The long non-coding RNA DKFZp434J0226 regulates the alternative splicing process through phosphorylation of SF3B6 in PDAC

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Abstract

Background: Long noncoding RNAs (lncRNAs), a type of pervasive genes that regulates various biological processes, are differentially expressed in different types of malignant tumors. The role of lncRNAs in the carcinogenesis of pancreatic ductal adenocarcinoma (PDAC) remains unclear. Here, we investigated the role of the lncRNA DKFZp434J0226 in PDAC.

Methods: Aberrantly expressed mRNAs and lncRNAs among six PDAC and paired non-tumorous tissues were profiled using microarray analysis. Quantitative real-time polymerase chain reaction was used to evaluate DKFZp434J0226 expression in PDAC tissues. CCK-8 assay, wound-healing assay, soft agar colony formation assay, and transwell assay were performed to assess the invasiveness and proliferation of PDAC cells. Furthermore, RNA pull-down, immunofluorescence, RNA immunoprecipitation, and western blotting assays were performed to investigate the association between DKFZp434J0226 and SF3B6. Tumor xenografts in mice were used to test for tumor formation in vivo.

Results: In our study, 222 mRNAs and 128 lncRNAs were aberrantly expressed (≥ twofold change). Of these, 66 mRNAs and 53 lncRNAs were upregulated, while 75 lncRNAs and 156 mRNAs were downregulated. KEGG pathway analysis and the Gene ontology category indicated that these genes were associated with the regulation of mRNA alternative splicing and metabolic balance. Clinical analyses revealed that overexpression of DKFZp434J0226 was associated with worse tumor grading, frequent perineural invasion, advanced tumor-node-metastasis stage, and decreased overall survival and time to progression. Functional assays demonstrated that DKFZp434J0226 promoted PDAC cell migration, invasion, and growth in vitro and accelerated tumor proliferation in vivo. Mechanistically, DKFZp434J0226 interacted with the splicing factor SF3B6 and promoted its phosphorylation, which further regulated the alternative splicing of pre-mRNA.

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Introduction
Pancreatic cancer is considered a rare type of cancer, with an estimated 57,600 newly diagnosed patients in 2020 in the United States according to the American Cancer Society, accounting for 3.2% (57,600 of 18,06,590) of all cancer cases (Siegel et al. 2020). Although the incidence rate of most cancers has been decreasing recently, the incidence and mortality rates of pancreatic cancer are gradually increasing (Simard et al. 2014). As the most common pancreatic malignant tumor, pancreatic ductal adenocarcinoma (PDAC) accounts for more than 85% of pancreatic cancer cases (Li et al. 2004). After diagnosis, only approximately 35% of the patients survive for > 5 years (Li et al. 2004). Surgical resection is the only available treatment for PDAC. However, only less than 20% of tumors can be surgically removed at diagnosis. In addition, these patients usually respond poorly to chemotherapy. Thus, it is necessary to determine the molecular mechanisms underlying PDAC development.

A recent study has reported that epigenetic alterations influence the regulation of gene function in pancreatic cancers (Omura et al. 2009). Non-coding RNAs (ncRNAs), DNA methylation, and histone modifications are the main causes of epigenetic dysregulation. Most mammalian transcriptome is composed of abundant ncRNAs (Ponting et al. 2009), which can be generally divided into long ncRNAs (lncRNAs; 200 nt to > 100 kb) and small (18–200 nt). Furthermore, lncRNAs have complex biological functions in multiple processes (Wang et al. 2011). Increasing evidence has shown that lncRNAs have a vital effect on oncogenesis in human cancers (Ponting et al. 2009). Aberrant expression of lncRNAs in different types of human malignant tumors has been widely documented (Wapinski et al. 2011; Gibb et al. 2011), promoting a common interest in the use of therapeutic targets and biomarkers such as MALAT-1 in non-small cell lung carcinoma, HOTAIR in breast cancer, and HEIH in hepatocarcinoma (Schmidt et al. 2011; Gupta et al. 2010; Yang et al. 2011).

Studies have reported that ncRNAs may regulate the process of precursor mRNA (pre-mRNA) splicing. Hu et al. (2016) discovered that ncRNAs can regulate gene expression through h5S-OT lncRNA during transcription and pre-mRNA splicing. They identified the splicing regulator U2AF65 as a cofactor of the h5S-OT-dependent alternative splicing pathway. U2AF65 is a core splicing regulator required for the binding of U2 snRNP to the pre-mRNA branch site and is essential for splicing machinery and intron excision. In addition, Tripathi et al. (2010) reported that the lncRNA MALAT1 can influence the distribution of splicing factors in nuclear speckles. In addition, they reported that depletion of MALAT1 can change the splicing pattern of similar endogenous pre-mRNAs by regulating the phosphorylated forms of splicing factors (Tripathi et al. 2010), indicating that phosphorylation of splicing factors may be an essential process in the activation of the spliceosome.

Alternative splicing of pre-mRNAs contributes to the diversification of human transcripts and gene functions. Changes in the phosphorylation of splicing factors can regulate alternative splicing patterns in pre-mRNAs. The regulation of alternative splicing patterns is generally considered to be associated with tumor progression, metastatic dissemination, and survival in patients with PDAC. However, the molecular mechanism underlying the activation and regulation of splicing factors in PDAC remains unclear.

In the present study, we detected lncRNA expression patterns in six pairs of PDAC tumor tissue samples and matched non-tumor tissue samples by microarray and in 109 pairs of PDAC tumor samples and non-tumor tissue samples by quantitative real-time polymerase chain reaction (RT-qPCR). We identified DKFZp434J0226 as a potential therapeutic target for PDAC. In addition, DKFZp434J0226 was found to contribute to the phosphorylation of the splicing factor SF3B6, which further regulates the alternative splicing of pre-mRNA.

