 0.25. Hallmark gene set collection was used to find relative signaling pathways of KLK8 from control and KLK8 overexpression group according to the genes presenting the strongest enrichment scores.

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA from 30 random pairs of fresh pancreatic cancer tissue and adjacent normal mucosa were isolated using the Trizol reagent (TaKaRa, Japan) according to the manufacturer's instruction. Primers for RT-qPCR were designed using Primer Express v2.0 software (Applied BioSystems). The primer sequences for KLK8 were as follows: forward 5'- AAG TGCACC GTC TCA GGC-3' and reverse 5'- TCC TCA CAC TTC TTC TGG GG-3'. β-actin was used as an internal control, and the primer sequences were as follows: forward 5'-CTA CGT CGC CCT GGA CTT CGA GC -3' and reverse 5'- GAT GGA GCC GCC GAT CCA CAC GG -3'. Real-time PCR was carried out using SYBR Green I (Applied BioSystems) and the relative expression was calculated using the $2^{-\Delta\Delta CT}$ method and normalized to β-actin (human) as the internal control gene.

Immunohistochemical (IHC) staining

The immunohistochemistry (IHC) staining of KLK8 was performed using a primary antibody against KLK8 (1:100 dilution; Abcam, Cambridge, UK) according to the manufacturer's instructions. Staining was independently examined by two experienced investigators blinded to the clinical characteristics of the patients. The score for KLK8 staining was based on the integrated staining intensity and the percentage of positive cells. Staining intensity was scored as follows[16]: 0 = no color; 1 = yellow; 2 = light brown; and 3 = dark brown. The proportion of immune-positive tumor cells (number of positively labeled tumor cells / number of total tumor cells) was scored as follows[17]: 0, positive cells < 5%; 1, 6%- 25% positive cells;2, 26%- 50% positive cells; 3, positive cells 51%- 75%; and 4 > 76%.). The comprehensive score was the product of staining intensity and average proportion of positive cells and expressed as follows[18]: negative staining (0–2); weak expression (3–5); moderate expression (6–9); and strong expression (10–12).

Cell culture and transient transfection

The human pancreatic cancer cell line Mia-paca-2, Panc-1 and human embryonic kidney cell line 293T (293T) were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Mia-paca-2 and Panc-1 cells were cultured in DMEM (Invitrogen). All medium was supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (Invitrogen).

To establish the cell lines stably overexpressing KLK8, hKLK8 Lentiviral vector and their negative controls were designed and synthesized by Shanghai Genechem Co. (Shanghai, China). Mia-paca-2 and Panc-1 cells were placed in 6-well plates at a density of 1 × 10^5cells/well the day before infection. The next day lentivirus were added in cell culture medium. Viruses were removed 24 h after infection and fresh cell culture medium was added. 72 h after transfection, puromycin (2 μg/ml; Roche, USA) was added into the cell culture medium to generate stable KLK8-overexpression cell line four weeks later. Antibiotic-resistant cells were pooled for subsequent analysis.

Western blot analysis

The cells were lysed, and proteins were extracted through standard protocols. The proteins were separated by SDS-polyacrylamide gel electrophoresis and subjected to western blot analyses. Protein bands were detected by the chemiluminescence method. Specific primary antibodies against

KLK8(1:1000 dilution),PI3K(1:1000 dilution),Akt(1:1000 dilution), mTOR (1:1000 dilution), p-PI3K(1:1000 dilution,P85 Y199), p-Akt(1:1000 dilution,Ser473),p-mTOR (1:1000 dilution,Ser2448),p-4EBP1(1:1000 dilution, Ser65),p-S6P-p70S6K (1:1000 dilution,Thr389),Notch1(1:1000 dilution),*c-myc* (1:1000 dilution) and cyclin D1 (1:1000 dilution; Abcam, Cambridge, UK) were used.β-Actin(1:5000 dilution; Santa Cruz, CA, USA) was used as a loading control. The chemiluminescent signals were detected with the chemiluminescence imaging system and quantified by Image J software (v1.37).

Cell counting kit-8 (CCK8) assay

The density of 1000 pretreated cells /well were seeded into a 96-well plate. The cells were incubated with CCK8 reagent (DOJINDO, Japan) at 37 °C for 1 h and absorbance at 450 nm were measured using a microplate reader (BioTek, Vermont, USA) for the appropriate time (1, 2, 3, 4 or 5 days).

Colony formation

Log phase Mia-paca-2 and Panc-1 cells were collected. The 500 cells were planted in each well of the 6-well plate and incubated at 37 °C exposed to 5% CO_2. After 14 days the colonies were counted visually, with > 100 cells/colony considered a clone.

Apoptosis assessment

Following transfected with KLK8 and vector plasmid, cells were washed with PBS 3 times and then stained using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) according to the instruction. Then cells were analyzed with a FACS flow cytometer (BD Biosciences).

Statistical analysis

Data were expressed as means ± standard error of the mean (SEM) from at least three experiments. All statistical analyses were performed using SPSS 13.0 (SPSS Inc.). Independent samples t-test was used to compare control and treatment groups and one-way ANOVA was performed to compare the data of multiple groups. The Kaplan Meier estimation method was used for overall survival analysis, and a log-rank test was used to compare differences. $P < 0.05$ was considered to be statistically significant.

Results

KLK8 was associated with pancreatic cancer progression and patients' outcome

Based on the indicated role of KLK8 in malignant disease found in GEPIA (http://gepia.cancer-pku.cn/detail.php?gene=KLK8) (Fig. 1A), we analyzed the expression of KLK8 in the independent public dataset from Oncomine (https://www.oncomine.org/resource/main.html) and found that KLK8 expression was elevated in the pancreatic cancer tissue samples in comparison to the normal pancreas (Fig. 1B, $P < 0.0001$). To further examine the potential relationship between KLK8 and PDAC, we analyzed data from the TCGA-PAAD cohort which was replenished by GTEX database, and found that KLK8 was

significantly upregulated in tumor tissues compared to normal tissues (Fig. 1C, $P < 0.0001$), suggesting that KLK8 might act as an oncogene in pancreatic cancer. Then we evaluated the relationship between KLK8 expression and patients' outcomes. The Kaplan–Meier curve analysis of the TCGA database indicated that higher KLK8 expression in PDAC was correlated with shorter OS ($n = 136$) and DFS rates ($P < 0.001$, Fig. 1D and E). These results suggest that KLK8 is not only involved in pancreatic initiation and progression but is also correlated with patient outcome.

