LncRNA KCNK15-AS1 inhibits pancreatic cancer progression by promoting the expression of its binding protein ACTR3B

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Research

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Abstract

Background

LncRNAs are reported to play an essential role in multiple tumors, including pancreatic cancer. LncRNAs could impact tumor growth via RNA-binding proteins, working as a coactivators of transcription factors or impacting their gene expression via posttranscriptional regulation. Our study aimed to elucidate the function and mechanism of lncRNA KCNK15-AS1 and its binding protein ACTR3B in PC progression. Our previous data indicated that KCNK15-AS1 is downregulated in PC tissues and cell lines compared to normal controls.

Methods

In this study, we overexpressed KCNK15-AS1 and ACTR3B in both BxPC-3 and Mia-PaCa-2 cells to detect the cellular phenotype in vitro and in vivo. RNA pulldown assays, mass spectrometry assays and RNA-binding protein immunoprecipitation assays were used to verify KCNK15-AS1 RNA binding protein ACTR3B. Luciferase reporter assay and ubiquitination assay were proceeded to detect the mechanism KCNK15-AS1 upregulated ACTR3B expression.

Results

Our results showed that overexpression of KCNK15-AS1 significantly inhibited the proliferation, colony formation and migration of PC cells. ACTR3B was screened by RNA pulldown and mass spectrometry assays. RNA-binding protein immunoprecipitation assays confirmed that KCNK15-AS1 physically bound to ACTR3B. Furthermore, mechanistic analyses demonstrated that KCNK15-AS1 promoted ACTR3B expression by inhibiting ACTR3B ubiquitin-mediated degradation and enhancing its promoter activity. Additionally, ACTR3B presented low expression in PC tissues and cell lines, and PC cell growth was significantly repressed when ACTR3B was overexpressed. Moreover, knockdown of ACTR3B in KCNK15-AS1-overexpressing cells reversed the effects of KCNK15-AS1 on PC cell growth via the cyclin D1/CDK4 axis.

Conclusion

Briefly, our study indicated that the lncRNA KCNK15-AS1/ACTR3B/cyclin D1/CDK4 axis may inhibit PC progression, which provides a potential therapeutic target for PC.

Background

Pancreatic cancer (PC) has been reported to be the seventh leading cause of cancer-related death worldwide[1]. It is often diagnosed at a late stage because of the lack of a functional diagnostic

approach. Although progress has been made in the treatment modalities for PC, including surgical resection, chemotherapy, immunotherapy, etc., its 5-year survival rate, which is lower than 9%, has not obviously improved[2, 3]. Therefore, more detailed research on the molecular mechanisms of PC is urgently needed.

Long noncoding RNAs (lncRNAs), which comprise a heterogeneous family of RNA molecules longer than 200 nucleotides with no or limited protein-coding capacity, have been observed to be aberrantly expressed in various types of cancers[4]. Multiple lncRNAs are involved in a variety of biological processes, including epigenetic control, nuclear import, cell cycle regulation, cellular differentiation, alternative splicing, RNA decay and RNA-binding protein modulation[5, 6]. Our previous study showed that lncRNA KCNK15-AS1 expression is decreased in PC and is regulated by the m6A methylation eraser ALKBH5[7]. This research aimed to elucidate the function of KCNK15-AS1 and its potential mechanism in PC.

Actin-related protein 3B (ACTR3B) is a protein-coding gene that is involved in the organization of the actin cytoskeleton and may decrease the metastatic potential of tumors[8]. Based on recent research, ACTR3B can be targeted by lncRNA AC009022.1 and miR-497-5p in lung cancer cells, which plays an important role in the tumorigenesis of lung cancer[9]. In our study, we first revealed that ACTR3B can inhibit cell proliferation, migration and invasion in PC. In addition, ACTR3B, a KCNK15-AS1 binding protein, is upregulated in KCNK15-AS1-overexpressing cells compared to control cells. Together, these data provide a deeper understanding of the exact functions of KCNK15-AS1 and ACTR3B and could provide new insights into the related clinical difficulties of PC pathogenesis.

Methods

Clinical samples

Fresh frozen PC tissues and their adjacent normal tissues were obtained from the Pancreas Biobank of the First Affiliated Hospital of Nanjing Medical University, which passed ISO9001:2015 certification in 2018. The samples were taken within 10 mins after tumor excision and immediately stored at −80°C until application in the experiments. Written informed consent was acquired from all patients, and this study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University.

Cell culture

Human PC cell lines (Mia-PaCa-2, BxPC-3 and ASPC-1) and human embryonic kidney cells (HEK-293T cells) were purchased from American Type Culture Collection (ATCC, Manassas, USA), and the normal human pancreatic duct epithelial cell line (HPNE) was obtained from Pancreas Institute, Nanjing Medical University. All cells were incubated at 37°C in humidified air with 5% CO_2 and maintained in Roswell Park Memorial Institute 1640 (RPMI1640, Gibco, CA, USA) or Dulbecco's modified Eagle medium (DMEM; Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin, with or without 2.5% horse serum (Gibco, CA, USA).

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells and tissues using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The NanoDrop ND2000 (Thermo Scientific Inc., USA) was used to determine the purity and quantify the concentration of RNA. The First Strand cDNA Synthesis Kit (TakaRa, China) was used to perform reverse transcription. Primers used for real-time quantitative polymerase chain reaction (qRT-PCR) were obtained from TsingKe (Nanjing, China). According to the manufacturer's instructions, qRT-PCR was performed using the SYBR Prime-Script RT-PCR kit (Roche, Germany) in an ABI LightCycler 96 fluorescent quantitative PCR system (Thermo Scientific Inc., USA). The reaction started at 95°C for 5 mins, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. ACTB was used as the internal control to normalize qRT-PCR results. Relative gene expression levels were measured using cycle threshold (CT) in the ΔΔCT calculation. All oligonucleotide sequences are listed in Supplementary Table 1.

Construction of cell lines stably expressing KCNK15-AS1 and ACTR3B

For stable overexpression of KCNK15-AS1, full-length KCNK15-AS1 cDNA was synthesized by TsingKe (Nanjing, China) and cloned into the pCDH retroviral vector, named KCNK15-AS1-pCDH, which was confirmed by sequencing (TsingKe, Nanjing, China). The retroviral particle concentration and transfection steps were described in our previous research[7, 10, 11]. Positive clones were screened with puromycin for 2-3 weeks to establish KCNK15-AS1 stable expression cell lines and the corresponding negative control for further study. ACTR3B stable overexpression cells were established in the same way but using pHAGE vectors. For transient knockdown experiments, small interfering RNAs (siRNAs) were purchased from Ribobio Co. (Guangzhou, China).

Cellular phenotype in vitro

Cell proliferation, migration and invasion assay methods have been described in detail in our previous articles[7, 11]. For the colony formation assay, cells cultured to 90% confluence were harvested and seeded in 6-well plates at a dose of 200 cells per well. Fourteen days later, the cells were rinsed in phosphate-buffered saline (PBS) and stained with 0.05% crystal violet for colony counting.

Cell cycle experiment

The cell cycle was exposed by flow cytometry assay using a Cell Cycle Analysis Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. After 48 h of transfection, the cells were harvested with PBS and then fixed in 75% alcohol overnight at -20°C. The fixed cells were washed three times with PBS and then stained with propidium iodide (PI) dyeing buffer, which contained 200 μg/ml RNase and 50 μg/ml PI, at room temperature for 30 mins in the dark. The cell cycle was examined by flow cytometry (LSR, BD Biosciences).

