Abstract. Adenylate kinase 4 pseudogene 1 (AK4P1) is a processed pseudogene whose function in cancer biology remains largely underexplored. Bioinformatics analysis suggested an association between the expression levels of adenylate kinase 4 (AK4) gene and AK4P1, as well as a clinical significance in relation to the increased transcription levels of AK4P1 in pancreatic adenocarcinoma (PAAD). In the present study, the expression levels of AK4P1 and AK4 were compared by RT-qPCR and western blotting between PAAD tissue and paired adjacent tissue. The level of AK4P1 transcript was compared between the circulating exosomes derived from patients with PAAD and those derived from healthy donors. Overall survival of the patients with PAAD with high or low expression levels of AK4P1 or AK4 was compared. AK4 gene expression level, in vitro cell viability and gemcitabine-induced apoptosis in PAAD cells with or without AK4P1 overexpression were also assessed using Cell Counting Kit-8 and TUNEL assays. It was identified that the transcription level of AK4P1 and the expression level of AK4 in PAAD tissue were significantly higher compared with those in paired non-cancerous tissue specimens. Transcription levels of AK4P1 and AK4 showed a significant relationship in PAAD. Circulating exosomes derived from patients with PAAD showed significantly higher level of AK4P1 transcript compared with that from circulating exosomes derived from blood samples of healthy donors. Patients with high expression of AK4P1 or AK4 exhibited significantly reduced overall survival compared with those with low expression. AK4P1 overexpression significantly upregulated the expression levels of AK4 in PAAD cells and rescued the viability and survival under gemcitabine challenge decreased by AK4 knockdown but not that by AK4 knockout. Treatment with exosomes secreted by AK4P1-overexpressing PAAD cells but not with those secreted by wild-type PAAD cells significantly increased the viability and survival under gemcitabine challenge of the recipient cells. These results suggested that AK4P1 affects the cellular biological functions of PAAD cells in vitro by upregulating the expression level of AK4. AK4P1 transcripts with elevated expression levels can be transmitted between PAAD cells through exosomes and exert pro-oncogenic effects in recipient cells.

Introduction

Pancreatic cancer is an exocrine tumor that originates mainly from glandular duct cells. Since its symptoms are non-specific, pancreatic cancer is often diagnosed only after the tumor has spread, which accounts for the 5-year survival rate being <10% (1). By the time it is diagnosed, ~90% of patients have locally advanced tumors that have invaded retroperitoneal structures, spread to local lymph nodes or have metastasized to the liver and the lungs, making pancreatic cancer one of the tumor types with the poorest prognosis for patients today (2,3). Treatment of pancreatic cancer is supplemented by chemotheraphy with gemcitabine or capecitabine, as well as radiotherapy in addition to pancreaticoduodenectomy (4). Pancreatic adenocarcinoma (PAAD) comprises 85% of pancreatic malignancies and is often poorly treated with or without these adjuvant therapies; by 2020, the 5-year survival rate for PAAD has increased to 10% (4,5).

Pseudogenes are non-functional gene fragments on the genome that are related to their parental genes but are themselves unable to be translated into functional proteins; they may be transcribed as long non-coding (Inc)RNAs or translated into non-functional polypeptides (6). The majority of the pseudogenes in the human genome are processed pseudogenes that originate from the retrotransposition of mature mRNAs; a number of these processed pseudogenes have lost the protein-coding capacity of their parental gene, and they function as IncRNAs that interact with other proteins or...
regulate the expression of their parental genes by competitively binding to microRNAs (miRNAs) (6), such as the cases of BRAF (7), KRAS (8) and PTEN (9) pseudogenes. These lncRNA-encoding pseudogenes have also been recognized as important regulators in cancer development, making them novel prognostic factors and potential targets for cancer management (10).