KLK8 was elevated in pancreatic cancer tissues at both the mRNA and protein levels

To further investigate the expression of KLK8 in pancreatic cancers, KLK8 protein expression was assessed in 20 pancreatic cancer tissues and para-cancerous pancreas by IHC staining. As showed in Fig. 2A,B and C, compared with normal tissues, the level of KLK8 were significantly increased in pancreatic cancer tissues ($p < 0.01$). Then, KLK8 mRNA expression was determined in 30 paired PADD tissues and matched para-cancerous pancreas. It was found that KLK8 mRNA levels were significantly increased in pancreatic cancer tissue samples as comparison to the adjacent non-tumor tissues (Fig. 2D, $p < 0.01$). These findings were consistent with the data obtained from the public datasets (Fig. 1).

KLK8 exerted pro-proliferation and anti-apoptotic functions in pancreatic cancer cells

Abnormal cell proliferation and apoptosis are characteristics of human malignant tumor [19]. We then determined whether elevated KLK8 expression could influence the proliferation and apoptosis of pancreatic cancer cells by using KLK8-overexpressed Mia-paca-2 and Panc-1 cell lines. The efficacy of KLK8 overexpression in two cell lines was confirmed by western blot analysis (Fig. 3A). We performed CCK-8 and colony formation assay to assess the effects of KLK8 in pancreatic cell proliferation. As shown in Fig. 3B and C, a significant promotion of cell proliferation was observed in the KLK8-overexpression group in comparison to the control group. In addition, the number of cell colonies were significantly increased in both Mia-paca-2 and Panc-1 pancreatic cancer cells overexpressed with KLK8 (Fig. 3D and E).

We then clarified the effect of KLK8 overexpression on pancreatic cancer apoptosis. As shown in Fig. 4A and B, compared with control group, the percentage of apoptosis cells was significantly reduced in KLK8 overexpressed pancreatic cancer cells.

KLK8 accelerated cell growth and inhibited apoptosis via PI3K-Akt-mTOR signaling pathway in pancreatic cancer cells

To gain an insight into the mechanisms by which KLK8 promoted PDAC progression, the gene expression in PDAC tissues with high expression of KLK8 and those with low expression of KLK8 was analyzed by gene set enrichment analysis (GSEA) based on the TCGA database. GSEA results showed that 14

enriched pathways were differentially expressed according to diverse KLK8 expression levels (Fig. 5A, $p < 0.05$). Notably, KLK8 was positively associated with PI3K-AKT-mTOR and Notch signaling pathways, both of which are known to play critical roles in cell proliferation and apoptosis (Fig. 5B and C). We observed significantly enhanced phosphorylated PI3K, phosphorylated Akt and phosphorylated mTOR expression in Mia-paca-2 and Panc-1 cells overexpressed with KLK8. KLK8 overexpression also led to significant increases in phosphorylated 4EBP1 and phosphorylated S6P-p70S6K, two of the most distinctive downstream targets of mTOR, in pancreatic cancer cell lines (Fig. 5D). In addition, KLK8 overexpression also led to significant increases in Notch-1 protein expression. C-myc and Cyclin D1, two downstream targets of Notch signaling, were increased in pancreatic cancer cells overexpressed with KLK8 (Fig. 5D). These findings suggest that KLK8 overexpression activates PI3K-Akt-mTOR and Notch pathways (Fig. 5D).

Next, we explored whether activation of PI3K-Akt-mTOR and Notch signaling pathways contributed to the pro-proliferation and anti-apoptotic functions of KLK8 in pancreatic cancer cells. Both CCK8 assay and colony formation assay showed that the pro-proliferation effects of KLK8 on pancreatic cells were counteracted by PI3K inhibitor LY294002, Akt inhibitor Deguelin, and mTOR inhibitor Rapamycin (Fig. 6). However, Notch inhibitor RO4929097 had no significant effect on KLK8-induced pro-proliferation in pancreatic cells (Fig. 6).

As shown in Fig. 7, the anti-apoptotic effects of KLK8 on pancreatic cells were blocked by PI3K inhibitor LY294002, Akt inhibitor Deguelin, and mTOR inhibitor Rapamycin. However, Notch inhibitor RO4929097 had no significant effect on KLK8-induced anti-apoptotic function in pancreatic cells (Fig. 7).

Taken together, these findings suggest that activation of PI3K-Akt-mTOR pathway, but not Notch pathway, contributes to the pro-proliferation and anti-apoptotic functions of KLK8 in pancreatic cancer cells.

Discussion

Pancreatic cancer continues to have a poor 5-year survival rate despite its rising incidence [20, 21]. By 2030, it is estimated to become the second leading cause of cancer related deaths [5]. Pancreatic resection is still the only curative intent therapy for PDAC patients. However, pancreatic resection is complex and carries with it the risk of major morbidity and mortality [22]. Thus, there is a desperate need for investigating the pathogenesis and identifying molecular biomarkers of PDAC to facilitate early diagnosis, prognosis prediction, and the development of effective therapeutic strategies for PDAC patients.

KLK8, also known as neuropsin, is a member of human kallikrein-related peptidase (KLKs) family which has been related to malignant behavior at multiple stages of tumor progression, including proliferation, migration and angiogenesis [23, 24]. Previous studies have found that abnormal expression of KLK8 was associated with several malignancies, including ovarian, cervical, gland and lung cancers [12, 25–28].However, the expression level and prognostic significance of KLK8 in PDAC are still unknown. In this study, we identified up-regulated KLK8 expression in pancreatic cancer compared with adjacent tissues

through TCGA database, which was further confirmed by using clinical samples. Furthermore, we found that high KLK8 expression predicts poorer OS and DFS in pancreatic cancer patients. These results indicated that KLK8 could be a prognostic marker for PDAC. Similar to our findings, several studies have confirmed that the upregulation of KLK8 was related to poorer cancer prognosis. For example, KLK8 has been recognized as a poor prognostic marker for lung and breast cancer [15, 25]. But in other tumors, such as ovarian cancer, the elevated expression of KLK8 is a favorable prognostic marker [29]. These results suggest that KLK8 may play different roles in different cancers, and the aberrant expression of KLK8 may serve as a potential clinical biomarker for cancer diagnosis or prognosis.

KLK family members have been implicated in the pathogenesis and progression of malignant tumor[30, 31]. For example, overexpression of KLK7 is found to stimulate colon cancer cell proliferation both *in vivo* and *in vitro*[32]. KLK13 enhances the invasiveness and motility of lung cancer via increasing laminin degradation and N-cadherin expression[33]. KLK5 promotes metastatic dissemination of Oral squamous cell carcinoma(OSCC) by promoting loss of junctional integrity through cleavage of desmoglein 1[34]. KLK14 acts at the cleavage site of PAR-2 to induce ERK1/2 activation, thus promoting colon cancer proliferation[35]. As for KLK8, it can facilitate colorectal cancer (CRC) cell proliferation, migration and invasion *in vitro* [9]. In this study, by using two pancreatic cancer cell lines, we demonstrated for the first time that overexpression of KLK8 significantly inhibited PDAC cell apoptosis, meanwhile profoundly promoted PDAC cell proliferation. These data suggest that KLK8 may promote tumor growth and suppress tumor apoptosis, and may be a potential molecular target in therapy for pancreatic cancer.