Western blot assay

Protein extracts from cells or immunoprecipitation samples were prepared using detergent-containing lysis buffer. Total protein (60 μg) was subjected to SDS-PAGE and transferred to a 0.45-μm PVDF membrane (Merck Millipore, Germany). Antibodies were as follows: anti-ACTR3B, anti-SDHA, anti-IMMT (Abcam, USA), and anti-GAPDH (Santa Cruz, USA). The bands were visualized with Immobilon Western Chemiluminescent HRP substrate (Merck Millipore, Germany) using the Fluorchem E System (ProteinSimple, CA, USA).

Immunohistochemistry (IHC)

Paraffin-embedded tissue sections were heated at 65°C for 3 h, dewaxed in xylene for 30 mins followed by a graded ethanol series (100%, 95%, 85%, 75% to water) and then subjected to antigen unmasking by autoclaving in citric acid retrieval buffer; endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 20 mins, then the slides were washed three times with PBS, incubated in 5% bovine serum albumin for 60 mins, and incubated with ACTR3B (1:100) antibody overnight in a humidified box. The slides were washed three times with PBS, incubated with HRP-conjugated secondary antibody for 120 mins, washed in PBS for 5 mins, developed using 3,3'-diaminobenzidine (DAB) reagent, stained with hematoxylin, rinsed in tap water for 5 mins, dehydrated in an ascending alcohol series (75%, 85%, 95%, 100%) and mounted. Images were captured using a light microscope.

RNA pulldown-coupled mass spectrometry analysis

In vitro biotin-labeled RNAs (KCNK15-AS1, its antisense RNA as unrelated control RNAs) were transcribed with Biotin RNA Labeling Mix and DIG RNA Labeling Kit (SP6/T7) (Roche) and purified with the RNeasy Mini Kit (QIAGEN). In advance, we mixed BxPC-3 cell protein with magnetic beads (Pierce™, Invitrogen) to remove the beads binding protein. Biotinylated RNAs were incubated with the remaining protein, and then streptavidin magnetic beads were added. Pulled down proteins were run on SDS-PAGE gels, and then gels were stained by silver staining. All pulldown protein samples were sent for mass spectrometry analysis (Shanghai Applied Protein Technology Co., Ltd.).

RNA immunoprecipitation assay

The RNA immunoprecipitation (RIP) assay was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Merck Millipore, USA) following the manufacturer's protocol. Briefly, BxPC-3 and Mia-PaCa-2 cells at 80-90% confluency were lysed in RIP lysis buffer, and 100 μl of cell extract was incubated with RIP buffer containing magnetic beads conjugated to human anti-ACTR3B or negative control normal IgG. The samples were incubated with proteinase K to digest proteins, and then the immunoprecipitated RNA was isolated and subjected to qRT-PCR.

Luciferase reporter assay

HEK-293T cells were cultured in 48-well plates and cotransfected with 50 ng of ACTR3B promoter luciferase reporter vector or pGL3-Basic control vector and 500 ng of KCNK15-AS1 vector using

Lipofectamine 2000. Twenty-four hours after transfection, the luciferase activities were assayed using the Dual-Luciferase® Reporter Assay System (Promega, USA).

Tumorigenesis assay

The animal care and experimental protocols were approved by the Nanjing Medical University Experimental Animal Welfare Ethics Committee. Four-week-old male BALB/c nude mice were purchased from the Animal Center of Nanjing Medical University and maintained under pathogen-free conditions. Mice (six in each group) were injected subcutaneously with 0.2 ml of cell suspension containing 1×10^7 cells in the back flank. When a tumor was visible, it was measured every week, and its volume was calculated according to the formula volume = 0.5 × length × width^2.

Statistical analysis

The statistical analysis was performed by SPSS software (Version 22.0) and GraphPad Prism (version 5.0). The quantitative data are presented as the mean ± SD. The differences between the means of two samples were analyzed by Student's t-test. The correlations between KCNK15-AS1 expression and various clinicopathological or serological variables were analyzed by the Mann-Whitney U test. Survival distributions and overall survival rates were determined using the Kaplan-Meier method, and the significance of the differences between the survival rates was calculated by the log-rank test. All data are representative of at least three independent experiments, and a difference was considered statistically significant at*p < 0.05, **p < 0.01, or ***p < 0.001.

Results

KCNK15-AS1 expression is associated with the clinicopathologic characteristics and prognosis of PC patients

Our previous article examined the expression of KCNK15-AS1, which was decreased in PC tissues compared to adjacent normal tissues[7]. In this study, we continued to explore the role of KCNK15-AS1 in PC cells and the clinical significance of KCNK15-AS1 in PC. Our results indicated that low KCNK15-AS1 expression was significantly associated with tumor size, lymph node metastasis, tumor-node-metastasis (TNM) stage (Fig. 1A-C), overall survival (log-rank test, p<0.001, Fig. 1D) and disease-free survival (Fig. 1E, Gene Expression Profiling Interactive Analysis [GEPIA] online database, http://gepia.cancer-pku.cn/index.html). Moreover, the univariate analysis showed that high TNM stage, high N stage, and low expression of KCNK15-AS1 were markedly associated with a high risk of cancer-related death (Table 1). Multivariate analysis revealed that TNM stage was a key prognostic factor (p = 0.001, Table 1). More importantly, the low expression of KCNK15-AS1 was considered an independent prognostic factor related to poor prognosis in PC (p = 0.003, Table 1).

KCNK15-AS1 inhibits the growth of PC cells in vitro and in vivo

To investigate the effect of KCNK15-AS1 on the proliferation, colony formation, migration, invasion and cell cycle of PC cells, we thawed and verified retroviral stable KCNK15-AS1-overexpressing BxPC-3 and Mia-PaCa-2 cells by qRT-PCR (Supplementary Fig. 1). KCNK15-AS1 overexpression resulted in a significant decrease in the cell viability and colony forming ability of BxPC-3 and Mia-PaCa-2 cells, as determined by Cell Counting Kit-8 (CCK-8), colony formation and wound healing assays (Fig. 2A, B). The wound healing assay also indicated that KCNK15-AS1 inhibited Mia-PaCa-2 cell migration (Fig. 2C). Moreover, KCNK15-AS1 arrested BxPC-3 and Mia-PaCa-2 cells entering S phase, which means PC cells were unable to complete mitosis (Fig. 2D). These results reveal that KCNK15-AS1 is crucial for cell growth in vitro. To further explore the function of KCNK15-AS1 in tumor progression, we injected nude mice subcutaneously with BxPC-3 and Mia-PaCa-2 cells stably overexpressing KCNK15-AS1 or pCDH vector control (Fig. 3A). Consistent with the in vitro results, six weeks after injection, both the tumor size and the tumor weight were substantially reduced in the KCNK15-AS1 overexpression group compared with the pCDH control group (Fig. 3B). Moreover, we verified the expression level of KCNK15-AS1 in tumors from the stable overexpression groups using qRT-PCR (Fig. 3C). Taken together, our data demonstrate that KCNK15-AS1 plays an essential role in PC tumor growth both in vitro and in vivo.