Materials and methods
Ethics statement
Ethical approval for human subjects was obtained from the research ethics committee of Zhongshan Hospital (Y2015-057), and written informed consent was obtained from each patient.

Patient samples and cell lines
Six paired tumor samples and non-tumor samples from patients with PDAC were randomly obtained from six patients who underwent surgical resection at Zhongshan Hospital, Fudan University, in 2016. Paired PDAC tumor samples and adjacent liver tissue samples were collected from 61 patients (Zhongshan Hospital, 2015–2019) for RT-qPCR analysis. For the clinical significance study, PDAC tissues were collected from 109 patients.
The pancreatic cancer cell lines AsPC-1, SW1990, MIAPaCa-2, CFPAC-1, Capan-1, and PANC-1 were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, CA, USA) supplemented with 10 U/mL penicillin G (Gibco, MA, USA), 10 U/mL streptomycin, and 10% fetal calf serum (Gibco, MA, USA). The cells were incubated at 37 °C with 5% CO₂.

**RNA extraction**

Total RNA was extracted from pancreatic cancer cells, snap-frozen PDAC samples, and matched normal non-tumor tissue samples using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA quality and quantification assurance were assessed by NanoDrop ND-1000, gDNA contamination and RNA integrity test were assessed by denaturing agarose gel electrophoresis.

**Microarray and computational analysis**

Samples (six PDAC tissues and six matched non-tumor tissues) were used to synthesize double-stranded cDNA, which was then labeled and hybridized to the Human LncRNA Array v2.0 (8 × 60 K, Arraystar, MD, USA). A total of 30,215 coding transcripts and 33,045 lncRNAs were collected from databases such as the Ensembl, UCSC Knowngenes, and RefSeq. After hybridization and washing, the processed array slides were scanned and analyzed using Agilent Scanner G2505C and Agilent Feature Extraction software (version 10.7.3.1), respectively. Subsequent data processing and quantile normalization were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies, CA, USA).

LncRNAs and mRNAs that were flagged in all six samples as Present or Marginal (“All Targets Value”) were chosen for further data analysis after quantile normalization of the raw data. Differentially expressed mRNAs and lncRNAs with statistical significance within the two groups were identified by volcano plot filtering. Gene ontology (GO) analysis and pathway analysis were performed to explore the roles of aberrantly expressed mRNAs in GO terms or biological pathways. The expression patterns of lncRNA and mRNA samples were examined by hierarchical clustering.

**Construction of the coding–non-coding gene co-expression network**

The coding–non-coding gene co-expression (CNC) network construction procedures included the following: (i) preprocessing data: taking the median value of the same coding gene with different transcripts to represent gene expression values without special treatment of lncRNA expression value, (ii) screen data: removing the subset of data according to the lists that show the differential expression of lncRNA and mRNA, (iii) calculating the Pearson correlation coefficient and using the R value to calculate the correlation coefficient of PCC between lncRNA coding genes, and (iv) screening by the Pearson correlation coefficient and selecting the part for which PCC ≥ 0.90 is considered meaningful and draw the CNC network using Cytoscape.

**RT-qPCR**

LncRNA and mRNA expression in PDAC tissues and cells was examined by RT-qPCR using SYBR Premix Ex Taq (Takara, Kusatsu, Japan) on an Eppendorf instrument. Additional file 1: Table S1 shows the primers used in this study. All experiments were performed in triplicates. All samples were normalized to GAPDH. Significance was examined by obtaining the average of the GAPDH-normalized 2−ΔΔCt values.

**LncRNA in situ hybridization**

A biotin-labeled antisense DKFZp434J0226 probe (ATG TCTAGAAGACTGTT) was synthesized using EXIQON. The paraffin-embedded tissues were treated with a peroxidase-quenching solution and incubated with a biotin-labeled probe. Then, streptavidin–horseradish peroxidase was reacted with the bound biotin-labeled probe. A TSA amplification kit (Perkin Elmer, Waltham, USA) was used to amplify the signal.

**RNA pull-down**

We performed an RNA pull-down assay as previously described (Zhang et al. 2017). Briefly, biotin-labeled RNA was synthesized using in vitro transcription, in which T7 RNA polymerase was used with biotin-UTP. A forward primer (TAA TAC GAC TCA CTA TAG GG) involving T7 RNA polymerase promoter and a reverse primer (GAT TTA GGT GAC ACT ATA G) involving the T3 RNA polymerase promoter were used to amplify the PCR fragments. In vitro transcription was performed using PCR products as DNA templates. Biotinylated RNA probes (approximately 20 pmol) were incubated with cell lysates for 30 min. Streptavidin Sepharose High-Performance beads (GE Healthcare, PA, USA) were used to isolate RNA-protein complexes. Liquid chromatography–mass spectrometry (LC–MS) was used to detect the isolated proteins.

**RNA immunoprecipitation**

RNA immunoprecipitation was performed as previously described (Zhang et al. 2017) Briefly, UV-cross-linking
of living cells \(1 \times 10^6\) was performed at 254 nm \(2000 \text{ J/m}^2\). Cells were washed with cold phosphate-buffered saline (PBS) and then lysed in 300 \(\mu\)l of lysis buffer \((200 \text{ U/ml RNase inhibitor; Thermo Fisher, MA, USA})\), 0.5% C24H39O4Na, 0.5% NP40, and protein inhibitor (Thermo Fisher) for 1 h. After treatment with DNase I (NEB, MA, USA) for 20 min, the cell lysate was incubated with anti-P14 antibody or IgG (Sigma) overnight at 4 °C. Then, 50 \(\mu\)l of protein A/G agarose beads (Santa Cruz, CA, USA) were added to the cell lysate. RNA binding with SF3B6 was recovered using Trizol-chloroform and detected using qPCR.