Phosphatidylinositide 3 kinases (PI3Ks) and their downstream mediators Akt and mammalian target of rapamycin (mTOR) are well-known to regulate cell proliferation, apoptosis, homeostasis and metabolism [36]. Previous studies have demonstrated that activation of PI3K/AKT/mTOR signaling pathway facilitates pancreatic cancer cell proliferation. In contrast, blockade of PI3K/AKT/mTOR signaling pathway promotes pancreatic cancer cell death [37–39]. Overexpression of KLK8 has been found to induce Akt activation under Hypoxia/Reoxygenation (H/R) stimulation in neonatal rat cardiomyocytes [8]. In the present study, GSEA analysis and western blot assay revealed that KLK8 overexpression resulted in the activation of PI3K/AKT/mTOR signaling pathway in pancreatic cancer cells. In addition, the pro-proliferation and anti-apoptotic functions of KLK8 were reversed by inhibitors targeting PI3K, Akt and mTOR. These findings suggest that elevated KLK8 may exert the pro-proliferation and anti-apoptotic effects in pancreatic cancer cells through activating PI3K-Akt-mTOR signaling pathway.

Notch signaling pathway also plays an important role in the occurrence and progression of pancreatic cancer [40–42]. In the present study, GSEA analysis and western blot assay revealed that KLK8 overexpression resulted in the activation of Notch signaling pathway. However, Notch inhibitor didn't influence the KLK8-induced effects in pancreatic cancer cells. These results suggest that the pro-proliferation and anti-apoptotic functions of KLK8 may not be dependent on activation of Notch signaling pathway. Notably, our GSEA analysis data showed that KLK8 overexpression might also lead to the activation of EMT (epithelial-mesenchymal transition), glycolysis and KRAS signaling pathway, which have been implicated in the pathogenesis and progression of pancreatic cancers[17, 43–45]. Whether

these processes and the related signaling pathways contribute to the KLK8-induced pro-proliferation and anti-apoptotic effects in pancreatic cancers merits further investigation.

Conclusions

In summary, our findings indicate that KLK8 overexpression exerts pro-proliferation and anti-apoptotic functions in pancreatic cancer cells via PI3K/AKT/mTOR signaling pathway. Positive KLK8 staining is associated with PDAC progression and predict poorer survival in patients, thus providing additional evidence for patient-tailored therapeutic strategies.

Abbreviations

PDAC: Pancreatic ductal adenocarcinoma

KLK8: Kallikrein-related peptidase 8

GSEA: gene set enrichment analysis

PI3K: Phosphatidylinositide 3 kinases

mTOR: mammalian target of rapamycin

KLKs: kallikrein-related peptidases

PAAD: Pancreatic Cancer

OSCC: Oral squamous cell carcinoma

CRC: Colorectal cancer

H/R: Hypoxia/Reoxygenation

EMT: epithelial-mesenchymal transition

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Fudan University Shanghai Cancer Center. (Shanghai, China).

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Qing Hua was responsible for conducting the study, under the supervision of Xiaoyan Zhu and Pingbo Xu and contributed to the experimental design; Qing Hua, Tianjiao Li and Yixuan Liu did the experiments and analyzed the data. Qing Hua and Xuefang Shen wrote the paper, and all authors read and approved the final manuscript. All authors read and approved the final manuscript.