KCNK15-AS1 upregulated ACTR3B expression at the mRNA and protein levels

Several reports have indicated that lncRNAs regulate gene transcription mainly by interacting with RNA-binding proteins. To identify proteins that interact with KCNK15-AS1, an RNA pulldown assay was conducted in BxPC-3 and Mia-PaCa-2 cells. RNA-associated proteins were harvested, and all samples were subjected to mass spectrometry analysis to identify KCNK15-AS1 binding proteins. Based on the differential protein report from mass spectrometry analysis (Supplementary Table 2), we selected ACTR3B, SDHA and IMMT via the cumulative protein score, peptides and peptide spectrum matches (PSMs). Among these proteins, ACTR3B was successfully validated in the RNA-bead eluate by western blotting, but SDHA and IMMT were found in the flow-through buffer. An obvious enrichment of ACTR3B was detected after pulldown assays were performed with KCNK15-AS1, but no enrichment was observed with the unrelated RNA as detected by the ACTR3B antibody (Fig. 4A). Conversely, we implemented anti-ACTR3B as a bait to carry out RIP assays with cell extracts from BxPC-3 and Mia-PaCa-2 cells. We observed marked enrichment of KCNK15-AS1 with the anti-ACTR3B versus a nonspecific antibody (IgG control) (Fig. 4B). A luciferase reporter gene assay demonstrated that compared with the ACTR3B-pCDH control group, the ACTR3B promoter transfected with the vector containing KCNK15-AS1 group had obviously higher relative luciferase activity (Fig. 4C). The ubiquitination of ACTR3B was suppressed in KCNK15-AS1-overexpressing BxPC-3 cells and significantly enhanced in KCNK15-AS1 knockdown Mia-PaCa-2 cells treated with the proteasome inhibitor MG132 (Fig. 4D). qRT-PCR and western blot assays demonstrated that ACTR3B was upregulated in KCNK15-AS1-overexpressing BxPC-3 and Mia-PaCa-2 cells (Fig. 4E). All these results indicated that KCNK15-AS1 upregulates ACTR3B expression by not only enhancing its promoter activity but also suppressing its ubiquitination. Additionally, correlation analysis revealed that ACTR3B mRNA levels positively correlated with KCNK15-AS1 levels in 45 PC tissues ($R = 0.782$, $P < 0.0001$, Supplementary Fig. 2).

ACTR3B plays an anticancer role in PC

To investigate the role of ACTR3B in PC cells, we measured ACTR3B expression in BxPC-3, Mia-PaCa-2, ASPC-1 and HPNE cells. The results showed that ACTR3B was expressed at lower levels in all three PC cell lines than in HPNE (Fig. 5A and B). The same results were observed in patient tumor samples by qRT-PCR and IHC (Fig. 5C and D). To examine whether ACTR3B can regulate cell growth in vitro, we overexpressed ACTR3B in BxPC-3 and Mia-PaCa-2 cells using the pHAGE vector (Fig. 6A). As shown in Fig. 6A, we overexpressed ACTR3B in BxPC-3 and Mia-PaCa-2 cells to investigate the influence of ACTR3B on tumor cells. The results indicated that ACTR3B plays a suppressive role in the migration, invasion, proliferation, colony formation and cell cycle progression of PC cells (Fig. 6B-F). Moreover, we subcutaneously injected ACTR3B-overexpressing cells into immunodeficient mice to explore whether ACTR3B can also inhibit tumor growth in vivo. Six weeks later, tumor size and weight were measured (Fig. 7A). We found that the tumors in the ACTR3B overexpression group were much smaller than those in the control pHAGE group, and ACTR3B expression remained higher (Fig. 7B-C). All these results revealed that ACTR3B can suppress PC growth both in vitro and in vivo.

The tumor suppressive function of KCNK15-AS1 is dependent on ACTR3B

The above results show that ACTR3B has an inhibitory effect on PC growth. As an RNA-binding protein, ACTR3B is upregulated by KCNK15-AS1. Hence, we assumed that the tumor suppressive function of KCNK15-AS1 is implemented through ACTR3B. To validate our hypothesis, stably KCNK15-AS1-expressing Mia-PaCa-2 and BxPC-3 cells and their corresponding pCDH control cells were transfected with si-ACTR3B or scramble control for 48 h, respectively. The Transwell assay showed that knockdown of ACTR3B attenuated the KCNK15-AS1 overexpression-mediated inhibition of cell migration and invasion ability (Fig. 8 A and B). In our CCK-8 assay, the ability of KCNK15-AS1 to inhibit tumor cell proliferation was significantly repressed when ACTR3B was knocked down (Fig. 8 C). Furthermore, colony formation was also markedly attenuated in the scramble group compared with the ACTR3B knockdown group (Fig. 8 D). Taken together, these data revealed that KCNK15-AS1 directly regulates the expression of ACTR3B, which in turn affects tumor cell growth. Additionally, we found that tumor cells were obviously arrested in G0/G1 phase after KCNK15-AS1 overexpression. We further detected whether KCNK15-AS1 impacted the PC cell cycle via the cyclin D1/CDK4 axis. Western blot assays were used to detect CDK4 and cyclin D1 expression. The results demonstrated that KCNK15-AS1 significantly inhibited CDK4 and cyclin D1 expression; however, this effect was rescued when we knocked down ACTR3B in Mia-PaCa-2 (Fig. 9 A) and BxPC-3 cells (Fig. 9 B). In general, we demonstrated that KCNK15-AS1 suppresses PC cell growth via the cyclin D1/CDK4 axis, which is dependent on its RNA binding protein ACTR3B.

Discussion

This study emphasized a novel lncRNA, KCNK15-AS1, that we identified in PC and explored its potential functions. It is well known that lncRNAs play important roles in modulating gene expression through various pathways and that their aberrant expression may induce uncontrolled tumor growth and cancer

progression[12–17]. Thus, increasing attention has been paid to the relationship between lncRNAs and malignant tumors. Differential expression of lncRNAs has been reported in several cancers, including colorectal cancer[18, 19], bladder cancer[20, 21], gastric cancer[22, 23] and liver cancer[24, 25]. Some of these lncRNAs have been identified as biomarkers or potential therapeutic targets[26–28]. Using ArrayStar lncRNA microarray assays, we found that KCNK15-AS1 is downregulated in PC tissues compared to adjacent tissues. KCNK15-AS1 is biologically active and acts by decreasing the proliferation, migration and invasion of PC cells in vitro. In a cohort of 94 PC patients, we determined that low expression of KCNK15-AS1 was associated with high T and N stages, which indicated that KCNK15-AS1 plays a suppressive role in PC. After analyzing clinical samples and conducting in vitro experiments, we performed in vivo experiments that confirmed that overexpression of KCNK15-AS1 obviously inhibits tumor growth.

Furthermore, we found that KCNK15-AS1 might suppress the proliferation, invasion and migration of PC by regulating ACTR3B. As reported, lncRNAs usually perform their functions by interacting with corresponding RNA-binding proteins, such as coactivators of transcription factors or gene promoters[29, 30]. ACTR3B has been identified to encode a member of the actin-related proteins in eukaryotic mRNA and plays an important role in regulating actin cytoskeleton, cell-shape change and motility[8]. In previous studies, ACTR3B expression was reported to correlate with lung and colorectal cancer[9]. However, the relationship between ACTR3B and PC has never been studied. In this study, we demonstrated that ACTR3B is physically bound by KCNK15-AS1 and is positively associated with the expression of KCNK15-AS1. In addition, our results indicate that KCNK15-AS1 can upregulate ACTR3B expression by enhancing its promoter activity and inhibiting its ubiquitin-mediated degradation.

