Key role of dual specificity kinase *TTK* in proliferation and survival of pancreatic cancer cells

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Background: Pancreatic ductal adenocarcinoma (PDAC) is among the most aggressive human malignancies with an overall 5-year survival rate of <5%. Despite significant advances in treatment of the disease during the past decade, the median survival rate (~6 months) has hardly improved, warranting the need to identify novel targets for therapeutic approaches.

Methods: Quantitative real time PCR, western blot analyses and immunohistochemical staining of tissue microarrays were used to analyse the expression of *TTK* gene in primary PDAC tissues and cell lines. To inhibit *TTK* kinase expression in a variety of pancreatic cancer cell lines, RNA interference was used. Functional roles of this kinase in the context of PDAC were studied using cell proliferation, viability and anchorage-independent growth assays. Western blotting, fluorescence-activated cell sorting analyses and fluorescence microscopy were used to gain mechanistic insight into the functional effects.

Conclusions: We show that the dual specificity kinase *TTK* (also known as Mps1), is strongly overexpressed in human PDAC. Functionally, cell proliferation was significantly attenuated following *TTK* knockdown, whereas apoptosis and necrosis rates were significantly increased. In addition, anchorage-independent growth, a hallmark of malignant transformation and metastatic potential, was strongly impaired in the absence of *TTK* gene function. Interestingly, immortalised normal pancreatic hTERT-HPNE cells were not affected by loss of *TTK* function. Mechanistically, these effects in cancer cells were associated with increased formation of micronuclei, suggesting that loss of *TTK* function in pancreatic cancer cells results in chromosomal instability and mitotic catastrophe. Taken together, our data show that *TTK* function is critical for growth and proliferation of pancreatic cancer cells, thus establishing this kinase as an interesting new target for novel therapeutic approaches in combating this malignancy.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive solid cancers and presently the fourth leading cause of cancer-related deaths in the western world (Hidalgo, 2010; Jemal *et al*, 2011; Malvezzi *et al*, 2013). Currently, PDAC is practically incurable with existing therapies. The vast majority of the patients are diagnosed at an advanced and often already metastasised stage, making curative resection impossible. Both, incidence and mortality are on the rise for PDAC, unlike for other cancer types. For the European Union, the death rate in pancreatic cancer is expected to rise from 7.86 in 2007 to 8.0 per 100 000 among men, and from 5.24 to 5.5 per 100 000 among women in 2013, and the trend is predicted to continue (Malvezzi *et al*, 2013). The median survival rate (~6 months) and the overall 5-year survival rate (<5%) has not improved despite significant research and diagnostic efforts over the

last decade, illustrating the urgent need to identify novel molecular targets and approaches to combat this devastating disease.

Protein kinases are critical regulators of diverse cellular functions. They are now widely acknowledged as effective druggable targets in a variety of pathological conditions. The chemistry and pharmacology of kinase inhibitors have also developed tremendously over the last two decades (Cohen, 2002), enabling most of the inhibitors to be available orally. Consequently, we have performed high-throughput screens for protein kinases, which are upregulated in pancreatic cancer and, which, when silenced, would impair the viability of pancreatic cancer cells.

TTK (also known as *Mps1*) is a dual specificity kinase, that is, it can act to phosphorylate both tyrosine as well as serine/threonine residues (Liu and Winey, 2012). First identified in budding yeast, it is highly conserved from yeast to humans (Mills *et al*, 1992; Liu and Winey, 2012). It is regarded as a key player in several steps of mitosis in a variety of eukaryotic cells (Liu *et al*, 2003) and is one of the main kinases involved in kinetochore localisation and the spindle assembly checkpoint (SAC), which inhibits metaphase-to-anaphase transition until proper spindle attachment to all chromosomes is achieved (Stucke *et al*, 2002; Liu and Winey, 2012; Nijenhuis *et al*, 2013). Several reports have indicated the role of *TTK* in a variety of disease patterns (Poss *et al*, 2002; Liu and Winey, 2012; Althoff *et al*, 2012; Morin *et al*, 2012), including, in an apparent paradox to its role as a checkpoint kinase, a growth promoting role in cancer cells of different origin. Here, we show for the first time that *TTK* is significantly overexpressed in pancreatic cancer and that it has a central role in maintaining the viability and proliferative potential of pancreatic cancer cells.