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Figures

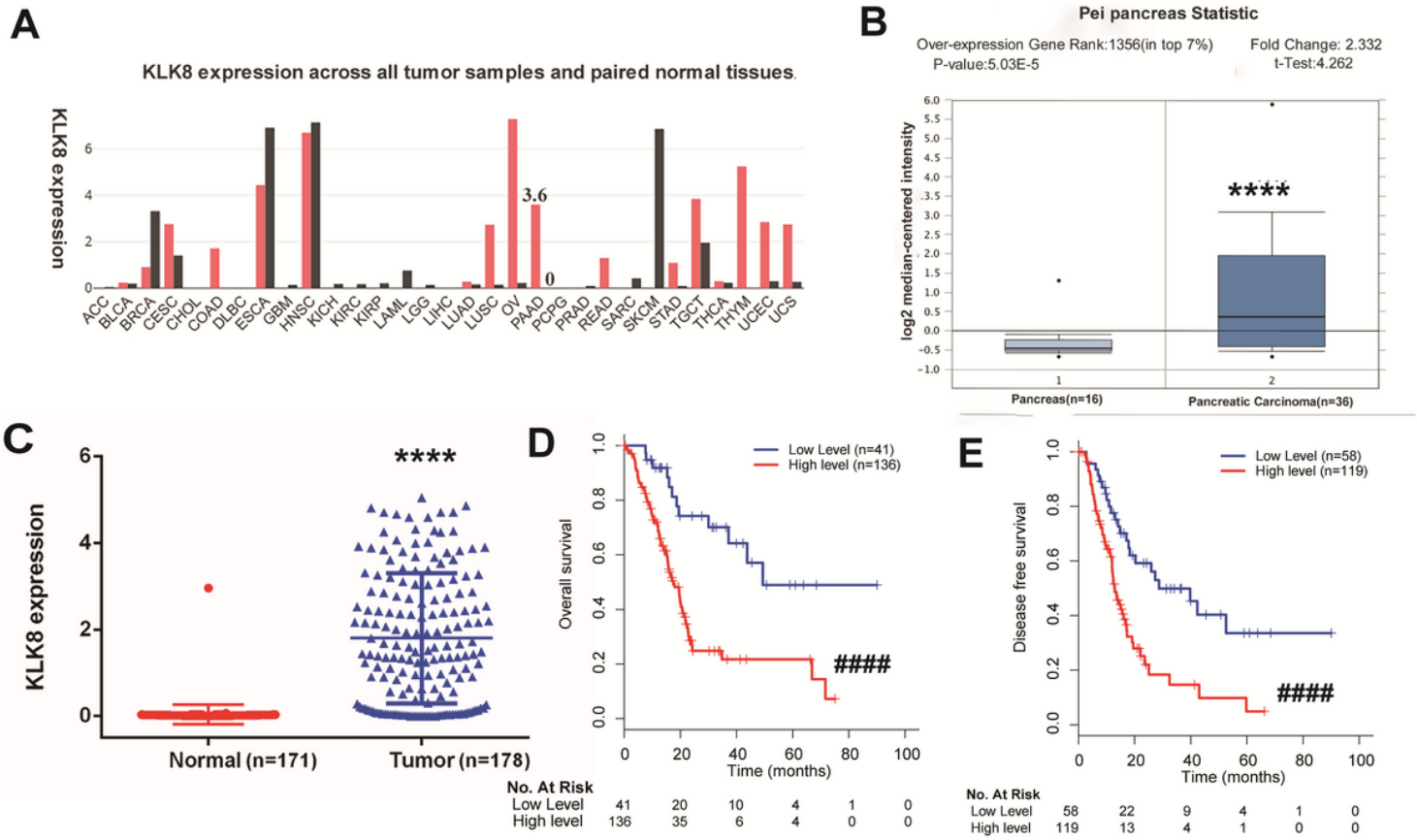

Figure 1

KLK8 was associated with PDAC tumorigenesis and prognosis in the TCGA-PAAD cohort. (A) KLK8 expression in different cancers and their paired normal tissues in the GEPIA. The height of bar represents the median expression of certain tumor type or normal tissue. (B) KLK8 expression of normal specimens and pancreatic carcinoma from an independent pancreatic dataset in the Oncomine database. The expression fold-change was 2.332. (C) In the TCGA-PAAD cohort, KLK8 was more significantly expressed in cancer patients (n=178) than in normal controls (n =171). (D-E) Overall survival (OS) and Disease-free survival (DFS) was compared between patients with low and high KLK8 expression in the TCGA-PAAD cohort. **** $p < 0.0001$ vs normal; ####$p < 0.001$ vs low level.

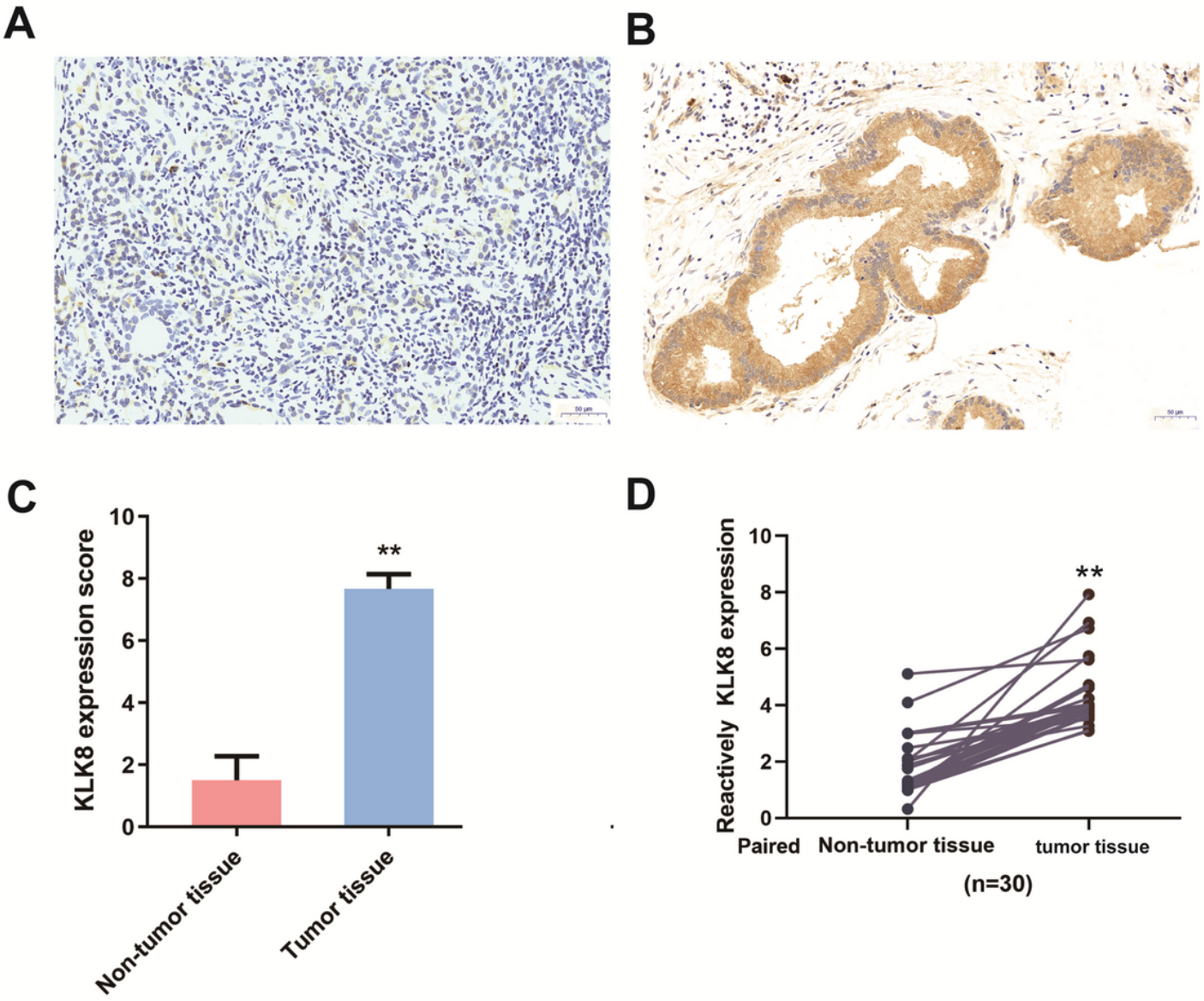

Figure 2

KLK8 expression in pancreatic cancer tissues and para-cancerous pancreas. (A-B) Representative immunohistochemistry staining of KLK8 in pancreatic cancer tissues and para-cancerous pancreas (n=20). (A) showed negative KLK8 staining in para-cancerous pancreas. (B) showed strong positive KLK8 staining in PDAC tissues. (C) Quantified data of the score for KLK8 staining. (D) Quantitative real-time PCR detection of KLK8 mRNA expression in paired human PDAC tissue samples and para-cancerous pancreas (n=30). Data were presented as the mean ± SEM. ** p <0.01 vs Non-tumor tissue.