**Construction of cell lines with knockdown or overexpressed DKFZp434j0226**

To knock down DKFZp434j0226, 50 nM DKFZp434j-0226 siRNA (SI05138511, si 1# and SI05138518, si 2#; Qiagen, Hilden, Germany) was transfected into MiaPaCa-2 cells and PANC-1 cells using Lipofectamine 2000 reagent, according to the manufacturer’s instructions (Wei et al. 2008). Control groups were transfected with the transfection agent, but not siRNA (mock) or scrambled control siRNA (negative control). To clone the full-length DKFZp434j0226, PCR was performed with primers 5′-CGGAATTCCGCTTTGTGTCTAAGAAGTTCCAG-3′ and 5′-CGGAATTCTTTATTTCTTACTACATAAGATCCAC-3′. Full-length DKFZp434j0226 was subcloned into the plVX-IRES-Puro lentiviral expression vector (Clontech, CA, USA). pLVX-IRES-Puro-DKFZp434j0226 or pLVX-IRES-Puro Packaging psPAX2 and envelope pMD2.G vectors were co-transfected into HEK 293T cells. Lentivirus-containing medium was collected from HEK 293T cells and filtered. In the presence of 8 \(\mu\)g/mL polybrene, AsPC-1 cells and CFPAC-1 cells were infected with the enveloped lentivirus. Stable cell lines were selected using puromycin \(10 \mu\)g/mL. For the knockdown of SF3B6, 50 nM SF3B6 siRNA (AM16708, Thermo Fisher) was transfected into AsPC-1 cells.

**Cell proliferation assay**

Cell proliferation was measured using cell counting kit-8 (Dojindo Co., Kumamoto, Japan) according to the manufacturer’s protocol. Cells were incubated with CCK-8 for 1 h in triplicate. We assessed the cell proliferation rate by measuring the absorbance at 450 nm using a universal microplate reader. Four biological replicates were analyzed.

**Colony formation assay**

Soft agar colony formation assay was performed to measure the anchorage-independent growth ability as described previously (Liu et al. 2013). A total of \(1 \times 10^3\) cells per well were suspended in DMEM containing 0.3% noble agarose (Takara) in six-well plates. The suspension was laid over DMEM containing 0.6% noble agarose and further overlaid with DMEM. After replenishing the medium every other day, the plates were incubated for 14 days in a 5% CO₂ incubator at 37 °C. A Nikon ECLIPSE TE300 microscope was used to image the colonies. Four biological replicates were analyzed.

**Cell migration and invasion assays**

Cell migration capacity was measured by a cell migration (wound-healing) assay, as described previously (Bao et al. 2012). Cells were seeded in a six-well plate and incubated at 37 °C until they reached 90% confluence. The confluent cells were then scratched with a 200 mL pipette tip and washed with PBS, followed by incubation with Mag in complete medium. After 24 h of incubation, the cells were fixed and stained with 2% ethanol containing 0.2% crystal violet powder (15 min), and randomly chosen fields were photographed under a light microscope. The number of cells that migrated into the scratched area was calculated. Cell invasive potential was studied by calculating the number of cells that invaded through Matrigel-coated transwell polycarbonate membrane inserts as described previously (Verma et al. 2006). In brief, transwell inserts with a pore size of 12 Am were coated with 0.78 mg/mL Matrigel in serum-free medium. Cells were recovered by trypsinization, washed, and resuspended in a serum-free medium. Then, 0.5 mL of the cell suspension \((0.5 \times 10^6 \text{ cells})\) was added to duplicate wells. After incubation for 48 h, the cells that passed through the filter were stained using the Hema-3 stain kit (Fisher Scientific, Houston, TX, USA). The cells in 10 random fields were counted under a microscope. Both assays were performed in four biological replicates.

**Subcellular fractionation**

Cells were resuspended in harvest buffer \((10 \text{ mM HEPE} [\text{pH } 7.9], 50 \text{ mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 0.5% Triton-100, phosphatase inhibitors, and PMSF})\) and incubated on ice for 10 min. Nuclei were obtained by centrifugation at 12,000 rpm for 10 min at 4 °C. Nuclei were washed three times with harvest buffer and dissolved in 1× loading buffer. The supernatant containing the cytosolic extract was also collected and dissolved in 5× loading buffer. The cytoplasmic and nuclear fractions were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The validity of fractionation was detected by western blotting using LaminB as
the nuclear protein control and β-tubulin as the cytosolic protein control.

**Co-immunoprecipitation and western blotting**

Cells were lysed with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM NaF, 1% NP-40, 1 mM EDTA, and 1X protease and phosphatase inhibitor solution) on ice for 30 min. Protein A/G agarose beads were used to pre-clear the supernatants. Anti-SF3B6 antibody (dilution: 1:500; Catalog number PA5-57077, Thermo Scientific, USA) was used for immunoprecipitation. The mixture was incubated with protein A/G-agarose beads (Santa Cruz Biotechnology) overnight on a mechanical shaker at 4°C. The beads were harvested and rinsed three times with a lysis buffer. Immunoblotting was used to detect bead-captured SF3B6. Proteins were separated by 10% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, MA, USA). The membrane was incubated with the indicated antibody at 4°C overnight after blocking with 5% non-fat dry milk. An enhanced chemiluminescence kit (Tiangen, Beijing, China) was used to develop the blots. Primary antibodies against P-tyrosine (dilution: 1:500; Catalog number sc-207232, Santa Cruz), SF3B6 (dilution: 1:500; Catalog number ARG40015, Arigo Biolaboratories, Taiwan, China), proliferating cell nuclear antigen PCNA, (dilution: 1:2000; Catalog number sc-56, Santa Cruz), GAPDH (dilution: 1:1000; Catalog number sc-47724, Santa Cruz), LaminB (dilution: 1:500; Catalog number ab32535, Abcam, Cambridge, MA), and β-tubulin (dilution: 1:500; Catalog number ab6046, Abcam, Cambridge, MA) were used as indicated. Each western blotting assay was replicated three times.