MATERIALS AND METHODS

Cell lines and primary tissues. The human pancreatic adeno-carcinoma cell lines Panc1, PaTu-8988 T and S2-028 were used in this study. Panc1 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). S2-028 cells were from T Iwamura (Miyazaki Medical College, Miyazaki, Japan) (Taniguchi *et al*, 1992). PaTu-8988 T cells were kindly provided by H P Elsässer (Cytobiology and Cytopathology Institute, Philipps University, Marburg, Germany). These cancer cell lines were maintained in Dulbecco's modified minimal essential medium (GIBCO, Grand Island, NY, USA) supplemented with 10% FCS (PAA, Cölbe, Germany) and cultured at $37\,^\circ$C/5% CO_2. The non-transformed pancreatic cell line hTERT-HPNE was kindly provided by C Schmees, NMI, Reutlingen, Germany. The basal medium for these cells consisted of 75% DMEM without glucose (GIBCO) with an additional 2 mM L-glutamine and $1.5\,g\,l^{-1}$ sodium bicarbonate and 25% M3 Medium (Incell Corp., San Antonio, TX, USA). For experiments, complete growth medium was used, which additionally contained 5% FCS, $10\,ng\,ml^{-1}$ human recombinant EGF (Sigma, Germany), 5.5 mM D-glucose and $750\,ng\,ml^{-1}$ puromycin (PAA). Immortalised human pancreatic ductal cells (HPDE) (Ouyang *et al*, 2000) were cultured in Defined Keratinocyte Serum-Free medium supplemented with human recombinant epidermal growth factor and bovine pituitary extract (Gibco/Life Technologies, Darmstadt, Germany).

Cancer cell line identities were verified using the GenomeLab Human STR Primer Set (Beckman Coulter, Krefeld, Germany) on a CEQ8800 sequencer (Beckman Coulter) according to the manufacturer's protocol. To confirm the identity of human cell lines, STR data were submitted to online verification tool of DSMZ (www.dsmz.de).

Surgically resected PDAC and chronic pancreatitis tissue samples were procured from the surgery departments of the University of Heidelberg and Ludwig Maximilian University of Munich. Samples of normal pancreatic tissue were obtained from the healthy donors. Informed consent in writing was obtained from all patients prior to using tissue samples. The study was approved by the ethics committee at the University of Heidelberg and Ludwig Maximilian University of Munich, Germany.

siRNA transfection of cell lines. PaTu-8988T, Panc1 and S2-028 cells were transfected with siRNAs using siLentFect Lipid Reagent (Bio-Rad, Munich, Germany) according to the manufacturer's protocol. Unless stated otherwise, for transfection, 150×10^3 cells were seeded per well in a 6-well plate. Three specific siRNAs targeting *TTK* gene were used: Hs_TTK_2, Hs_TTK_6 and Hs_TTK_7 (FlexiTube, Qiagen, Hilden, Germany); henceforth only distinguished as si1, si2 and si3, respectively. Accell Control siRNA #1 (Thermo Scientific-Dharmacon, Lafayette, CO, USA) was used as a non-silencing control (siC). Untreated cells, henceforth designated NT, were used in all experiments as additional control.

RNA extraction, cDNA sysnthesis and qRT-PCR. RNA from cell lines was extracted using the peqGold Total RNA Kit (PEQLAB GmbH, Erlangen, Germany) according the manufacturer's protocol. Tissue samples were homogenised in liquid nitrogen using a mortar and pestle. Extraction of RNA was done using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. One microgram total RNA was used for first-strand cDNA synthesis using the Omniscript RT Kit (Qiagen) as per the manufacturer's protocol. Quantitative real time reverse transcription PCR (qRT-PCR) was performed using SYBR Green MasterMix (Applied Biosystems, Foster City, CA, USA) on a 7500 Fast Realtime PCR system (Applied Biosystems). The following primer pairs were used for qRT-PCR: ribosomal protein, large, P0; RPLP0_For: 5′-TGGGCAAGAACACCATGATG-3′; RPLP0_Rev: 5′-AGTTTCTCCAGAGCTGGGTTGT-3′; TTK_For: 5′-TGGCCAACCTGCCTGTTT-3′; TTK_Rev: 5′-AATGCATT CATTTGCTGAAGAAGA-3′.