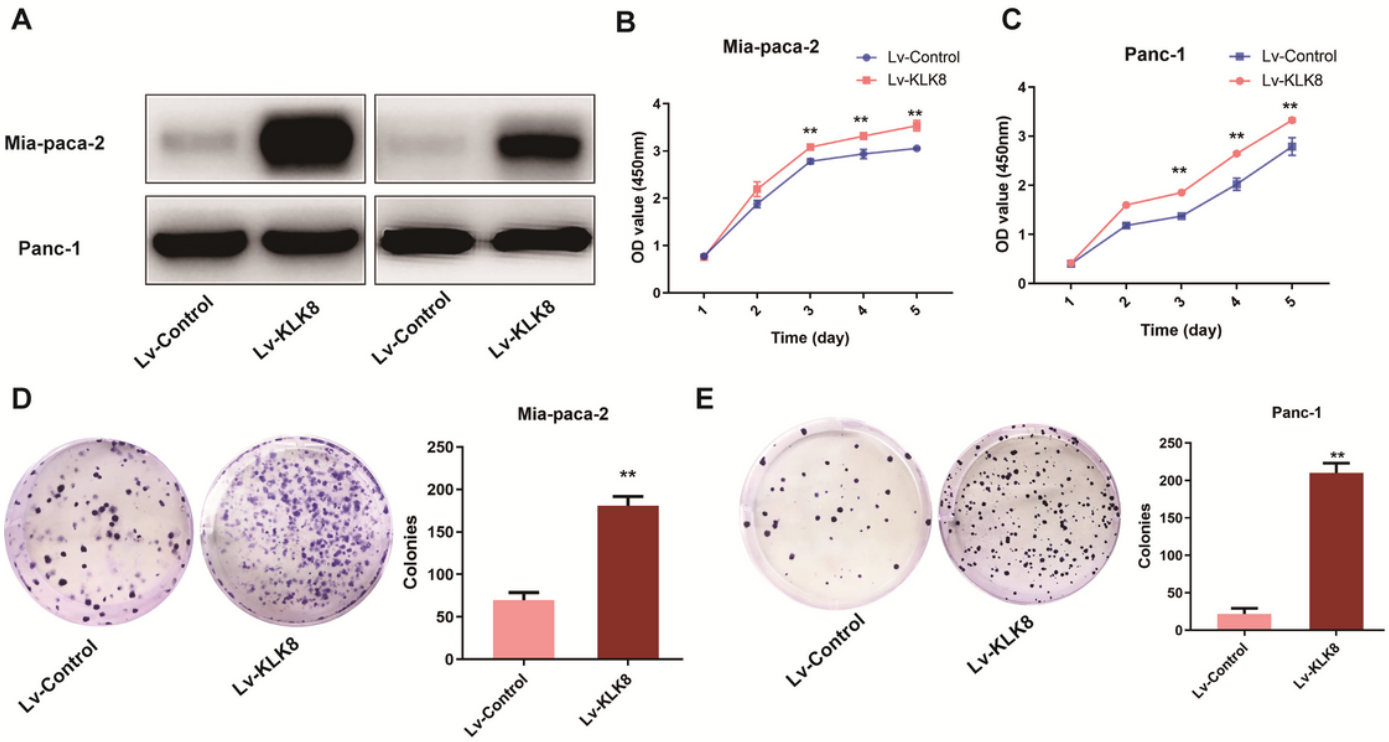

Figure 3

KLK8 overexpression promoted the proliferation of PDAC cells. KLK8 overexpression was induced with recombinant Lentivirus infection (Lv-KLK8) in human pancreatic cancer cell lines Mia-paca-2 and Panc-1, and an empty adenovirus served as control (Lv-Control). (A) Mia-paca-2 and Panc-1 cells transfected with KLK8 overexpression vectors were validated at protein level by western blot analysis. (B-C) Cell proliferation was detected by CCK8 Assay in Mia-paca-2 (B) and Panc-1 (C) cells. (D-E) Colony formation was detected in Mia-paca-2 (D) and Panc-1 (E) cells. Data were presented as the mean ± SEM (n=3). ** p <0.01 vs Lv-control.