**Immunofluorescence**

Cells were incubated on glass coverslips pre-coated with poly-l-lysine, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked in 10% bovine serum albumin in PBST for 30 min at 25°C. After staining with secondary antibodies, the primary antibodies were incubated at 4°C overnight. Primary antibodies against SF3B6 (dilution: 1:500; Catalog number PA5-57077, Thermo Scientific, USA) and DAPI (Beyotime, Shanghai, China) were used to identify SF3B6 and cell nuclei. A Leica confocal microscope (Leica TCS SP8, Germany) was used to capture the images.

**Animal studies**

Animal studies were approved by the Animal Use Committee at Fudan University. Four-week-old female BALB/c nude mice were obtained from the Experimental Animal Center of Fudan University. Animals were housed with a 12-h light/dark cycle, and MiaPaCa-2 cells infected with control lentivirus and DKFZp434J0226 lentivirus (2 × 10⁶ viable cells/mouse) were resuspended in PBS (0.1 mL) and injected subcutaneously into the right dorsal flank of BALB/c-nu/nu mice. After the tumor volume reached 200 mm³, control group (n = 6) and DKFZp434J0226 overexpressed group (n = 7) were analyzed. The tumor volume was calculated as

\[ V(\text{mm}^3) = (a b^2)/2, \]

where “a” indicates the tumor length and “b” indicates the tumor width. The mice were anesthetized by intra-peritoneal administration of pentobarbital (75 mg/kg) and tumors were harvested by surgery once tumor size reached 1200 mm³. Tumors were measured every 3–4 days. Mice bearing large tumors were carefully monitored for any signs of discomfort.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Prism Software Inc., San Diego, CA). Unpaired Student’s t-test or the Mann–Whitney U test was used for comparing two groups. Paired data were compared using a paired t-test. Analysis of variance (ANOVA) was used to compare three or more groups. Ordinary one-way ANOVA with the Sidak test was used for multiple comparisons between different groups. Categorical variables were compared using the χ² test. Independent two-sample t-tests were used to compare continuous variables. Univariate and multivariate analyses were performed using the Cox proportional hazards model. Survival curves were obtained using the Kaplan–Meier method with the log-rank test. Data are presented as mean ± standard deviation (SD) of three independent experiments. All tests were two-tailed, and a P-value of < 0.05 was considered statistically significant.

**Results**

**LncRNA expression profile in PDAC**

Systematic variations in the expression of lncRNAs between six PDAC samples and paired non-tumor samples were demonstrated by hierarchical clustering (Fig. 1A). Additional file 1: Table S2 shows the clinical characteristics of the patients. The expression levels of lncRNAs in paired samples were clearly shown by calculating the log fold change Tumor/Normal (T/N). We identified 128 aberrantly expressed lncRNAs (≥ twofold, \(P < 0.05\)) in RefSeq_NR, Fantom, lncRNA, UCR Ensemble, misc_lncRNA, Fantom_stringent, UCSC_knowngen, H-invDB, NRED, and RNAdb, with 53 upregulated lncRNAs and 75 downregulated lncRNAs (Fig. 1B and Additional file 1: Table S3).
LncRNAs were classified as Enhancer lncRNAs, Rinn lncRNAs, HOX lncRNAs, and LincRNAs nearby coding genes (Ørom et al. 2010; Rinn et al. 2007). Human Homeobox transcription factor (HOX) clusters have been found to contribute to the formation of numerous lncRNAs (Rinn et al. 2007). The lncRNAs expressed in site-specific fashion may have a general regulating ability as the HOX and used similar enhancers as HOX genes. In our study, approximately 281 HOX lncRNAs were detected, four of which were aberrantly expressed in human HOX loci in PDAC (Additional file 1: Table S4). Eleven of the 2341 lncRNAs were found to be aberrantly expressed, and the profiling data of Rinn lncRNAs are provided in Additional file 1: Table S5. Nine of the 1m133 enhancer lncRNAs were detected with different expressions, and the profiling data are shown in Additional file 1: Table S6. However, we did not find any enhancer lncRNAs nearby coding genes (distance, 300 kb), except for one lncRNA (distance, 300 kb; lncRNA-AK000839 and coding gene-NM_001075099). The classification of the detected lncRNAs and differentially expressed lncRNAs in paired samples is summarized in Fig. 1C, D, respectively.

Overview of mRNA profiles
Systematic variations in the expression of protein-coding mRNAs in the six PDAC samples and paired non-tumor tissues are shown by hierarchical clustering (Fig. 2A). A
Fig. 2 Microarray analysis of differentially expressed mRNAs between six pancreatic ductal adenocarcinoma (T) samples and paired non-tumor (N) samples. A Hierarchical cluster analysis of differentially expressed (≥ twofold change) mRNAs between six pancreatic ductal adenocarcinoma (T) samples and paired non-tumor (N) samples. The red color indicates upregulation, while the green color indicates downregulation. B A total of 222 mRNAs, with the upregulation of 66 mRNAs and downregulation of 156 mRNAs, were selected for clustering. C Gene ontology analysis of the differentially expressed genes (DE) between six PDAC tumors and paired non-tumor samples. The upregulated mRNAs are mainly aggregated in (a) the “spliceosome,” (b) “metabolic pathway,” and (c) “glycosaminoglycan and keratan sulfate” (framed in red). The downregulated mRNAs mainly associated with (d) “Staphylococcus aureus infection,” (e) “graft-versus-host disease,” and (f) “type I diabetes mellitus” (framed in green).
total of 222 mRNAs were differentially expressed among the six pairs of samples, with the upregulation of 66 mRNAs and downregulation of 156 mRNAs in PDAC compared to that in the corresponding normal samples (Fig. 2B and Additional file 1: Table S7). GO analysis indicated the upregulated mRNAs were mostly aggregated in the “spliceosome,” the “metabolic pathway,” and “glycosaminoglycan and keratan sulfate” (Fig. 2C), and the downregulated mRNAs were mainly associated with “Staphylococcus aureus infection,” “graft-versus-host disease,” and “type 1 diabetes mellitus” (Fig. 2D).