Protein extraction and western blotting. For protein extraction, cells were collected together with medium and centrifuged at 1600 rpm at $4\,^\circ$C for 5 min. Pellets were washed twice with ice-cold PBS and then resuspended in $200\,\mu l$ lysis buffer (PBS containing protease inhibitors (Protease Arrest, GBiosciences, St Louis, MO, USA) and phosphatase inhibitors (PMSF 1 mM, EDTA 0.5 mM, sodium pyrophosphate 25 mM, sodium orthovanadate, 10 mM, sodium luoride 50 mM)). Cells were sonicated (LabSonic, BBraun, Melsungen, Germany) and protein content was assessed using Protein Assay Reagent (Thermo Scientific). For western blotting, $10\,\mu g$ proteins were electrophoresed on SDS-polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes (Optitran, GE Healthcare Life Sciences, Little Chalfont, UK). Membranes were blocked in 5% non fat dry milk in TBST (10 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.1% Tween 20) for 2–4 h at room temperature and then probed with appropriate primary and secondary antibodies (anti-Phospho-Chk1 (Ser345): Cell Signaling, Boston, MA, USA; cat# 2341; anti-Phospho-Histone H2A.x (Ser139): Cell Signaling, cat# 9718; anti-Cyclin B1: Cell Signaling, cat# 4135; anti-Mps1 (*TTK*): Millipore; cat# 05–682; anti-PARP: Cell Signaling, cat# 9532 and; anti-Caspase-3: Cell Signaling, cat# 9664 and 9665).

Immunohistochemistry. Two tissue microarrays (TMAs) including a retrospective series of 40 FFPE surgically treated PDACs and 8 non-neoplastic controls were retrieved from the ARC-Net biobank at Verona University Hospital. Four 1-mm-tissue cores per case were analysed. The materials have been collected under Program 853 protocol 298CE 15/02/02 and revised Program 1885 protocol 52438 23/11/2010, approved by the Verona University Hospital ethics committee. The protocols include informed consent from the patient.

Immunohistochemical stainings for *TTK* (clone 3-472-1; Upstate Biotechnology, Lake Placid, NY, USA; 1:100) were obtained on 4-μm-thick FFPE sections using an automated instrument (Bond-maX, Menarini, Rome, Italy). Sections were lightly counterstained with hematoxylin. Appropriate positive and negative controls were run concurrently. TTK cytoplasmic immunostaining was considered positive. In all the considered tissue samples, granulocytes and endothelia always featured TTK cytoplasmic expression and were assumed as positive internal control (not retained for IHC scoring).

BrdU cell proliferation assay. DNA synthesis as a direct measure of cell growth was measured using the Cell Proliferation ELISA, BrdU chemiluminescence Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, 5000 cells were re-seeded 24 h after transfection into a 96-well plate (ViewPlate Black Clear Bottom, PerkinElmer, Rodgau, Germany). On the next day, cells were incubated for 4 h with BrdU-containing medium, then fixed at room temperature for 1 h and incubated with the peroxidase-conjugated anti-BrdU antibody for 1.5 h. The chemiluminescence intensity was measured as relative light units per second (rlu s^{-1}).

MTT cell viability and cell survival assay. 24 hours post transfection, 30×10^3 cells per well were re-seeded into 12-well plates. After additional 48 h of incubation, cells were incubated for 1.5 h with MTT-containing medium prepared by dissolving thiazolyl blue (Carl Roth, Karlsruhe, Germany). The cells were then solubilised using 10% Triton X-100 (Carl Roth)/0.1 M HCl (Fisher Scientific, Schwerte, Germany) in isopropanol (Sigma-Aldrich, Taufkirchen, Germany) and the extinction was then measured at 570 nm on a plate reader. For the cell survival assay, 10×10^3 cells were seeded per well in seven 24-well plates and transfected with siRNAs next day. Over a period of 7 days, MTT assays were performed and cell survival was plotted as relative cell number by measuring the MTT extinction coefficient for each day.