Additionally, we found that ACTR3B markedly decreased the proliferation, invasion and migration of PC cells in vitro and in vivo. We further carried out a phenotypic rescue experiment to verify whether the function of KCNK15-AS1 in PC was dependent on ACTR3B. Moreover, cell cycle data showed that KCNK15-AS1 arrested PC cells in G0/G1 phase. The mammalian cell cycle is driven by complex interactions between cyclins and their associated cyclin-dependent kinases (CDKs), and the dysregulation of these interactions is a vital hallmark of cancers. Several studies indicate that dysregulation of the cyclin D1/CDK4 axis has an essential role in rapid cancer proliferation, and some tumors overexpress cyclin D1[31]. In our study, we found that the expression of cyclin D1 and CDK4 was suppressed by KCNK15-AS1. However, when we knocked down ACTR3B, this phenomenon was partially reversed. These results suggest that knocking down ACTR3B can moderately reverse the effects of KCNK15-AS1 in vitro.

Nevertheless, some questions remain to be explained in our future studies. We found that KCNK15-AS1 increased the expression of ACTR3B by impacting the promoter region and enhancing its activation. However, the details, such as which field of KCNK15-AS1 affects the promoter of ACTR3B, still need further research. We found that KCNK15-AS1 directly binds to ACTR3B and inhibits its ubiquitin and proposed that this interaction may prevent ubiquitin activator E1, ubiquitin binding protein E2, or ubiquitin ligase E3 from binding with ACTR3B. However, more evidence is needed on this topic. Next-generation sequencing technology was also used to research the changes in the transcriptome of BxPC-3 and Mia-

PaCa-2 cells after ACTR3B overexpression. These results showed that over 100 genes were significantly upregulated or downregulated in these ACTR3B overexpressed cell lines (Supplementary Fig. 3), and most of these genes were involved in metabolism pathways, which provided us with some clues to further study the mechanism of ACTR3B in the future (Supplementary Fig. 4).

Conclusions

Our studies aimed to explore the regulatory role and potential molecular mechanisms of lncRNA KCNK15-AS1 in the progression of PC (Fig. 10). The results indicated that KCNK15-AS1 and ACTR3B are novel tumor suppressors in PC. Based on the Luciferase reporter assay and ubiquitination assay, KCNK15-AS1 facilitated ACTR3B expression by enhancing ACTR3B promotor region and suppressing its ubiquitin-mediated degradation. Furthermore, KCNK15-AS1 inhibited tumor cell cycle progress by cyclin D1/CDK4 axis, which could be rescued via knocking down ACTR3B. In general, we summarized that the KCNK15-AS1/ACTR3B/cyclin D1/CDK4 axis might offer a potential target for developing an anti-PC therapeutic strategy.

Abbreviations

CCK-8: Cell Counting Kit-8; HPNE: Human pancreatic duct epithelial cell line; IHC: Immunohistochemistry; lncRNAs: Long noncoding RNAs; ACTR3B:

Actin-related protein 3B; PC: Pancreatic cancer; qRT-PCR: Quantitative real-time PCR; TNM: Tumor-node-metastasis.

Declarations

Availability of data and material

All data generated or analyzed during this research are included in this published article and supplementary data.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University and all patients provided written informed consent. All animal experiments were performed in accordance with animal protocols approved by the Nanjing Medical University. Our study was completed based on the Declaration of Helsinki.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Pengfei Wu, Hao Yuan and Xiangya Ding authors contributed equally to this work. Kuirong Jiang and Zipeng Lu conceived the project, planned the experiments, and analyzed the data with support from WPF and YH.

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Tables

Table 1. Univariate and multivariate analysis of overall survival in PC patients (n = 92)

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-Value	HR	95% CI	p-Value
Gender	1.382	0.616-3.100	0.433			
Age	0.618	0.335-1.140	0.123			
TNM stage			0.002**			
II vs I	2.117	1.120-4.001	0.024*	1.7611	0.9121-3.4005	0.092
III vs I	4.342	1.187-15.880	< 0.001***	4.7418	1.8384-12.2306	0.001**
T stage			0.472			
T2 vs T1	1.737	0.745-4.051	0.201			
T3 vs T1	1.324	0.387-4.525	0.655			
Lymph node metastasis			0.033*			
N1 vs N0	1.775	0.934 to 3.371	0.069			
N2 vs N0	3.237	0.8183 to 12.800	0.008**			
KCNK15-AS1 expression						
High vs Low	0.345	0.192 to 0.621	< 0.001***	0.3956	0.2141-0.7312	0.003**

Abbreviations: HR Hazard ratio; 95% CI = 95% confidence interval
Cox regression analysis, *P < 0.05, **P < 0.01, ***P < 0.001

Figures

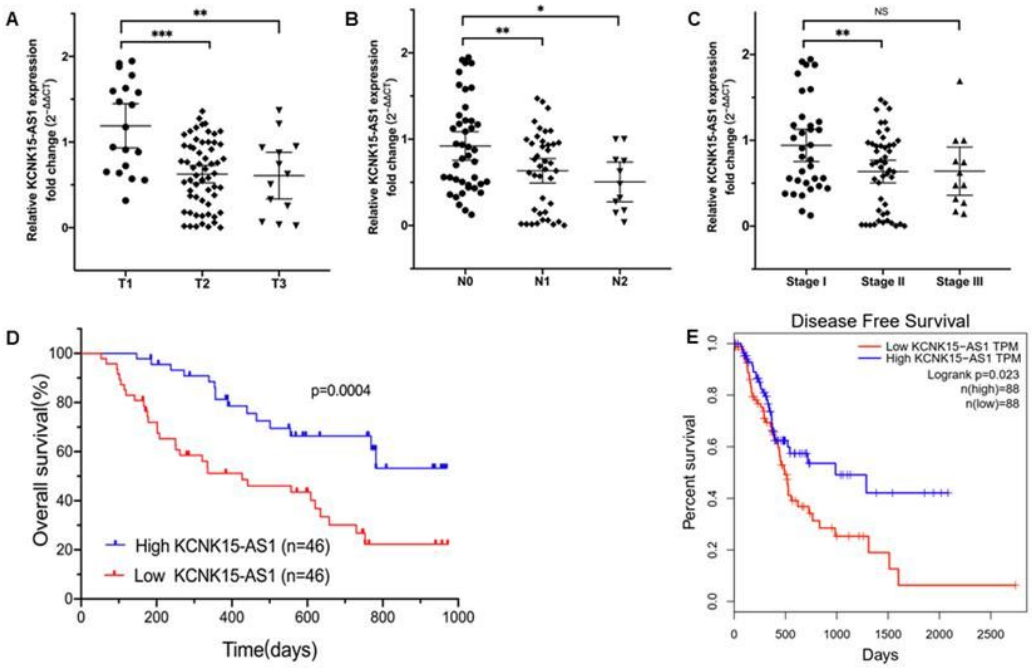

Figure 1

Associations between KCNK15-AS1, clinicopathologic characteristics and prognosis after surgery. A: KCNK15-AS1 expression in different TNM stages of PC: T1, T2, and T3 (n = 19, n = 62 and n = 13, respectively). B: KCNK15-AS1 expression in the lymph node metastasis N0 group (n = 42), N1 group (n = 41) and N2 group (n = 11). C: KCNK15-AS1 expression in different TNM stages of PC: stage I, II, and III (n = 34, n = 48 and n = 12, respectively). The qRT-PCR data are shown as the fold change (2−ΔΔCT). When assessing expression in tissues, the levels were first normalized to those of ACTB as ΔCT and then compared with one of the tissues and converted to the fold change (2−ΔΔCT). D: Overall survival curve of the high-level and low-level groups according to KCNK15-AS1 expression. E: The relationship between KCNK15-AS1 and disease-free survival of PC was also analyzed by the GEPIA online database. The P-values are shown with the log-rank test (two-sided). NS (not significant). *P < 0.05, **P < 0.01, ***P < 0.001.