**DKFZp434J0226 can be an independent prognostic factor in patients with PDAC**

We investigated the relationship between DKFZp434J0226 (one with the greatest changes in expression) and the protein-coding genes using an absolute correlation coefficient cutoff of >0.85 and an FRD of <0.1. We found that hundreds of coding genes involved in cancer growth and metastasis were significantly associated with the lncRNA DKFZp434J0226 (Fig. 3A). We further examined the expression of DKFZp434J0226 in 109 pairs of PDAC and corresponding non-tumor tissue samples using RT-qPCR. Our data confirmed that DKFZp434J0226 was overexpressed in PDAC tissues compared to non-tumor tissues (Fig. 3B). Additionally, similar expression patterns were observed in the six PDAC cell lines compared to the immortal human pancreatic ductal epithelial (HPDE) cell line (Fig. 3C). Thus, our data demonstrated a strong consistency between the microarray data and qPCR results.

In the CNC network, DKFZp434J0226 was connected to a variety of protein-coding genes involved in cancer metastasis and growth (Fig. 3A). Therefore, we studied the role of the lncRNA DKFZp434J0226 in PDAC. In situ hybridization confirmed that the expression of DKFZp434J0226 was increased in PDAC tissues and was localized in both the nucleus and cytoplasm (Fig. 3D). We further examined the association between DKFZp434J0226 expression and clinical characteristics of 109 PDAC tissue samples from patients. Additional file 1: Table S8 presents the clinical characteristics of the patients. Clinicopathological analysis showed that high DKFZp434J0226 levels in PDAC tissues were significantly correlated with tumor grade, perineural invasion, and tumor-node-metastasis (TNM) stage. However, DKFZp434J0226 levels failed to match other clinicopathological data (Additional file 1: Table S9). Kaplan–Meier curves showed that high DKFZp434J0226 expression in tumor samples was positively correlated with shorter overall survival (OS) and time to progression (TTP) \((P=0.003 \text{ and } P=0.002, \text{ respectively; Fig. 3E}).\)

Univariate analysis indicated that high DKFZp434J0226 expression was correlated with both OS \((P=0.002)\) and TTP \((P=0.003)\). In addition, other factors indicative of shorter OS was tumor grading, tumor size, perineural invasion, vascular invasion, lymph node metastasis, and TNM stage. The factors associated with lymph node metastasis, tumor grading, vascular invasion, and TTP included age, tumor size, perineural invasion, and TNM stage, as shown in Additional file 1: Table S10. Multivariate analyses revealed that high DKFZp434J0226 levels were independently associated with worse OS \((P=0.014)\) and higher PDAC recurrence \((TTP, P=0.024; \text{ Table 1}).\)

**Effect of DKFZp434J0226 on PDAC cell proliferation, invasion, and migration**

To estimate the effect of DKFZp434J0226 on cell biological behaviors, we established MIAPaCa-2 cell line with DKFZp434J0226 knockdown (Fig. 4A) and AsPC-1 cell line with DKFZp434J0226 overexpression (Fig. 4B). Cell growth was inhibited by DKFZp434J0226 knockdown using cell counting kit-8 assays (Fig. 4C) and elevated by DKFZp434J0226 overexpression (Fig. 4D). Soft agar colony formation assay indicated that the colony formation ability was decreased by DKFZp434J0226 knockdown (Fig. 4E), and increased by DKFZp434J0226 overexpression (Fig. 4F). Consistent with the results of cell proliferation, DKFZp434J0226-knockdown MIA-PaCa-2 and PANC-1 cells and DKFZp434J0226-over-expressing AsPC-1 and CFPAC-1 cells showed lower and higher expression of PCNA, respectively, compared to control cells (Fig. 4G). Cell scratch assay demonstrated that the capacity of cells to migrate was attenuated by DKFZp434J0226 knockdown and enhanced by DKFZp434J0226 overexpression (Fig. 4H, I). Tran-swell invasion assay indicated that the invasiveness of cells was inhibited by DKFZp434J0226 knockdown and...
Fig. 3 (See legend on previous page.)
overexpression (Additional file 2: Fig. DKFZp434J0226 knockdown and CFPAC-1 cell line with DKFZp434J0226 behaviors were also observed in PANC-1 cell line with the similar effects of DKFZp434J0226 on cell biological (S1A–I).

Assays demonstrated an endogenous association between PCNA protein (a negative control). Cross-linked RIP in contrast, was not associated with DKFZp434J0226 (Fig. 5B). That SF3B6 interacted with DKFZp434J0226 (Fig. 5A). Mass spectrometry analysis revealed that a 14-KDa protein, SF3B6, interacted with DKFZp434J0226 (Fig. 5A). Mass spectrometry (RAP-MS) using MIAPaCa-2 cells performed native RNA pull-down assay combined with -interacting proteins, we identified DKFZp434J0226 and promotes its phosphorylation