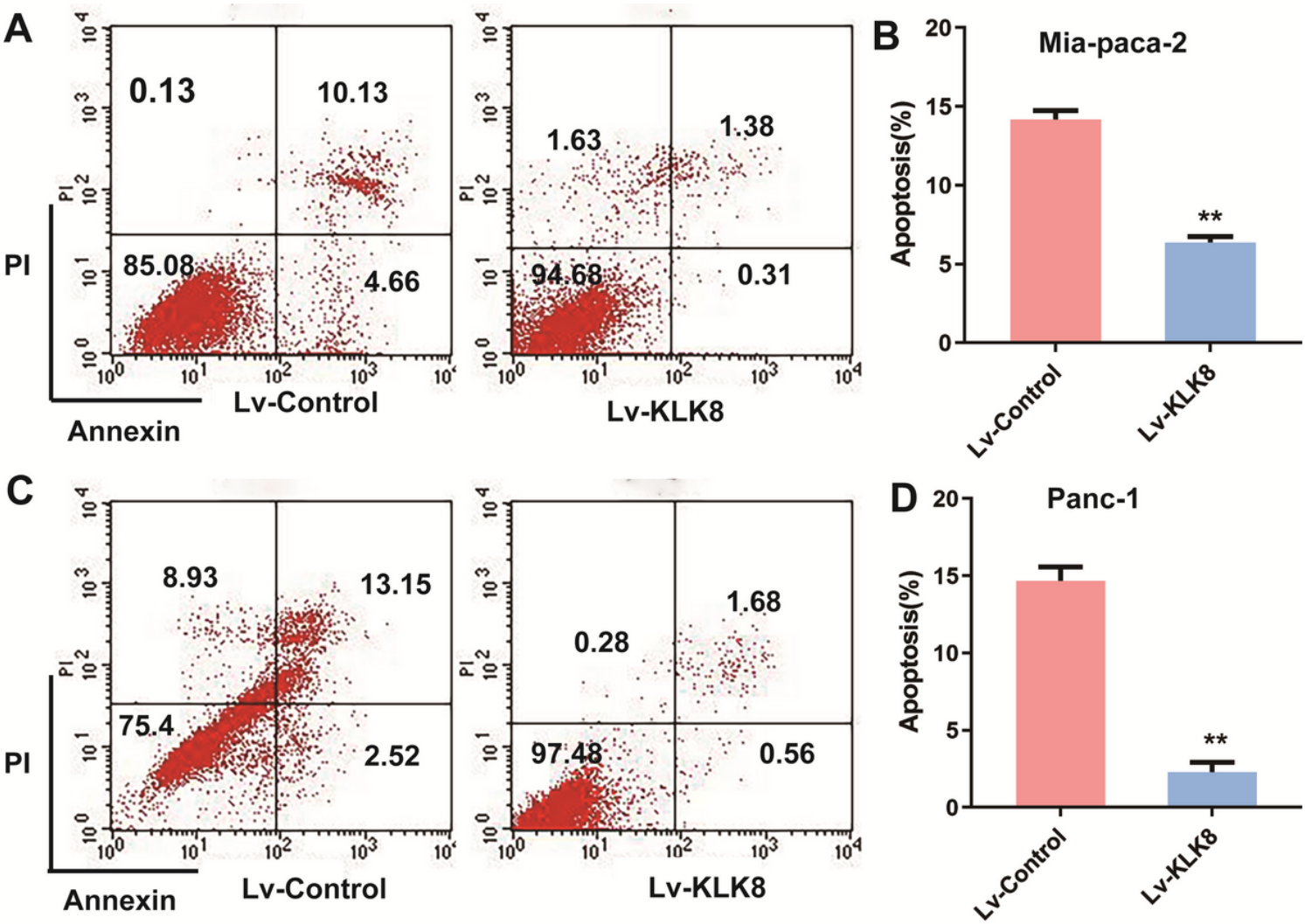

Figure 4

KLK8 overexpression inhibited apoptosis of PDAC cells. KLK8 overexpression was induced with recombinant Lentivirus infection (Lv-KLK8) in human pancreatic cancer cell lines Mia-paca-2 (A and B) and Panc-1 (C and D), and an empty adenovirus served as control (Lv-Control). Cell apoptosis was determined by Annexin V-FITC and PI double staining analysis performed by flow cytometry. Representative flow cytometry images were shown (A and C). (B and D) demonstrated the quantified data of cell apoptosis. Data are expressed as the mean ± SEM (n=3). ** p <0.01 vs Lv-control.

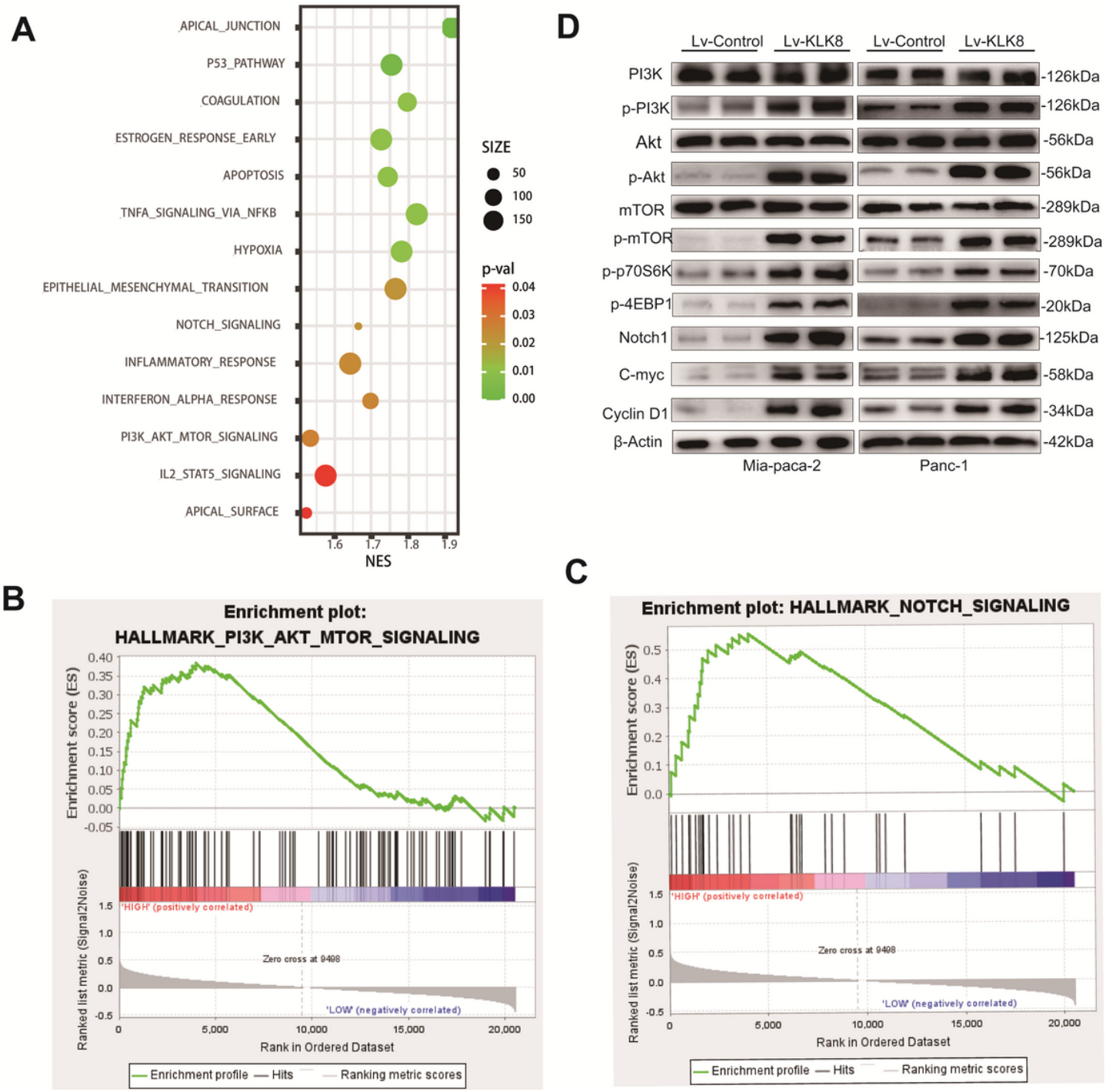

Figure 5

Significantly-altered pathways were predicted in PDAC and verified in KLK8-overexpressed pancreatic cancer cells. (A) Gene sets enriched in the transcriptional profiles of tumors belonging to the top KLK8 high-expression group, compared with the bottom-expression group in the TCGA dataset. Shown are the NES (normalized enrichment score) values for each pathway using the Hallmark gene sets. The functional annotations of KLK8 positive and negative expression in PDAC was predicted. A nominal p value of <0.05 is considered statistically significant. (B-C) GSEA highlighted positive association of increased KLK8 expression levels with PI3K-Akt-mTOR (B) and Notch (C) signal pathways. (D) levels of

key proteins in PI3K-AKT-mTOR and Notch signaling pathways were examined in KLK8-overexpressed Mia-paca-2 and Panc-1 cells using western blot. NES = normalized enrichment score. (n=3).