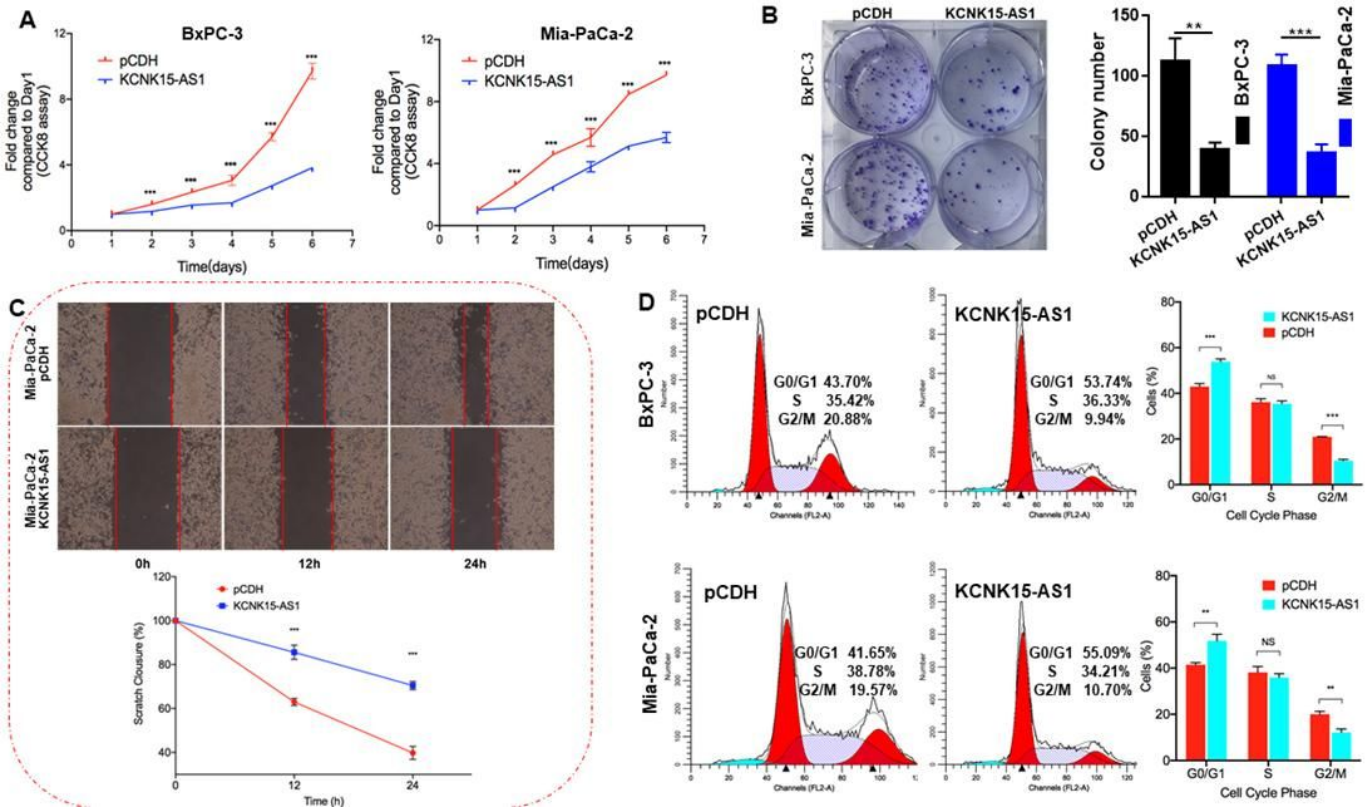

Figure 2

Influence of KCNK15-AS1 on PC cell proliferation, colony formation, migration and cell cycle progression. A: The proliferative activity of the two cell lines detected by CCK-8 assay. B: The colony formation ability of the two cell lines was significantly different from that of the control cells. C: Analysis of the wound healing assay in the two cell lines and corresponding control cells. D: Cell cycle data are shown by flow cytometry assay. *P < 0.05, **P < 0.01, ***P < 0.001.

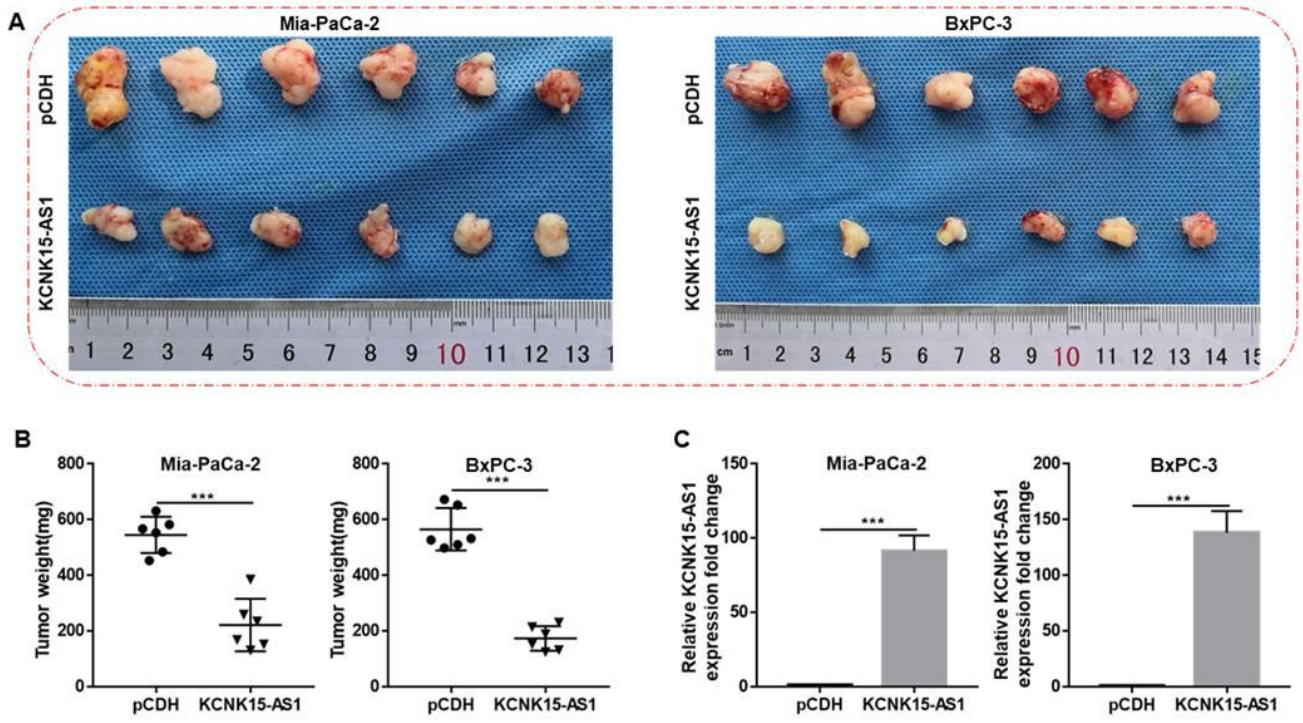

Figure 3

Stable overexpression of KCNK15-AS1 obviously reduces tumor growth in vivo. A: Tumors removed from the mice 6 weeks after injection of Mia-PaCa-2 or BxPC-3 cells stably transfected with KCNK15-AS1-pCDH or pCDH, respectively. B: Tumor weights are shown as the mean ± SD when the tumors were harvested. C: The expression of KCNK15-AS1 in paired tumor tissues was analyzed by qRT-PCR. ***P < 0.001.