To identify DKFZp434J0226-interacting proteins, we performed native RNA pull-down assay combined with mass spectrometry (RAP-MS) using MIAPaCa-2 cells (Fig. 5A). Mass spectrometry analysis revealed that a 14-KDa protein, SF3B6, interacted with DKFZp434J0226. Western blotting with anti-SF3B6 antibody confirmed that SF3B6 interacted with DKFZp434J0226 (Fig. 5B). In contrast, DKFZp434J0226 was not associated with PCNA protein (a negative control). Cross-linked RIP assays demonstrated an endogenous association between SF3B6 and DKFZp434J0226 (Fig. 5C). The expression of DKFZp434J0226, but not MEG3 (a negative control), was upregulated in samples immunoprecipitated with anti-SF3B6 antibodies compared to that in samples immunoprecipitated with IgG. These results demonstrated that the SF3B6 protein can bind to DKFZp434J0226RNA. The splicing pathway in eukaryotes contains the U2- and U12-dependent pathways, splicing-specific pre-mRNA introns differing in the branch point and splice site consensus sequences. SF3B6 is a member of the SF3B spliceosome and is present in the U2- and U12-dependent pathways, responsible for the first catalytic step of the splicing reaction (Perea et al. 2016). Next, we examined the protein levels of SF3B6 in DKFZp434J0226 overexpressed and knockdown cells (Fig. 5C). No significant difference in SF3B6 protein levels was observed in

Table 1 Multivariate analyses of factors associated with OS and TTP

|                | Hazard ratio (95% CI) | P     |
|----------------|-----------------------|-------|
| OS             |                       |       |
| Tumor differentiation (I vs. II vs. III) | 0.940 (0.488–1.811) | 0.853 |
| Tumor size (T1 vs. T2 vs. T3) | 1.817 (0.668–4.944) | 0.243 |
| Lymph node metastasis (negative vs. positive) | 1.263 (0.722–2.209) | 0.414 |
| Perineural invasion (negative vs. positive) | 0.684 (0.336–1.392) | 0.295 |
| Vascular invasion (negative vs. positive) | 1.336 (0.770–2.318) | 0.002 |
| TNM stage (I vs. II) | 1.913 (0.470–7.795) | 0.365 |
| DKFZp434J0226 expression (low vs. high) | 2.017 (1.154–3.527) | 0.014 |

TTP

|                | Hazard ratio (95% CI) | P     |
|----------------|-----------------------|-------|
| Age, years (<60 vs. >60) | 1.523 (0.877–2.647) | 0.135 |
| Tumor grade (I vs. II vs. III) | 1.040 (0.566–1.912) | 0.899 |
| Tumor size (T1 vs. T2 vs. T3) | 1.966 (0.812–4.764) | 0.134 |
| Lymph node metastasis (negative vs. positive) | 1.481 (0.866–2.534) | 0.151 |
| Perineural invasion (negative vs. positive) | 0.567 (0.289–1.111) | 0.098 |
| Vascular invasion (negative vs. positive) | 1.385 (0.790–2.428) | 0.006 |
| TNM stage (I vs. II) | 1.683 (0.474–5.970) | 0.421 |
| DKFZp434J0226 expression (low vs. high) | 1.875 (1.085–3.237) | 0.024 |

Variables were adopted for prognostic significance using univariate analysis (P < 0.05). Bold P values less than 0.05, indicating statistical significance
Fig. 4 (See legend on previous page.)
overexpressed or knockdown cells (Fig. 5D). As previous studies have reported the importance of phosphorylation in alternative splicing, we examined the level of SF3B6 phosphorylation in both overexpressed and knockdown cells and found that SF3B6 phosphorylation levels were significantly increased in \textit{DKFZp434J0226}-overexpressed cells and decreased in \textit{DKFZp434J0226}-knockdown cells (Fig. 5E).

\textbf{DKFZp434J0226 promotes translocation of SF3B6 from the cytoplasm to the nucleus and modulates alternative splicing}

To identify the effect of \textit{DKFZp434J0226} on splicing factor SF3B6, we examined the expression of SF3B6 in the nucleus and cytoplasm of \textit{DKFZp434J0226}-overexpressed AsPC-1 cells using western blotting. \textit{DKFZp434J0226} overexpression promoted the translocation of SF3B6 from the cytoplasm to the nucleus (Fig. 6A). Furthermore, we performed immunofluorescence...
analysis using DKFZp434J0226-overexpressed and control cells. In DKFZp434J0226-overexpression AsPC-1 cells, SF3B6 signals aggregated in the nucleus (Fig. 6B). Together, these results indicated that the lncRNA DKFZp434J0226 may contribute to the translocation of SF3B6 from the cytoplasm to the nucleus in PDAC cells.

To investigate the effect of DKFZp434J0226 on alternative splicing, we analyzed the alternative splicing of the MDM4 transcript, which has been recently reported as an SF3B6 target mRNA (Siebring-van Olst et al. 2017). RT-qPCR indicated that DKFZp434J0226 overexpression promoted the expression of the MDM4-S transcript and decreased the expression of the MDM4-FL transcript in AsPC-1 cells (Fig. 6C). These results indicated a potential role of DKFZp434J0226 in modulating alternative splicing events.

SF3B6 is required in DKFZp434J0226-induced cell proliferation and migration

To study whether DKFZp434J0226 increases cell migration and proliferation via SF3B6, we knocked down SF3B6 in DKFZp434J0226-overexpressed AsPC-1 and CFPAC-1 cell lines. We found that DKFZ-induced cell proliferation was rescued by SF3B6 knockdown, as evidenced by the results of the cell growth curve assay (Fig. 7A and (Additional file 2: Fig. S2A), soft agar colony formation assay (Fig. 7B and (Additional file 2: Fig. S2B), and western blotting of PCNA (Fig. 7C and (Additional file 2: Fig. S2C). In addition, SF3B6 silencing inhibited DKFZp434J0226 overexpression-induced cell migration, as measured by migration invasion assays (Fig. 7D and (Additional file 2: Fig. S2D). Furthermore, SF3B6 silencing reduced DKFZp434J0226 overexpression-induced alternative splicing of MDM-4 (Fig. 7E and (Additional file 2: Fig. S2E). These results indicated that SF3B6 was necessary for DKFZp434J0226-induced cell growth and migration.
**Discussion**