Cancer cells communicate with other tumor cells, tumor associated fibroblasts and immune cells to shape the tumor microenvironment to a cancer-supportive status by secreting exosomes (11-13). lncRNAs loaded in the cancer cell-secreted exosomes can influence gene expression in the recipient cells by interacting with protein, mRNA and miRNA molecules that have significant impact on the biology of these cells (14). In a preliminary study, exosomal lncRNAs derived from the blood samples of cancer patients were investigated in comparison with those derived from the blood samples of healthy individuals documented in the exorBase website (15).

It was identified that the level of lncRNA encoded by the adenylate kinase 4 pseudogene 1 (AK4P1) was significantly increased in the exosomes from the blood samples of patients with PAAD compared with the levels in circulating exosomes from healthy individuals. According to the records in the Ensemble database (https://asia.ensembl.org/index.html), the pseudogene AK4P1 is located on human chromosome 17 and is 2,386 base pairs long. It is transcribed into a lncRNA of 672 bases in length from the positive strand; its expression and function in cancer biology have not yet been examined. Adenylate kinase 4 (AK4), the parent gene of AK4P1, has been proposed as a novel cancer-promoting gene in various types of solid tumors, such as lung (16), breast (17), ovarian (18) and bladder cancer (19), but the function of this gene in pancreatic cancer has not been evaluated. In the present study, The Cancer Genome Atlas (TCGA)-PAAD data were downloaded from the Xena platform (20), and the association between the increased transcription levels of AK4 and AK4P1 with the development of PAAD and the decrease in overall survival of patients was identified. The clinical significance of the expression level of these two genes was further confirmed in the present study, and AK4P1 was identified as a PAAD-driving pseudogene, and this function may be through regulating the expression of AK4.

Materials and methods

Cell culture and transfection. Primary PAAD cells (cat. no. GPC0158) derived from pathological tissue samples of human pancreatic cancer obtained by surgery were purchased from China Center for Type Culture Collection. The present study was approved by the ethical review committee of Suizhou Hospital affiliated to Hubei University of Medicine; written informed consent was provided by each participant or the legal representative/guardian of the participant. The transcriptomic and survival data of the patients in the Genomic Data Commons (GDC) and the Cancer Genome Atlas (TCGA)-PAAD dataset were downloaded from Xena platform (20). The data from the exorBase database (http://www.exorbase.org/exorBase/download/toIndex) were downloaded to explore exosomal lncRNAs with significant differences in their abundances in circulating exosomes derived from patients with PAAD and from healthy volunteers. PAAD pathological tissue specimens and the paired adjacent non-cancerous tissue specimens (~2 cm from the tumoral tissue) were collected during surgery from 31 patients with PAAD who received radical surgery before adjuvant chemo/radiotherapy in our facility between January 2019 and June 2020. The inclusion criteria were as follows: i) Patients were 19-75 years old; ii) patients with PAAD were diagnosed by CT and MRI before surgery, and confirmed by pathological examinations after surgery; iii) Patients with PAAD had not received radiotherapy and chemotherapy before surgery; and iv) the basic information of the patients was complete. The exclusion criteria were as follows: i) Metastatic pancreatic cancer; ii) pancreatic tissue that had not been confirmed by pathological examination; and iii) the data were incomplete and difficult to be included in statistical analysis. The tissue specimens were stored in liquid nitrogen before analysis. Circulating exosomes were extracted from frozen plasma