Soft agar assay. Soft agar clonogenic assays were performed in a 12-well plate format as described before (Hann *et al*, 2011). Briefly, 6×10^3 siRNA-transfected cells were re-seeded in a 0.33% DMEM-bacto-agar per well onto a bottom layer of 0.5% DMEM-bacto-agar. One microlitre DMEM medium was added after the top agar had solidified. Medium was replaced every third day. Quantification was done by counting the number of colonies formed after 10 days of culturing.

Flow cytometric analysis. For the cell cycle analyses, 48 h after transfection, cells were collected, washed with PBS and then were fixed overnight at 4 °C with ice-cold 70% ethanol. Fixed cells were washed again once with ice-cold PBS and incubated with FxCycle Violet Stain (1:1000, Molecular Probes, Invitrogen, Darmstadt, Germany) for 30–45 min in the dark. Measurement was done at the central fluorescence-activated cell sorting (FACS) facility of the Medical Faculty, Marburg University, on a FACS LSR-II (BD Biosciences, Heidelberg, Germany) machine by acquiring a minimum of 20 000 cells in the Pacific Blue channel. For the Annexin-V/Propidium iodide (PI) staining, 48 h after transfection, the cells were collected, washed with PBS, resuspended in the binding buffer (10 mM HEPES/NaOH pH7.4, 140 mM NaCl, 2,5 mM CaCl$_2$) and then stained with a FITC-coupled-Annexin-V antibody (BD Biosciences, cat# 556419) for 15 min in the dark. Shortly before measurement, PI was added to the cells. A minimum of 20×10^3 cells were acquired for the experiment at the FACS LSR-II machine.

Fluorescence microscopy. 24 h after transfection, 20×10^3 cells per well were re-seeded onto coverslips coated with collagen (type I, Sigma-Aldrich) in 12-well plates. After additional 24 h of incubation, the coverslips were washed with PBS twice, fixed on microscope slides using DAPI containing mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA) and observed under the microscope.

Statistical analysis. Each experiment was repeated independently at least three times. Values are expressed as mean ± s.e.m. of the triplicates, unless stated otherwise. Student's *t*-test was used to analyse the difference between samples of interest *vs* control. A *P*-value of < 0.05 was considered to be statistically significant.

RESULTS

***TTK* kinase is upregulated in pancreatic cancer.** The mRNA levels of the *TTK* gene in primary pancreatic cancer and chronic pancreatitis tissues were determined using qRT-PCR and compared with the levels in healthy pancreas samples. A total of 11 cancer tissues, 9 chronic pancreatitis and 8 healthy pancreatic tissues were analysed. A modest increase in the mRNA expression of *TTK* was seen in chronic pancreatic tissues as compared with the healthy tissues (Figure 1A), whereas pancreatic cancer tissues showed significant overexpression of *TTK* compared with both chronic pancreatitis as well as healthy pancreatic tissues (Figure 1A). *TTK* mRNA expression was also readily detectable in all pancreatic cell lines tested, including transformed (IMIM PC1 & PC2, S2-007, S2-028, PaTu-8988 T and Panc1) as well as non-transformed (HPDE and HPNE) lines. However, no systematic difference in expression levels was observed between cancer *vs* the normal (immortalised) cell lines (Figure 1B). These findings were further validated by analysing TTK protein levels in primary human tissues as well as cultured cells. Immunohistochemical detection of TTK was performed on TMAs comprising a series of 40 different pancreatic cancer lesions. In normal pancreatic parenchyma, TTK expression was negative/faint in both pancreatic acini and duct epithelia; Langerhans islets showed a moderate TTK expression. A significant overexpression was observed in PDAC lesions. Twenty-eight out of 40 (70%) PDACs showed a moderate/ strong cytoplasmic immunoreaction in the epithelial cancer cells (Figure 1C). In congruence to the mRNA data, no systematic difference was seen in the TTK protein levels in the normal human pancreatic cell line (HPNE) *vs* the pancreatic cancer cell lines used for the functional experiments (Figure 1D).

***TTK* function is indispensible for pancreatic cancer cell proliferation and viability.** To assess the functional relevance of this upregulation of *TTK* in pancreatic cancer, the kinase was transiently silenced using three different specific siRNAs. All three siRNAs led to significant downregulation of the *TTK* gene product at both transcriptional and protein levels (Figure 2A) in all the three pancreatic cancer cell lines tested (Panc1, S2-028 and PaTu-8988T). Downregulation of *TTK* mRNA after siRNA transfection was measurable as early as 24 h post transfection and remained stable for over 96 h (data not shown).