Over the past decades, several studies have shown that ncRNAs have regulatory potential both in transcription and post-transcription and play important biological roles in human diseases (Mercer et al. 2009; Wilusz et al. 2009). Furthermore, IncRNAs have been shown to be associated with a spectrum of biological processes, such as modulation of alternative splicing, protein activity, and epigenetic regulation (Sana et al. 2012; Werner et al. 2017). In this study, we studied the profiles of IncRNA expression between PDAC tissues and adjacent normal tissues using human IncRNA/mRNA expression microarray analysis. The IncRNA *DKFZp434J0226* was found to be aberrantly increased in PDAC tissues, and increased *DKFZp434J0226* expression was positively associated with poor prognosis and aggressive phenotypes in patients with PDAC. Furthermore, the study demonstrated that *DKFZp434J0226* promotes the phosphorylation and translocation of SF3B6, an important splicing factor, and modulates the alternative splicing process in PDAC cells.

Using human IncRNA/mRNA expression microarray analysis, we identified 106 differentially expressed IncRNAs and 222 aberrantly expressed mRNAs in 33,045 IncRNAs and 30,215 coding transcripts, respectively. GO analysis suggested that the upregulated mRNAs mainly aggregated in the “spliceosome,” the “metabolic pathway,” and “glycosaminoglycan and keratan sulfate.” These data are consistent with the viewpoint that PDAC is a disease of metabolic aberration and immune imbalance (Stolzenberg-Solomon et al. 2013; Matsuda et al. 2013; Sideras et al. 2013); glycosaminoglycan and keratan sulfate are important components of the extracellular matrix, which broadly participate in extracellular signal transduction and are closely associated with the metastasis and invasion of tumor cells (Shrimali et al. 2013; Heinemann et al. 2014). Aberrant expression of genes included in glycosaminoglycan and keratan sulfate biosynthesis in PDAC may provide insights into the molecular pathogenesis and may explain the highly aggressive behavior of PDAC.

The expression pattern of IncRNAs has been documented in different types of human malignant tumors (Gupta et al. 2010; Yang et al. 2011; Ji et al. 2003; Schmitt et al. 2016), but only a few studies have reported the roles of some specific IncRNAs in pancreatic cancer. Liu et al. (2014) reported that MALAT1 could be an independent predictor of disease-specific survival of PDAC (Tahira et al. 2011). They studied the role of intronic and intergenic IncRNAs in PDAC and found that loci harboring intronic IncRNAs were aberrantly expressed in PDAC metastases. We found that *DKFZp434J0226*, a 1635-bp intergenic IncRNA located in the region of chromosome 19q13.3, is upregulated in PDAC. To date, only one study has reported the function of *DKFZp434J0226*; the results showed a possible association between *DKFZp434J0226* expression and prognosis of colorectal cancer (Zhao et al. 2018). In our study, high *DKFZp434J0226* levels were positively correlated with tumor phenotypes such as tumor grading, TNM stage, and perineural invasion. Loss-and gain-of-function assays in vitro revealed that *DKFZp434J0226* promoted cell migration, invasion, and cell proliferation in PDAC cells.

Recent studies have suggested that IncRNAs could regulate alternative splicing (Wang et al. 1998; Eto et al. 2010; Misteli et al. 1997). The main mechanisms can be categorized into three types: (i) IncRNAs interact with specific splicing factors, (ii) IncRNAs bind to pre-mRNA molecules, and (iii) IncRNAs affect chromatin remodeling. A subset of IncRNAs has been reported to bind splicing factors and affect their activity by (i) regulating their post-translational modification (such as phosphorylation) or (ii) modulating their interactions with other splicing factors (Tripathi et al. 2010). Analyses have revealed that phosphorylation of SF3B1, an important member of SF3b spliceosome, is essential for splicing (Wang et al. 1998). Phosphorylated SF3B1 has been reported in nuclear structures, whereas non-phosphorylated SF3B1 has been found in the nucleoplasm, suggesting that phosphorylation of SF3B1 may play a vital role in pre-mRNA splicing on chromatin concomitant with transcription (Eto et al. 2010). In addition, SR proteins and some other snRNP proteins were found to be phosphorylated to form functional spliceosomes (Misteli et al. 1997). These findings indicate that phosphorylation
of some splicing factors might not only contribute to the assembly of the spliceosome but also to intranuclear transportation. Consistent with these studies, we found that *DKFZp434J0226* promotes the phosphorylation of the splicing factor SF3B6, elevates its nuclear translocation, and consequently regulates alternative splicing.

As a multiprotein complex component of the spliceosome, the splicing factor SF3B complex is necessary for branch site selection and recognition in the splicing process. It is made of seven proteins: SF3B6/p14, SF3B1/SAP155, SF3B2/SAP145, SF3B3/SAP130, SF3B4/SAP49, SF3B5 and SF3B14b (Sun and Sun 2020). In the spliceosome, SF3B proteins in contact with pre-mRNA around
the branch site strengthen the U2 snRNA/BS base-pairing interaction, thereby playing a vital role in branch site recognition and splicing. Among them, SF3B6 is located near the catalytic center, associated with the first step of the splicing reaction (Will et al. 2001; Spadacini et al. 2006), indicating that SF3B6 plays a vital role in the early splicing process (Perea et al. 2016). Although SF3B6 plays a crucial role in alternative splicing, there