Analysis of PAAD tissue specimens and clinical data of patients. The present study was approved by the ethical review committee of Suzhou Hospital affiliated to Hubei University of Medicine; written informed consent was provided by each participant or the legal representative/guardian of the participant. The transcriptomic and survival data of the patients in the Genomic Data Commons (GDC) and the Cancer Genome Atlas (TCGA)-PAAD dataset were downloaded from Xena platform (20). The data from the exorBase database (http://www.exorbase.org/exorBase/download/toIndex) were downloaded to explore exosomal lncRNAs with significant differences in their abundances in circulating exosomes derived from patients with PAAD and from healthy volunteers. PAAD pathological tissue specimens and the paired adjacent non-cancerous tissue specimens (~2 cm from the tumoral tissue) were collected during surgery from 31 patients with PAAD who received radical surgery before adjuvant chemo/radiotherapy in our facility between January 2019 and June 2020. The inclusion criteria were as follows: i) Patients were 19-75 years old; ii) patients with PAAD were diagnosed by CT and MRI before surgery, and confirmed by pathological examinations after surgery; iii) Patients with PAAD had not received radiotherapy and chemotherapy before surgery; and iv) the basic information of the patients was complete. The exclusion criteria were as follows: i) Metastatic pancreatic cancer; ii) pancreatic tissue that had not been confirmed by pathological examination; and iii) the data were incomplete and difficult to be included in statistical analysis. The tissue specimens were stored in liquid nitrogen before analysis. Circulating exosomes were extracted from frozen plasma
samples derived from each of the 31 PAAD patient or 25 healthy individuals who received routine medical check-ups at our facility by ultracentrifugation following a previously described procedure (21) and were verified by transmission electron microscopy before cryopreservation (TEM image of the exosomes is shown in Fig. S2). In short, peripheral blood was centrifuged at 2,000 x g for 10 min at 4˚C to collect plasma, and then centrifuged at 4,000 x g for 20 min at 4˚C, 12,000 x g for 30 min at 4˚C, filtered with a 0.22-µm filter to collect the plasma and centrifuged at 100,000 x g for 60 min at 4˚C twice, and finally the pellet was resuspended in PBS for use in follow-up analysis. Exosomes (20 µl) were pipetted onto the copper net dropwise and left for 5 min at RT. The excess liquid was absorbed at the side using filter paper, then 30 µl of 2% phosphotungstic acid (cat. no. C0681090235; Nanjing Reagent) was added dropwise, and negative staining conducted for 3 min at RT. After the negative staining, the excess liquid was again absorbed with filter paper, and baking was performed under an incandescent lamp for 10 min. The shape and size of the exosomes was examined with a transmission electron microscope at 80 kV acceleration voltage. Clinicopathological characteristics of the patients are shown in Table SI. Paraffin-embedded specimens of 83 patients diagnosed with PAAD in Suzhou Hospital between March 2012 and July 2018 were collected. qPCR was performed to detect the expression levels of AK4 and AK4P1. These patients were followed up by telephone for survival analysis. The clinicopathological characteristics of these patients are presented in Table SII. The patients were divided into high and low groups based on the median of the transcription levels of AK4P1 and AK4 as detected by RT-qPCR in their tissue specimens and the overall survival of these patients was compared.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from all samples was extracted by TRizol® method (Invitrogen; Thermo Fisher Scientific, Inc.). Primers were designed for the specific detection of AK4 or AK4P1 gene transcript and were constructed by GeneCopoeia, Inc. The primer sequences were as follows: AK4 forward, 5'-CTT GTGCCAGTCTCCCGGCTC-3' and reverse, 5'-CCTCGC GAAGGCCATTGGCT-3'; AK4P1 forward, 5'-GTTGTCCTTA AAGATCCTCCTCTCCCCCTGT-3' and reverse, 5'-CCT CGCGAAAGGCAATGGCT-3'; and β-actin forward, 5'-CTC TTCACAGCTTCTCTCTC-3' and reverse, 5'-AGC ACTTGTTTGGCGTGTCAG-3'. qPCR was performed using BlazeTaq One-Step SYBR Green RT-qPCR Kit (GeneCopoeia, Inc.) and a thermocycler using 1 µg cDNA as the template in each assay. The thermocycling conditions were as follows: Pre-denaturation at 95˚C for 10 min, denaturation at 95˚C for 15 sec and extension at 60˚C for 30 sec (40 cycles); and melting curve: 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec). β-actin was used as housekeeping gene for the comparison of AK4P1 or AK4 gene transcription levels among tissue specimens or cells cultured in vitro and AK4P1 transcription levels in the exosome samples. Data were analyzed by the 2^−∆∆Cq method (22).