We next used BrdU incorporation assays to analyse the impact of a loss of *TTK* expression on the proliferative potential of pancreatic cancer cells. In all three cell lines tested, *TTK* knockdown by the three independent siRNAs led to a significant reduction in proliferation rates compared with cells treated with non-silencing control siRNA (Figure 2B), with the exception of si3 in Panc1 cells, where the proliferation was also lower but did not reach statistical significance. These results were confirmed in MTT assays, where knockdown of *TTK* expression with any of the three independent siRNAs in any of the three pancreatic cancer cell lines led to statistically highly significant decrease in the viability of the cells as compared with controls (Figure 2C). Interestingly, transient loss of *TTK* function in the non-transformed human pancreatic cell line hTERT-HPNE had no effect on the viability of these cells despite reaching very good knockdown efficiencies
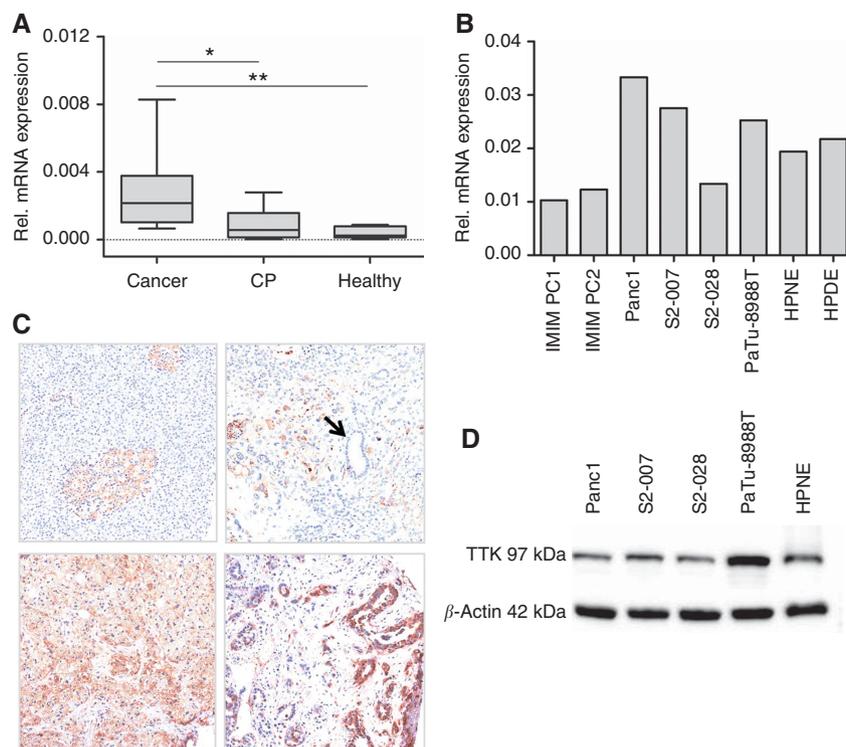
Figure 1. *TTK* expression in primary pancreatic tumour tissues and cell lines. *TTK* mRNA and protein levels were examined in primary pancreatic cancer and control tissues as well as in cultured pancreatic cancer and control cell lines. (A) Box-and-whisker plot showing *TTK* mRNA expression in primary human pancreatic tumour tissue samples, chronic pancreatitis and normal pancreas as analysed by quantitative real time reverse transcription PCR (qRT-PCR). Expression was normalised to ribosomal protein, large, P0 (RPLP0) mRNA levels. Data in figure represent median and 2nd and 3rd quartiles (boxes) as well as minimum and maximum values (whiskers). CP = Chronic pancreatitis. *$P<0.05$; ** $P<0.01$ (Student's *t*-test). (B) *TTK* mRNA expression levels in a variety of different pancreatic cancer and control cell lines. (C) Immunohistochemical staining of tissue microarrays (TMA) for TTK expression. In normal pancreatic tissue, Langerhans islets were stained positive for TTK, whereas normal acinar and ductal cells showed only very faint signal (top left panel). Enhanced expression was seen in the PDAC tissues (top right as well as bottom panels). Normal ducts stained negative for TTK (see arrow, top right panel). (D) Western blot analyses showing TTK expression in the different pancreatic cancer and control cell lines used for the functional experiments. Beta-actin served as loading control.