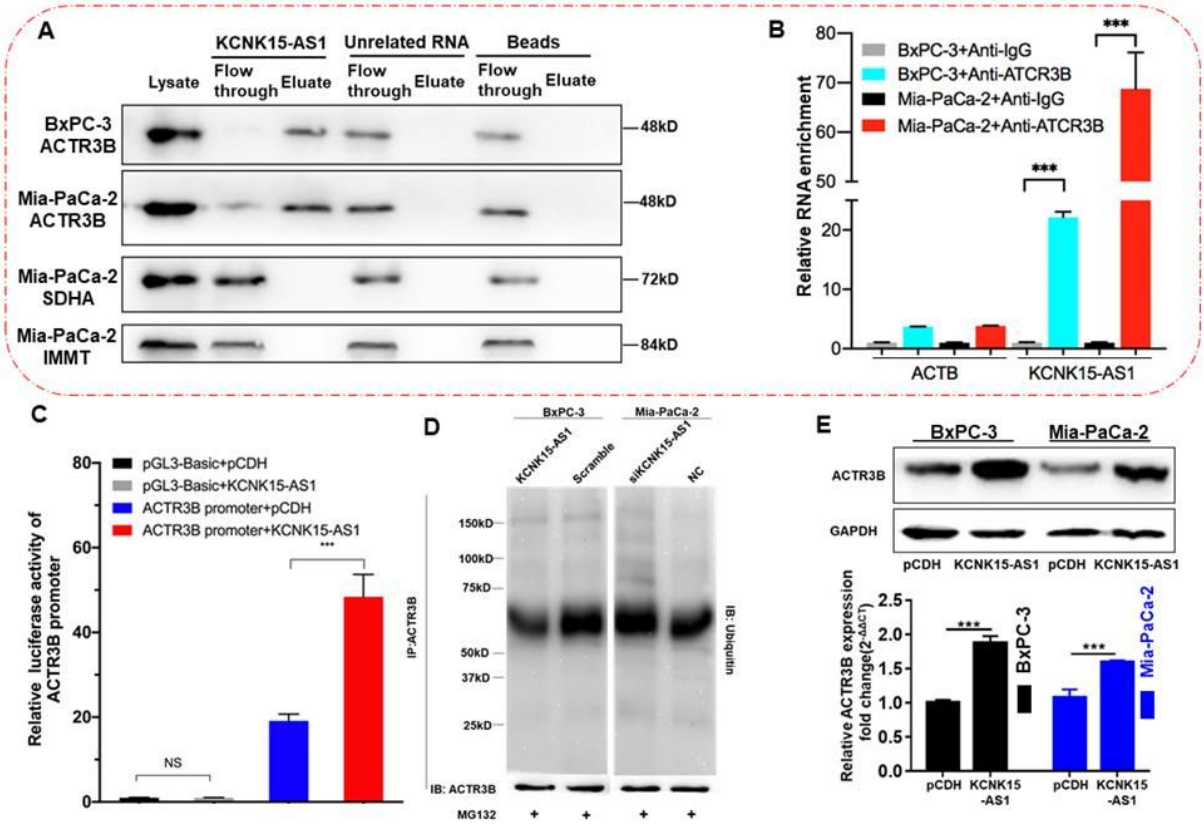

Figure 4

KCNK15-AS1 interacts with ACTR3B and upregulates its expression. A: RNA pulldown assays were performed with BxPC-3 cells using the full-length KCNK15-AS1 transcript (KCNK15-AS1 antisense transcript as unrelated RNA). The harvested samples were identified by mass spectrometry. ACTR3B was detected in the eluate lane, which was from KCNK15-AS1 binding proteins. B: In turn, compared with the corresponding IgG control, anti-ACTR3B enriched lncRNA KCNK15-AS1. C: KCNK15-AS1 acts on the ACTR3B promoter region. D: Cell lysates from KCNK15-AS1-overexpressing BxPC-3 cells and KCNK15-AS1-knockdown Mia-PaCa-2 cells were immunoprecipitated (IP) with ACTR3B antibody and then immunoblotted for ubiquitin and ACTR3B. Tumor cells were treated with the proteasome inhibitor MG132. E: KCNK15-AS1 can upregulate ACTR3B at the mRNA and protein levels. ***P < 0.001

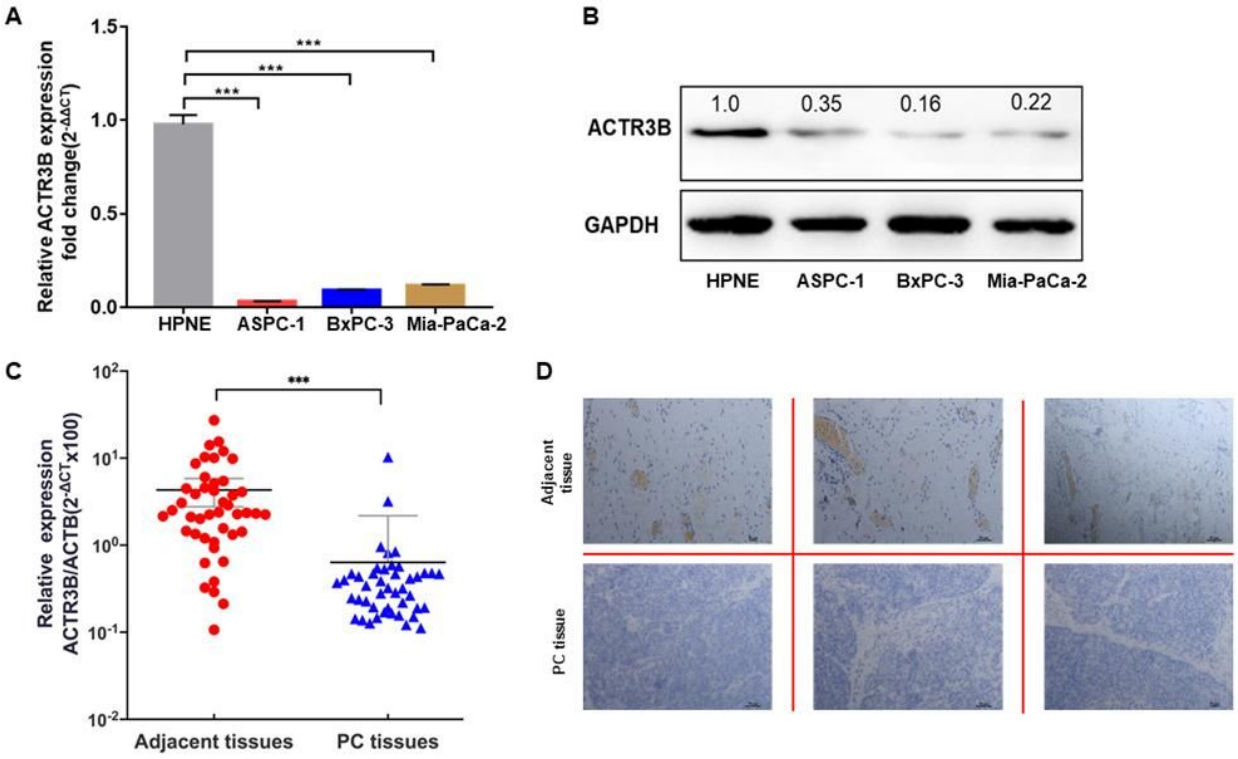

Figure 5

Expression of ACTR3B in PC cell lines and tissues. A-B: Relative ACTR3B expression level in cell lines by qRT-PCR and western blotting. C: Relative ACTR3B expression in tissues (n = 96). PC tissues versus corresponding adjacent tissues. D: Representative images (×200) IHC staining for ACTR3B in paraffin-embedded PC and corresponding adjacent tissues. ***P < 0.001.