Western blotting. Protein levels of AK4 in the tissue specimens or cells cultured in vitro were assessed by western blotting using β-actin as internal reference gene. In brief, total protein from tissue specimens or cells cultured in vitro was extracted using harsh RIPA lysis buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.). The protein concentration was determined using a BCA protein concentration assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.). A total of 30 µg protein was used in each western blot assay. The protein sample was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and the protein was transferred to a PVDF membrane (cat. no. ab133411; Abcam). The PVDF membrane was incubated for 2 h in a blocking solution (TBS with 0.1% Tween-20 solution containing 5% BSA (cat. no. A8010; Beijing Solarbio Science & Technology Co., Ltd.; pH 7.4) at room temperature. Then, the PVDF membrane was incubated with antibody at 4˚C overnight. The membrane was then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:3,000; cat. no. ab6721; Abcam) at room temperature for 3 h. Digital imaging and signal quantification were performed using the ECL Plus Western Blotting Substrate (cat. no. 32134; Thermo Fisher Scientific, Inc.) using Bio-Rad Image Lab Software (version 4.0; Bio-Rad Laboratories, Inc.). Primary antibodies used in western blot assays were as follows: Anti-AK4 mouse monoclonal antibody (dilution, 1:1,000; cat. no. sc-271161; Santa Cruz Biotechnology, Inc.) and anti-β-actin rabbit monoclonal antibody (dilution, 1:1,000; cat. no. ab115777; Abcam).

Cell viability and apoptosis assays. PAAD cell viability was evaluated by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Inc.). Briefly, the cells were inoculated on a 96-well-plate at an initial density of 5x10^4 cells/well and were cultured in serum-free complete culture medium for human pancreatic cancer primary cells at 37˚C for 48 h; after which, the cells were incubated with 10 µl CCK-8 working solution at 37˚C for 1 h. Absorbance was measured at an optical density of 450 nm using a microplate reader.

Apoptosis of the PAAD cells in response to gemcitabine challenge was evaluated using TUNEL method. The cells were inoculated on a 96-well-plate at a density of 2x10^4 cells/well, before they were treated with gemcitabine (LY-188011; Abmole Bioscience Inc.) at a final concentration of 10 nM for 24 h. Apoptosis was assessed by TUNEL staining using a One Step TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer’s protocol and fluorimetry using a fluorescence microscope (excitation wavelength, 450-500 nm; emission wavelength, 515-565 nm). Briefly, the cells were washed with PBS, fixed with immunostaining fixative (P0098; Beyotime Institute of Biotechnology) for 30-60 min and washed with PBS, and PBS containing 0.1% Triton X-100 was added. The cells were incubated in an ice bath for 2 min. After washing with PBS, 50 µl TUNEL detection solution was added to the cells. The cells were incubated for 60 min in the dark at 37˚C. Subsequently, the section was counterstained with 4',6'-diamidino-2-phenylindole (DAPI). After washing with PBS, the cells were re-suspended with 250-500 µl PBS and detected by fluorescence microscope (Olympus) at an objective magnification of x800, with filter blocks for DAPI and fluorescein. At least 300 cells in each group were randomly selected for quantification, and the apoptosis rate was evaluated by the number of TUNEL staining positive cells.

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Statistical analysis. GraphPad Prism software (version 9.02; GraphPad Software, Inc.) was used for statistical analysis. All data were normalized to the mean value of the data in the control group under the same experimental settings and are presented as the mean ± SD, unless otherwise indicated. An unpaired Student's t-test was used for comparing two groups of data. One-way analysis of variance was used for multi-group comparison followed by Tukey's post hoc test. Association analysis was performed by simple linear regression. Kaplan-Meier survival curve and log-rank test was used for survival analysis. A difference was considered statistically significant when P<0.05.