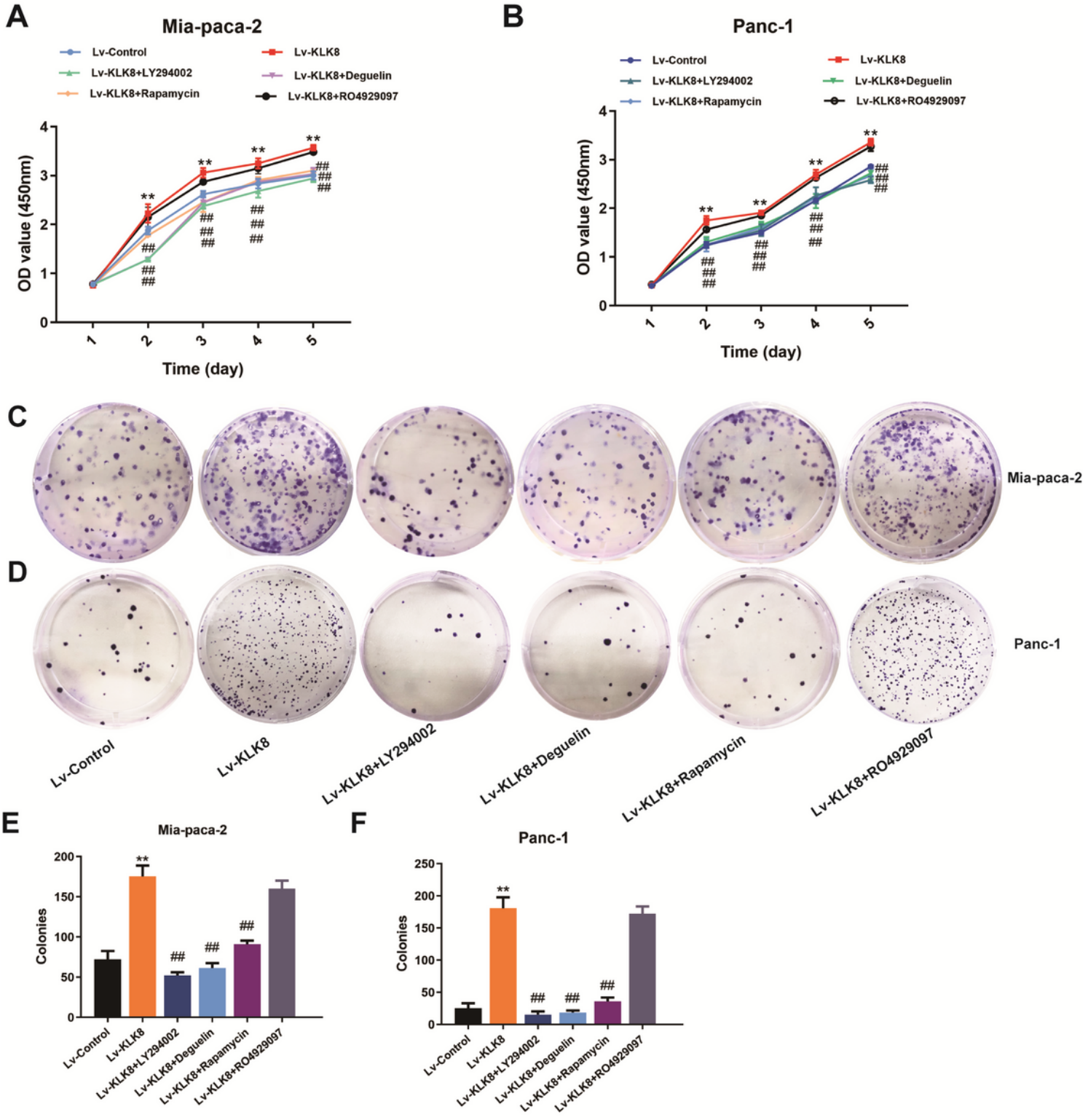

Figure 6

KLK8 promoted pancreatic cancer cells proliferation via the activation of the PI3K-Akt-mTOR pathway. KLK8 overexpression was induced with recombinant Lentivirus infection (Lv-KLK8) in the presence or absence of PI3K inhibitor LY294002 LY294002(75 μM), Akt inhibitor Deguelin(500 nM), mTOR inhibitor

Rapamycin (100 nM) or Notch inhibitor RO4929097 (10 μM) in Mia-paca-2 and Panc-1 cells. (A-B) Cell proliferation was detected by CCK8 Assay in Mia-paca-2 (A) and Panc-1 (B) cells. (C-D) Colony formation was detected in Mia-paca-2 (C) and Panc-1 (D) cells. (E-F) demonstrated the quantified data of cell colonies. Data were presented as the mean ± SEM (n=3). * p<0.05, ** p <0.01 vs Lv-control; #p < 0.05, ## p <0.01 vs Lv-KLK8.