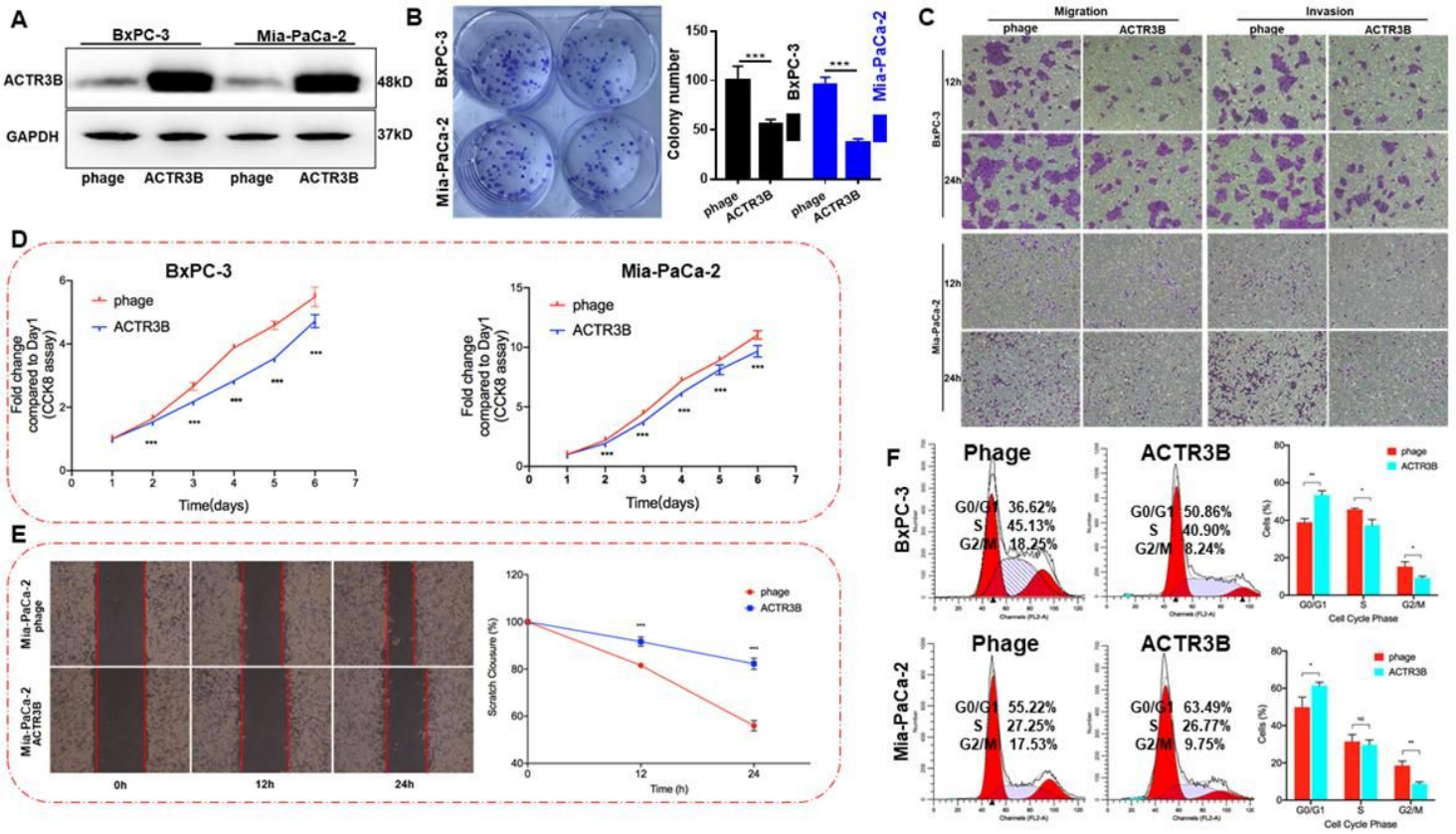

Figure 6

Influence of ACTR3B on PC cell progression. A: Stable overexpression of ACTR3B was established in BxPC-3 and Mia-PaCa-2 cells. B-E: Overexpressed ACTR3B suppressed tumor cell progression in terms of colony formation, migration, proliferation and invasion. F: The tumor cell cycle was also influenced by high ACTR3B levels. *P < 0.05, **P < 0.01, ***P < 0.001.

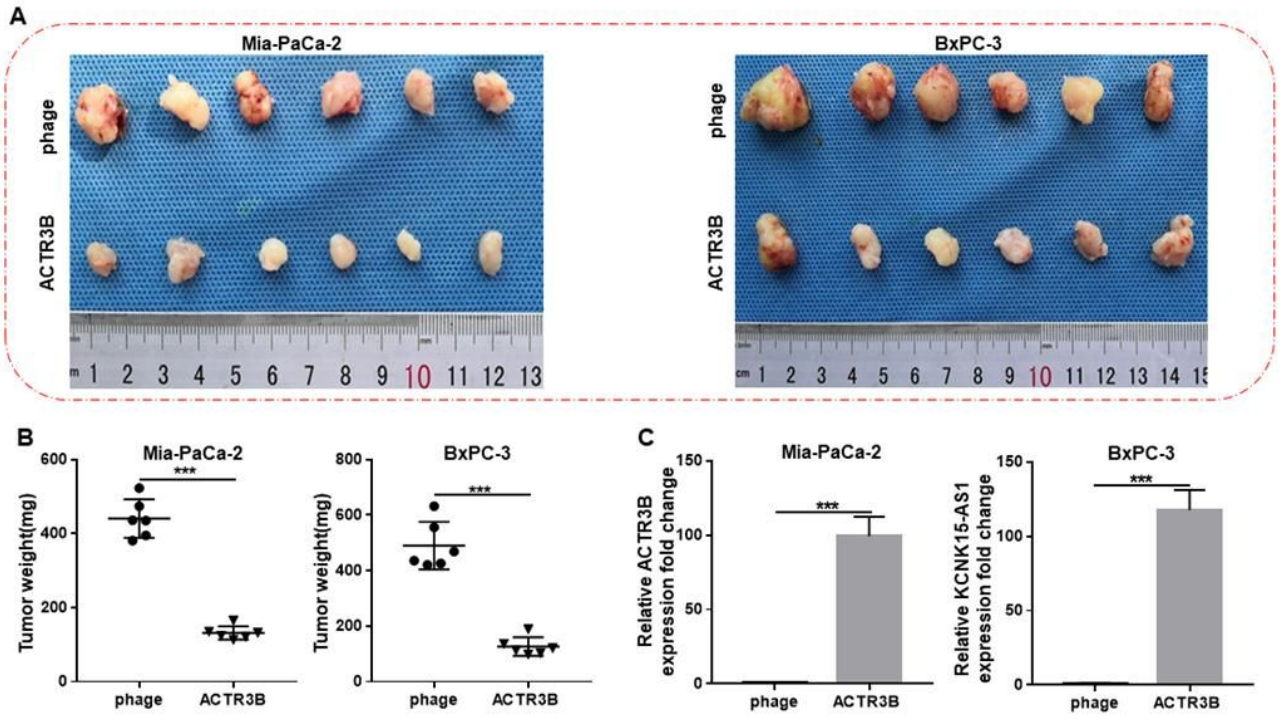

Figure 7

Stable overexpression of ACTR3B obviously reduces tumor growth in vivo. A: We injected ACTR3B-overexpressing Mia-PaCa-2 or BxPC-3 cells and corresponding control cells into immunodeficient mice and harvested the tumor tissue after six weeks. B: Tumor weights are shown as the mean ± SD when the tumors were harvested. C: The expression of ACTR3B in paired tumors. ***P < 0.001.