(Supplementary Figure 1A and B), indicating that dependency on *TTK* expression is cancer cell specific.

Long-term survival studied using the PaTu-8988T cell line demonstrated that efficient and sustained growth inhibition persisted over a period of 7 days following *TTK* knockdown, thus providing no indication of compensatory mechanisms that would allow cells to successfully escape growth inhibition (Figure 2D).

The ability to continuously proliferate in the absence of adhesion to a solid matrix is a hallmark feature of malignantly transformed epithelial cells and correlates directly with high invasive and metastatic potential. Soft agar assays with Panc1 and S2-028 cells with and without transient *TTK* knockdown demonstrated that anchorage-independent growth was severely attenuated in the absence of *TTK* expression in both cell lines (Figure 2E).

TTK knockdown induces apoptotic and necrotic cell death. Western blot analyses of pancreatic cancer cells with and without RNAi-mediated knockdown of *TTK* expression demonstrated strongly increased PARP (Figure 3A) and/or Caspase-3 (Figure 3B) cleavage in *TTK* knockdown cells, indicating strongly increased levels of apoptosis in the absence of *TTK* expression. Moreover, flow cytometry analysis of cells after annexin-V/PI staining further corroborated the western blot findings by demonstrating a strong increase in the numbers of early (annexin-V signal only) and late (annexin-V plus PI signal) apoptotic cells as well as necrotic cells (PI staining only) (Figure 3C) as compared with untreated or control siRNA-treated cells, indicating that cell death can occur via different routes.

Loss of TTK function causes chromosomal missegregation, but not DNA damage or cell cycle arrest in pancreatic cancer cells. Flow cytometric cell cycle analyses of PaTu-8988T cells revealed only a modest influence of *TTK* knockdown on the relative distribution of cells across different phases of the cell cycle, thus producing no indication of induction of a cell cycle arrest following loss of *TTK* function (Figure 4A). Concurrently, western blot experiments revealed no evidence of induction of the cellular DNA damage response (DDR). Phosphorylation of the ATR/p53 targets H2AX and Chk1 was not changed, and Cyclin B1 levels were not systematically altered in *TTK* knockdown cells (Figure 4B), indicating that mitotic exit and cell cycle progression were not attenuated following *TTK* knockdown.

However, fluorescence microscopy of DAPI-stained cell nuclei demonstrated that genomic integrity was severely compromised in cells lacking functional *TTK*. In addition to an overall tendency towards increased abnormal nuclear morphology, formation of large numbers of micronuclei, a hallmark feature of massive aneuploidy and chromosomal missegregation, was readily apparent in all three cell lines examined (Figure 5).