Results

Analysis of the transcription levels of AK4 and AK4P1 in publicly available data. AK4P1 was identified as an exosomal lncRNA whose abundance in the circulating exosomes derived from patients with PAAD was significantly higher compared with that in the circulating exosomes derived from healthy individuals (Fig. 1A). It was also found that the abundance of AK4 mRNA in the circulating exosomes derived from patients with PAAD was significantly higher when compare with that in the circulating exosomes derived from healthy individuals (Fig. 1B). To further explore the expression levels of AK4P1 and AK4 mRNA in publicly available data, the transcriptomic and survival data of patients in the GDC TCGA-PAAD dataset were downloaded from the Xena platform (Fig. 1C and D). The high transcription levels of either AK4P1 or AK4 mRNA were significantly associated with the decrease in the overall survival and tumor progression of patients with PAAD (Fig. 1E-H). The transcription levels of AK4P1 were significantly associated with those of AK4 mRNA in the tissue specimens of 179 patients with PAAD (Fig. 1I).

Upregulation in AK4P1 and AK1 gene expression in PAAD is associated with shortened overall survival of patients. The expression levels of AK4 gene or AK4P1 pseudogene in 31 pairs of PAAD pathologic tissue specimens and para-carcinoma tissue specimens were compared. RT-qPCR results demonstrated that the transcription levels of AK4 and AK4P1 were significantly higher in PAAD tissue specimens compared with those in the para-carcinoma tissue specimens (Fig. 2A and B, respectively). Western blot results also showed a significant increase in AK4 protein expression levels in the PAAD tissue specimens compared with those in the para-carcinoma tissue specimens (Fig. 2C and D).
Figure 2. Upregulation in AK4P1 and AK1 expression in PAAD is related to decreased overall survival of patients. Increased transcription levels of (A) AK4P1 pseudogene and (B) AK4 gene were revealed by RT-qPCR. (C and D) Upregulated protein expression level of AK4 in PAAD tissues compared with the paired adjacent normal tissue specimens were revealed by western blotting. (E) Association between AK4P1 and AK4 expression levels in the PAAD tissue specimens was revealed using the data presented in A and B. (F) Upregulation in the transcription level of AK4P1 in the circulating exosomes derived from patients with PAAD compared with that in the exosomes isolated from the blood of healthy volunteers was revealed by RT-qPCR. Significant associations between the increased transcription levels of (G) AK4P1 or (H) AK4 and the decrease in the overall survival of patients with PAAD was revealed by Kaplan-Meier analysis. **P<0.01 and ***P<0.001. AK4, adenylate kinase 4; AK4P1, adenylate kinase 4 pseudogene 1; PAAD, pancreatic adenocarcinoma; RT-qPCR, reverse transcription-quantitative PCR.
The expression levels of AK4 mRNA or AK4P1 pseudo-gene were further divided into high and low groups based on the median value, and it was observed that the mRNA expression levels of AK4 significantly associated with the transcription levels of lncRNA AK4P1 in the 31 PAAD tissue specimens (Fig. 2E). In addition, it was found that the exosomes obtained from patients with PAAD contained significantly higher levels of AK4P1 transcripts compared with those in the circulating exosomes derived from healthy individuals, as revealed by RT-qPCR (Fig. 2F). By integrating the AK4 and AK4P1 transcription levels in cryo-preserved PAAD tissue specimens and the survival of each patient, it was identified that high expression levels of AK4 and AK4P1 in PAAD tissue specimens were significantly associated with decreased overall survival of patients (Fig. 2G and H).