Fig. 8  DKFZp434J0226 (DKFZ) inhibits xenograft tumor growth. MiaPaCa-2 cells infected with control lentivirus (control, n = 6) and DKFZ lentivirus (DKFZ, n = 7) were injected subcutaneously into nude mice. A Tumor size. B In vivo subcutaneous tumor growth curves. Data are shown as mean ± SD; *P < 0.05 vs. Mock and NC; **P < 0.01 vs. control. C Total tumor weight of each group of mice. D Western blotting analysis and quantification of SF3B6 phosphorylation levels in indicated tumors (n = 4). E The alternative spliced transcripts (MDM4-FL, MDM4 full-length transcript; MDM4-S, MDM4 S transcript) in the indicated tumors verified by RT-qPCR. The percent spliced in index was quantified for the alternative splicing events (n = 4). C–E Data are shown as mean ± SD; *P < 0.05, **P < 0.01
are only a few studies on the target pre-mRNA of SF3B6. Recently, MDM4 was identified as a target pre-mRNA of SF3B6 (Siebring-van Olst et al. 2017). Its alternative splicing product, the MDM4-S transcript, encodes a truncated Mdm4 protein with the N-terminal 114 amino acid p53-binding domain and C-terminal 26 aa residues (Rallapalli et al. 1999). Previous overexpression studies have shown that MDM4-S lacks an internal autoinhibitory sequence. Moreover, nuclear-localized MDM4-S can act as a strong p53 inhibitor; therefore, it likely functions as an oncogene (Rallapalli et al. 2003). We found that DKFZp434J0226 increased the level of MDM4-S transcript, indicating a role of DKFZp434J0226 in the promotion of oncogene production by regulating alternative splicing.

Conclusions
Taken together, our results, for the first time, identified DKFZp434J0226 as an oncogenic lncRNA in PDAC. DKFZp434J0226 promotes oncogenesis of PDAC by interacting with SF3B6 and regulating alternative splicing.

Abbreviations
IncrNA: Long noncoding RNA; PDAC: Pancreatic ductal adenocarcinoma; ncRNAs: Non-coding RNAs; pre-mRNA: Precursor mRNA; RT-qPCR: Quantitative real-time polymerase chain reaction; GO: Gene ontology; CNC: Coding–non-coding gene co-expression; PCR: Polymerase chain reaction; PBS: Phosphate-buffered saline; SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; ANOVA: Analysis of variance; SD: Standard deviation; T/N: Tumor/normal; HOX: Human Homeobox transcription factor; HPDE: Human pancreatic ductal epithelial; OS: Overall survival; TTP: Time to progression; CCK-8: Cell counting kit-8; RAP-MS: RNA pull-down assay combined with mass spectrometry; DE: Differentially expressed genes; RIP: RNA immunoprecipitation; IP: Immunoprecipitation; PSI: Percent spliced in index.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s10020-021-00347-7.

Additional file 1: Table S1. Primer sequences used in this study.
Table S2. Clinical characteristics of 6 patients with PDAC for microarray. Table S3. 128 differentially expressed IncRNAs (≥ 2-fold; P < 0.05) between six PDAC samples and paired non-tumor samples. Table S4. 281 HOX IncRNAs detected in PDAC. Table S5. 2241 Rinn IncRNAs detected in PDAC. Table S6. 1133 Enhancer IncRNAs detected in PDAC. Table S7. 222 differentially expressed mRNAs (≥ 2-fold; P < 0.05) between six PDAC samples and paired non-tumor samples. Table S8. Clinical characteristics in 109 patients with pancreatic cancer. Table S9. Correlation between DKFZp434J0226 and clinical characteristics. Table S10. Univariate analysis of factors associated with survival and recurrence.

Additional file 2: Figure S1. DKFZp434J0226 (DKFZ) promotes PDAC cell proliferation and migration. (A, B) Verification of DKFZ knockdown (A) and DKFZ overexpression (B) efficiency in Panc-1 and CFPA-1 cells (n = 3). Data are shown as mean±SD; *P<0.05, **P<0.01. (C) Cell growth curves of lipofectamine-treated Panc-1 (Mock) cells, negative siRNA-transfected Panc-1 (NC) cells, and two DKFZ siRNA-transfected Panc-1 (s1 and s2; n = 4). Data are shown as mean±SD; *P<0.05 vs. Mock and NC; **P<0.01 vs. Mock and NC. (D) Cell growth curves of control lentivirus-infected CFPA-1 cells (control) and DKFZ lentivirus-infected CFPA-1 cells (DKFZ) (n = 4). Data are shown as mean±SD; *P<0.05 vs. Mock and NC; **P<0.01 vs. Mock and NC. (F–I) Data are shown as mean±SD; *P<0.05 vs. Mock and NC. (F) Western blotting analysis of PCNA expression in the indicated PDAC cells. (G) Wound healing assay (A, B) and soft agar colony formation assay (B) using the indicated PDAC cells. (n = 4). (F–I) Data are shown as mean±SD; *P<0.05, **P<0.01. Scale bars refer to 100 μm. Figure S2. (A–C) Cell growth curves assay (A), soft agar colony formation assay (B), and western blotting of PCNA (C) demonstrating that SF3B6 knockdown rescues DKFZ-induced cell proliferation in CFPA-1 cells. (D) Migration assay demonstrating that SF3B6 knockdown rescues DKFZ-induced cell migration in CFPA-1 cells. (E) RT-qPCR demonstrating that SF3B6 knockdown rescues DKFZ-induced alternative splicing of MDM-4. (A) Data are shown as mean±SD; *P<0.05 vs. DKFZ; **P<0.01 vs. DKFZ. (B–E) Data are shown as mean±SD; *P<0.05, **P<0.01. Scale bars refer to 100 μm.

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Authors’ contributions
JL, XQ, SZ, RX and HT performed the experiments and wrote the manuscript. QJ and DL participated in the cell experiments and data analysis. WT, YZ, SC and DJ provided technical supports. XQ, SZ and RX conceived and designed the experiments. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
Ethical approval for human subjects was obtained from the research ethics committee of Zhongshan Hospital (Y2015-057), and written informed consent was obtained from each patient.

Consent for publication
We would like to submit the enclosed manuscript entitled “The long non-coding RNA transcriptomes. PLoS ONE. 2011;6(10):e25915.

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