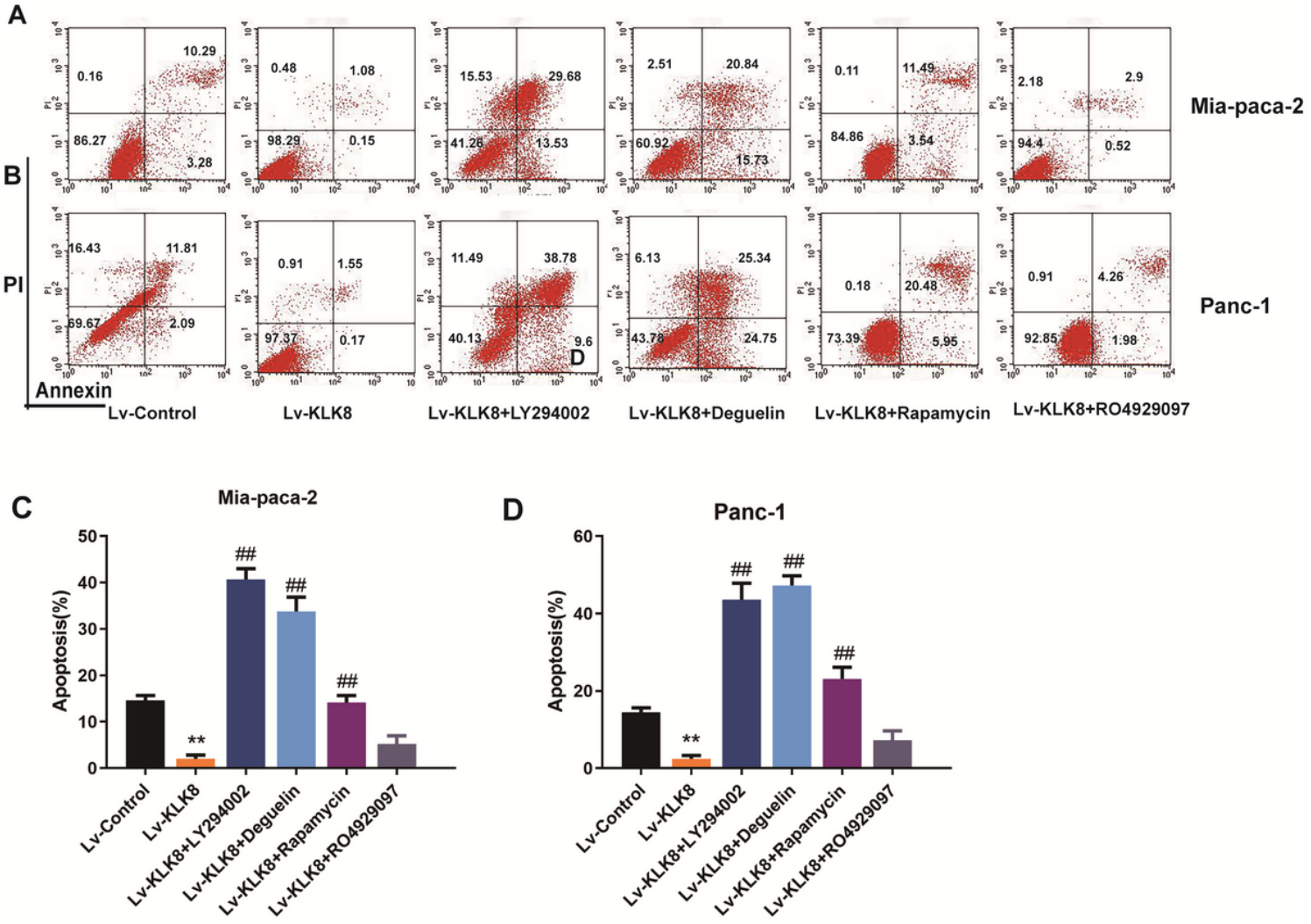

Figure 7

KLK8 suppressed pancreatic cancer cells apoptosis through the activation of the PI3K-Akt-mTOR pathway. KLK8 overexpression was induced with recombinant Lentivirus infection (Lv-KLK8) in the presence or absence of PI3K inhibitor LY294002(75 μM), Akt inhibitor Deguelin(500 nM), mTOR inhibitor Rapamycin (100 nM) or Notch inhibitor RO4929097 (10 μM) in Mia-paca-2 (A and B) and Panc-1 (C and D) cells. Cell apoptosis was determined by Annexin V-FITC and PI double staining analysis performed by flow cytometry. Representative flow cytometry images were shown (A and C). (B and D) demonstrated the quantified data of cell apoptosis. Data are presented as means ± SEM (n = 3). * p<0.05, ** p <0.01 vs Lv-control; #p < 0.05, ## p <0.01 vs Lv-KLK8

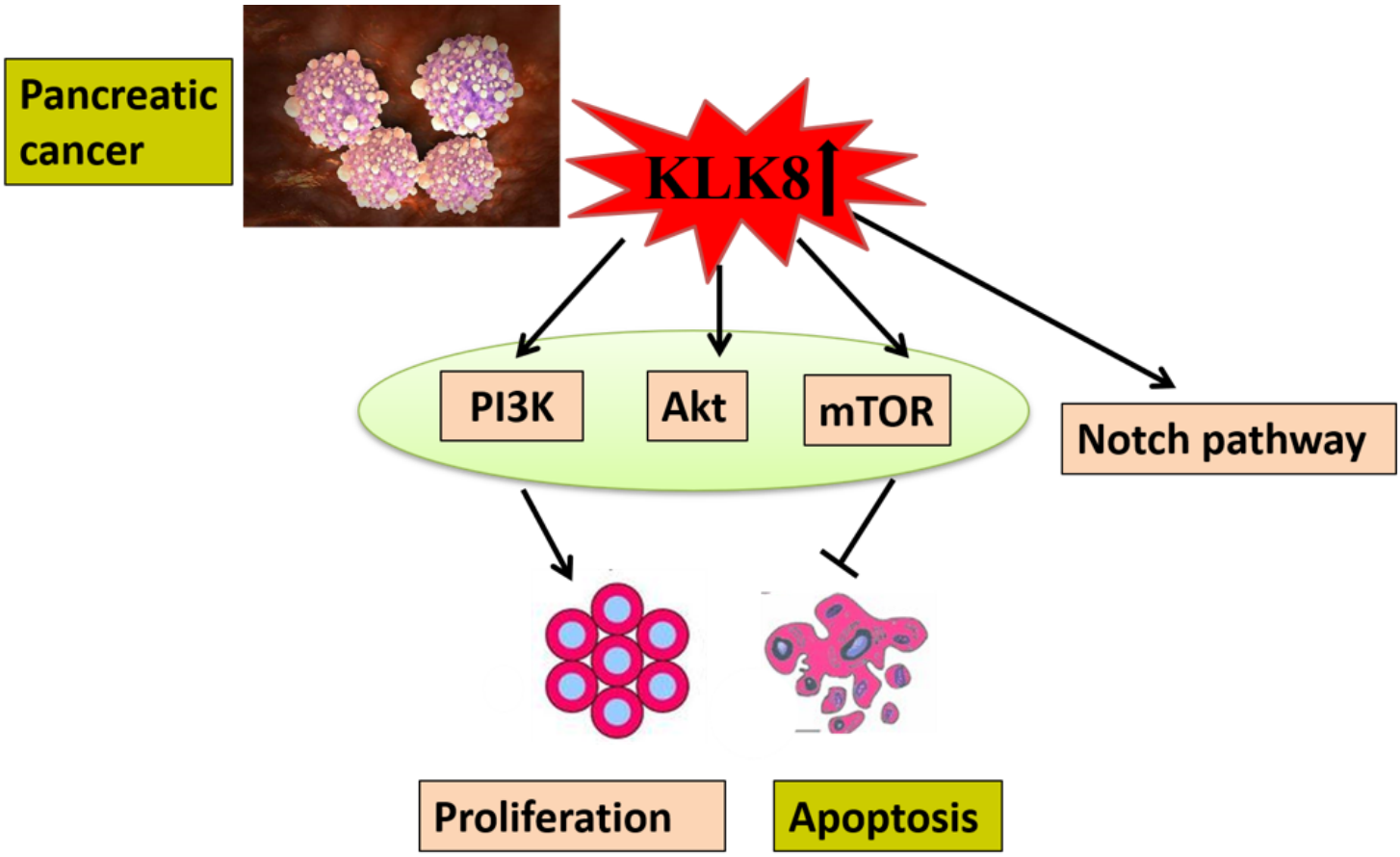

Figure 8

KLK8 was up-regulated in oancreatic cancer tissues compared to para-concerous pancreas. Upregulated KLK8 in pancreatic cancer predict poor prognosis and may be a potential therapeutic target for PDAC. KLK8 was positively associated with PI3K-Akt-mTOR and Notch Pathway. KLK8-induced pro-proliferation and anti-apoptotic effects in mia-paca-2 and Panc-1 cell were attenuated via PI3K-Akt-mTOR but not Notch pathways.