DISCUSSION

Despite considerable efforts, the biology of pancreatic cancer is not clearly understood, and this malignancy continues to pose a significant challenge to clinicians and researchers alike. To date,
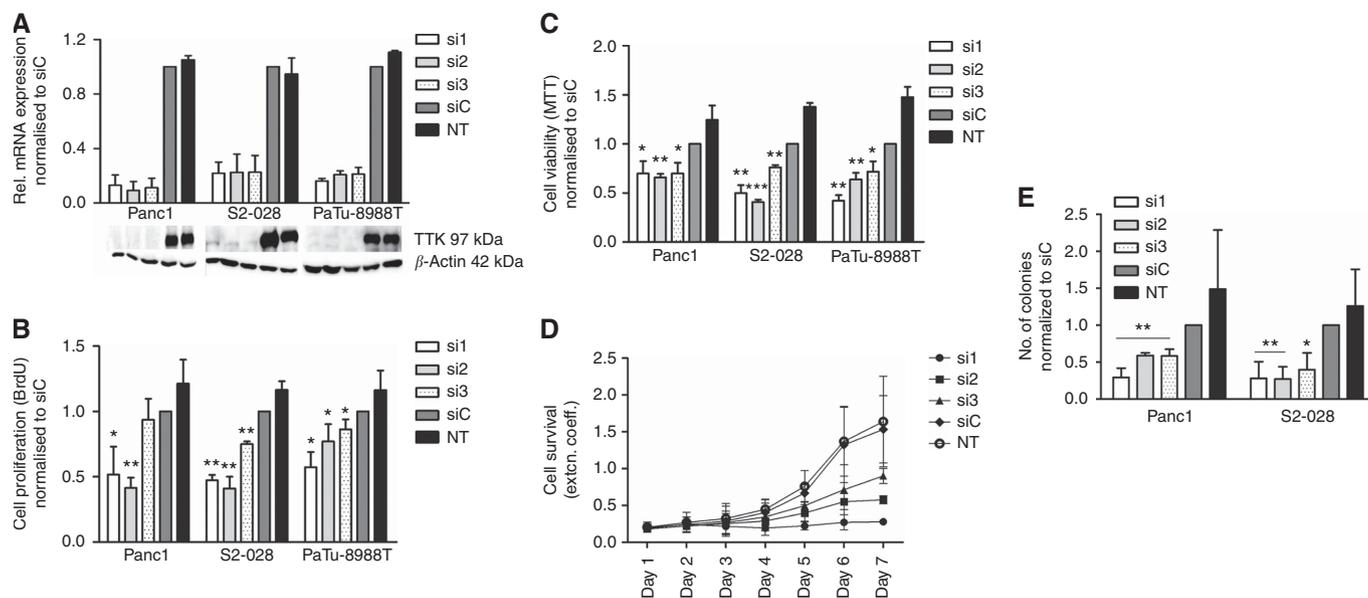
Figure 2. Impaired proliferation and viability of pancreatic cancer cells following RNAi-mediated inhibition of *TTK* expression. (**A**) Relative levels of *TTK* mRNA and protein after transient transfection of TTK-specific and control siRNAs. qRT-PCR analyses demonstrated that all three TTK-specific siRNAs led to ∼70% reduction in *TTK* mRNA levels compared with non-silencing control siRNA in three different pancreatic cancer cell lines (bar graphs). Expression was normalised to control siRNA-treated cells. Similarly, TTK protein levels were nearly undetectable 72 h after siRNA transfection as determined by western blot analyses (lower panels). Beta-actin served as loading control. Loss of TTK protein significantly impaired proliferation and viability of pancreatic cancer cell lines as measured by DNA BrdU incorporation (**B**) and MTT assays (**C**), respectively. (**D**) Long-term growth inhibition following transient silencing of *TTK* in PaTu-8988T cells was stable over at least 7 days as assessed by survival assay. (**E**) Soft agar assays established significantly reduced numbers of colonies in *TTK* knockdown populations of Panc1 and S2-028 cells compared with controls after 10 days of continuous culture under non-adherent conditions. All data represent mean ± s.e.m. of at least three independent experiments. siC = cells transfected with non-silencing control siRNA; NT = untreated cells. *P < 0.05; ** P < 0.01; *** P < 0.001 (Student's *t*-test).
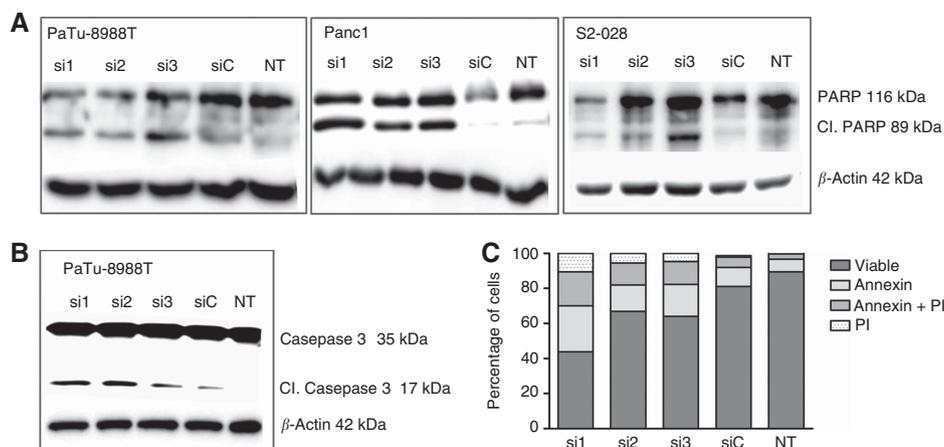
Figure 3. *TTK* knockdown leads to apoptotic and necrotic death of pancreatic cancer cells. Western blot analysis for induction of apoptosis showed enhanced PARP (**A**) and/or Caspase-3 (**B**) cleavage 72 h after siRNA transfection in all three pancreatic cancer cell lines used in the experiments. Data are representative of at least three independent experiments. Beta-actin served as loading control. (**C**) Flow cytometry analyses via Annexin-V/PI staining in PaTu-8988 T cells revealed significantly increased proportions of early (Annexin-V signal only) and late (Annexin-V plus PI signal) apoptotic cells as well as necrotic cells (PI staining only) following transient *TTK* knockdown. Data represent an average of three experiments. siC = cells transfected with non-silencing control siRNA; NT = untreated cells.