Overexpression of AK4P1 pseudogene increases AK4 gene expression and affects the cellular biological functions of PAAD cells in vitro. Overexpression of AK4P1 pseudogene increases AK4 gene expression and affects the cellular biological functions of PAAD cells in vitro. To investigate the function of AK4P1 pseudogene in PAAD cells, the cells were transfected with AK4P1 overexpression vectors (Fig. 3A). It was observed that AK4P1 overexpression significantly upregulated the mRNA and protein levels of AK4 in PAAD cells in vitro (Fig. 3B and C, respectively). It was also found that AK4P1 overexpression significantly increased PAAD cell viability in vitro (Fig. 3D), and this upregulation was weakened by siRNA-mediated AK4 gene knockdown or abrogated by AK4 gene knockout achieved using CRISPR/Cas9 machinery. The cell apoptosis rate was normalized to the PAAD cells treated with gemcitabine (which could induce tumor cell apoptosis), and the cell apoptosis rate of different groups was compared (Fig. 3E), showing that AK4P1 overexpression significantly...
rescued PAAD cells from gemcitabine-induced cell apoptosis. In addition, when AK4P was overexpressed, the increase in apoptosis caused by AK4-KD was rescued, compared with the PAAD cells with siRNA-mediated AK4 gene knockdown. However, when AK4 gene was completely knocked out in PAAD cells, the increase in apoptosis level could not be rescued by AK4P1 overexpression (Fig. 3E). The representative TUNEL staining images are shown in Fig. S3.

Exosomal AK4P1 regulates cell proliferation and apoptosis in recipient PAAD cells in vitro. Since the upregulation in AK4P1 levels in the circulating exosomes of patients with PAAD compared was confirmed, it was hypothesized that pancreatic cancer cells with high AK4P1 expression may deliver AK4P1 transcripts through exosomes, thereby affecting recipient cell growth and apoptosis. To investigate this, exosomes in the cell culture media conditioned by PAAD cells with or without AK4P1 overexpression were collected and used to incubate wild-type PAAD cells. RT-qPCR and western blot results showed that treatment with exosomes derived from AK4P1-overexpressing PAAD cells significantly increased the mRNA and protein expression levels of AK4 in the recipient PAAD cells compared with those treated with exosomes derived from donor cells without AK4P1 overexpression (Fig. 4A and B). It was further demonstrated that treatment with AK4P1-overexpressing PAAD cell-derived exosomes resulted in a significant increase in cell viability and a significant decrease in gemcitabine-induced cell apoptosis in the recipient cells compared with the recipients treated with exosomes derived from donor cells without AK4P1 overexpression (Fig. 4C and D). The representative TUNEL staining images are shown in Fig. S4.

Discussion

AK4 has been proposed as a novel cancer-promoting gene in several types of solid tumors. In breast cancer cells, for instance, high AK4 expression levels have been reported to be
associated with the progression of Her-2 positive breast cancer, and its knockdown significantly reduced the colony formation, proliferation and migration of breast cancer cells in vitro as well as the growth of xenografted tumors on nude mice (17). Similar findings were also reported in lung (16), ovarian (18) and bladder cancer (19). Upregulated AK4 protein expression was also found to be associated with the development of therapeutic resistance of cancer cells, such as tamoxifen resistance in breast cancer cells (23), platinum-based drug resistance in HeLa cells (24) and radio-resistance in oral squamous cell carcinoma cells (25), possibly by modulating mitochondrial metabolism and disrupting the mitochondria-mediated activation of the intrinsic apoptotic pathway (24,26). Hypoxia responsive transcription factor HIF-1α was found to initiate the transcription of AK4 in colorectal cancer cells (27), and AK4 protein was shown to interact with and stabilize HIF-1α protein in lung cancer cells (28). This positive feedback loop may underlie the hypoxia tolerance conferred to cancer cells by the increased expression of AK4 (24). The meta-analysis by Atay using publicly available data proposed AK4 as one possible prognostic marker in pancreatic ductal adenocarcinoma (29). However, the relationship between the expression level of this gene and PAAD progression has neither been evaluated in clinic nor in the laboratory.