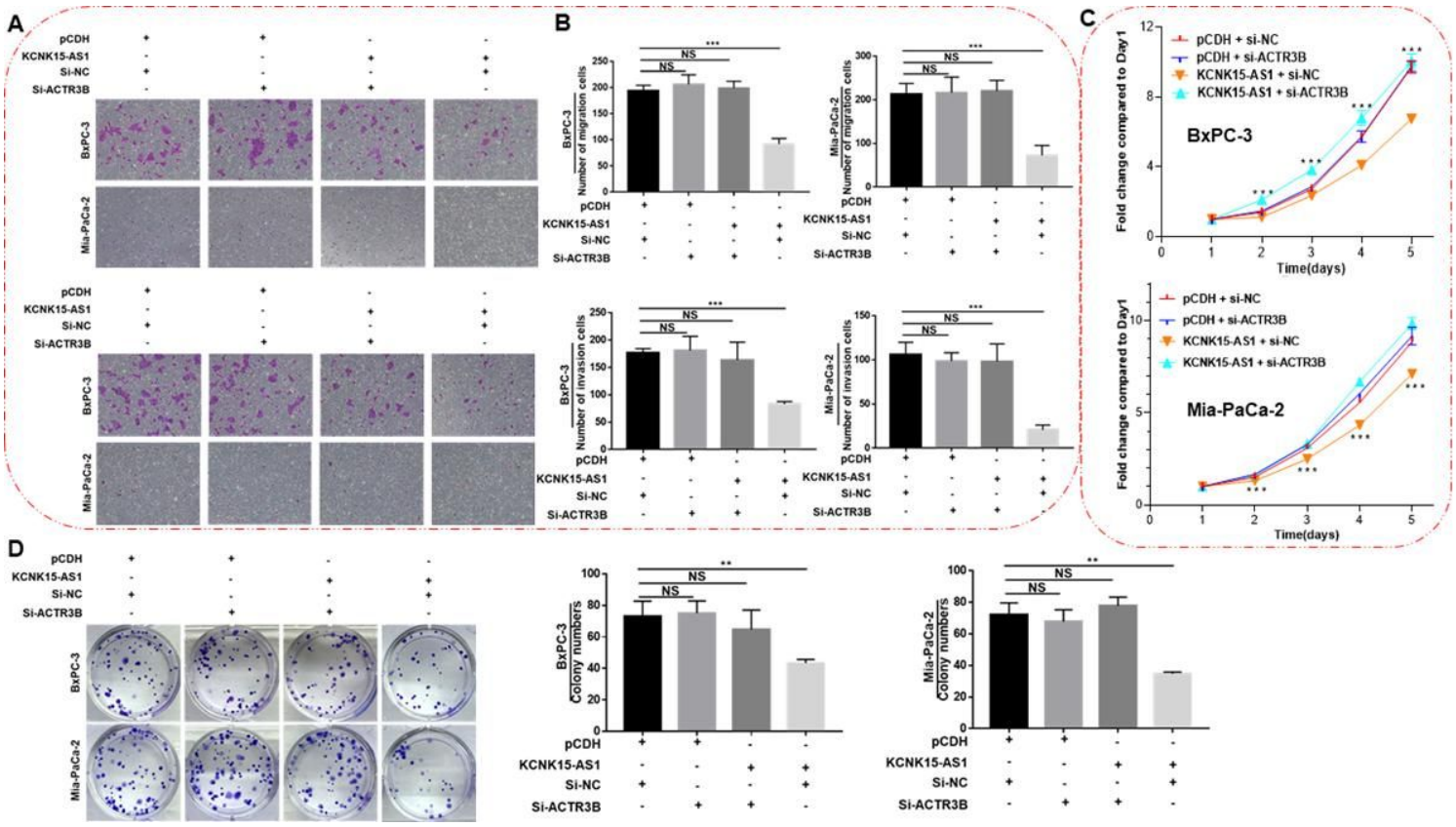

Figure 8

KCNK15-AS1-mediated inhibition of PC cells is reversed by ACTR3B knockdown. A-B: The invasion and migration of KCNK15-AS1-overexpressing cells transfected with si-ACTR3B compared with that in controls by Transwell assays. Values represent the mean ± SD from three independent experiments. C: Cell proliferation ability as shown by CCK-8 assay in KCNK15-AS1-overexpressing or ACTR3B knockdown cells. D: Colony formation assay indicated the influence of ACTR3B on KCNK15-AS1-mediated cell colony formation. *P < 0.05, **P < 0.01, ***P < 0.001

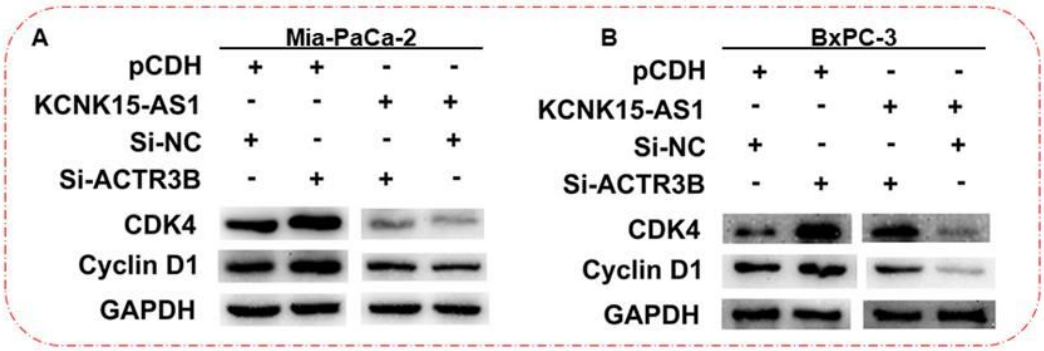

Figure 9

KCNK15-AS1 suppresses CDK4 and cyclin D1 expression, which is reversed by ACTR3B knockdown in Mia-PaCa-2 cells (A)and BxPC-3 cells (B).

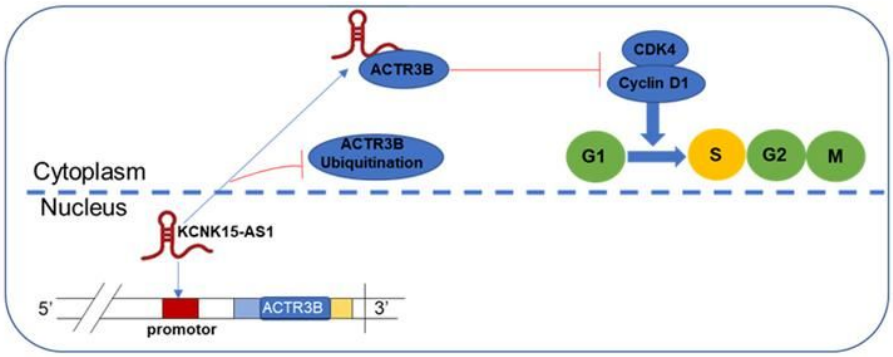

Figure 10

KCNK15-AS1 suppresses CDK4 and cyclin D1 expression, which is reversed by ACTR3B knockdown in Mia-PaCa-2 cells (A)and BxPC-3 cells (B).

Supplementary Files

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