Eukaryotic-derived exosomes contain receptors on their lipid bilayer membranes and carry inside them a variety of biomolecules obtained from parent cells, such as proteins, nucleic acids and lipids (30). Exosomes mediate short- and long-range communication between cells (30). An increasing number of studies have shown that exosomes secreted from tumor tissues can serve as potential biomarkers for cancer diagnosis, as they can reflect intracellular alterations in the parent cells (31,32). The exosomal membrane protein, Glypican-1, was found to be markedly more highly expressed in exosomes from cancerous than non-cancerous tissue, implying its clinical value as an exosomal biomarker for early diagnosis of pancreatic cancer (33). In the present study, bioinformatics analysis results and clinical findings showed a significant increase in the expression levels of both AK4 and AK4P1 in pathological tissue specimens and in circulating exosomes of patients with PAAD as well as its association with the decreased overall survival of patients. These results not only suggested AK4 and AK4P1 as potential biomarkers for the prognosis of PAAD but also implied their participation in its development. Based on the high degree of transcript sequence identity, pseudogenes such as BRAFp1, KRASP1 and PTEP1 are all considered to assist in the expression of their parental genes in tumor cells by competitively binding to and sequestering miRNAs (10). Considering the significant association in the transcription levels between AK4P1 and AK4, as found in the present study, it was speculated that AK4P1 may facilitate the expression of AK4 gene in PAAD cells through a similar competing endogenous RNA mechanism; considering that gemcitabine is frequently used in clinical practice as adjuvant chemotherapy for pancreatic cancer; in the present study this drug was used as an apoptosis inducer. The data showed that AK4P1 overexpression could significantly increase the expression of AK4 gene in PAAD cells in vitro; siRNA-mediated AK4 knockdown significantly inhibited the growth of PAAD cells and increased their apoptotic rates induced by gemcitabine treatment, which were significantly attenuated by AK4P1 overexpression. Interestingly, it was observed that AK4P1 failed to restore the proliferation and survival of PAAD cells with AK4 gene knockout, showing that the pro-malignant role of AK4P1 requires the intact AK4 gene in PAAD cells, which suggested that the AK4P1 transcript regulated the expression of AK4 gene in a post-transcriptional fashion. It was further identified that treatment with exosomes derived from AK4P1-overexpressing PAAD cells significantly increased the expression of AK4 gene in the recipient cells, which indicated that PAAD cell-derived exosomes may shape the malignancy of the recipient cells by transferring AK4P1, similar to the mechanisms previously reported (34-36). However, certain limitations exist in the present study. It was not possible to verify the function of cytobiology, such as apoptosis, in PAAD cells with AK4P1 knockdown, which will be the focus of our future research.

Collectively, in the present study, the cancer-promoting role of AK4P1 transcript was reported for the first time, to the best of our knowledge. The significant upregulation of AK4P1 expression levels and its parental gene AK4 in PAAD was identified, which significantly was associated with the decreased overall survival of patients; elevated levels of AK4P1 transcripts in peripheral blood exosomes of patients with PAAD may contribute to the early screening and intervention of this disease, consequently improving prognosis of patients. The effect of AK4P1 on PAAD cells required intact AK4 gene transcription, and AK4P1 transcript from the donor cells may exert cancer-promoting effects in the recipient cells through exosome delivery.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
MX conceptualized and designed the study. LL, TD and QZ analyzed pancreatic adenocarcinoma tissue specimens and clinical data of patients. YY and YL performed cell viability and apoptosis assays. LY and LL conducted cell culture and transfection assays. LL and MX confirm the authenticity of all the raw data. LL was the major contributor in writing the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
This research was approved by the Ethical Review Committee of Suizhou Hospital Affiliated to Hubei University of Medicine (Suizhou, China). All research complied with The Declaration
of Helsinki and the relevant rules of the Ethics Committee. Written informed consent was provided by each participant or the legal representative/guardian of the participant.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References