erlotinib, a small molecule inhibitor of EGFR pathway, remains the only targeted therapeutic substance that has been demonstrated to be effective in a subset of pancreatic cancer patients. There is thus a pressing need to identify new targets for novel approaches to the therapy of PDAC. In this context, we focussed on protein kinases, which are especially well suited for pharmacological inhibition, in an effort to identify novel candidates with specific overexpression and essential functional roles in PDAC.

In this report, we show that *TTK* is overexpressed in human pancreatic cancer tissues as compared with normal pancreas or chronic pancreatitis. Our results are in conformity with previous reports from high-throughput analyses listing *TTK* among the genes with increased expression in PDAC and mucinous neoplasms of the pancreas, respectively (Grutzmann *et al*, 2004; Sato *et al*, 2004). At present, it remains unclear though if *TTK* expression is a prognostic factor in pancreatic cancer. Moreover,
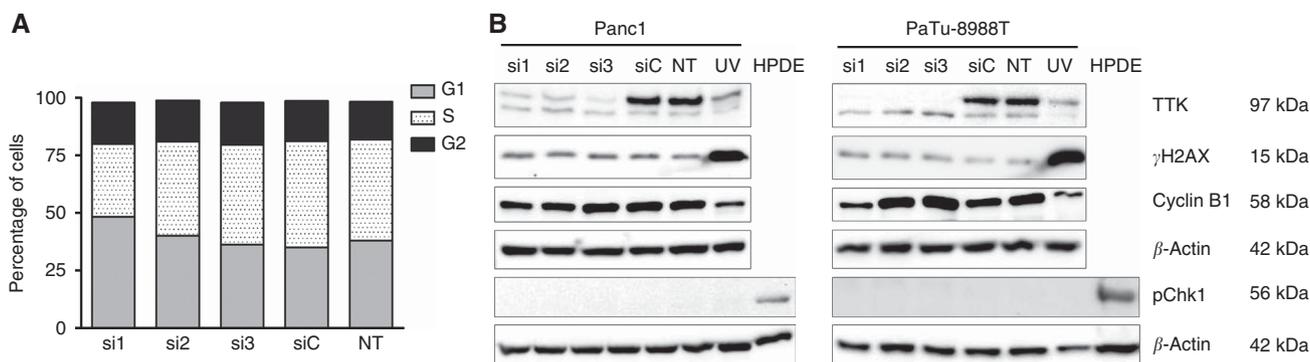
Figure 4. No evidence for cell cycle arrest or DNA damage following *TTK* knockdown. Cell cycle analysis was done after transient inhibition of *TTK* expression using FxCycle Violet Stain and flow cytometry of PaTu-8988T cells. *TTK* inhibition did not result in consistently altered cell cycle distribution (**A**). DNA damage, as assessed by western blot analyses using phospho-specific antibodies, did not reveal increased phosphorylation levels of the ATR/p53 targets H2AX and Chk1 after *TTK* silencing. Likewise, cellular Cyclin B1 levels were also not systematically altered (**B**). UV-treated cancer cells ('UV') served as positive controls for γH2AX detection; UV-treated normal human pancreatic ductal epithelial (HPDE) cells were used as positive controls for phospho-Chk1 detection. Beta-actin was used as a loading control in these experiments. siC = cells transfected with non-silencing control siRNA; NT = untreated cells.
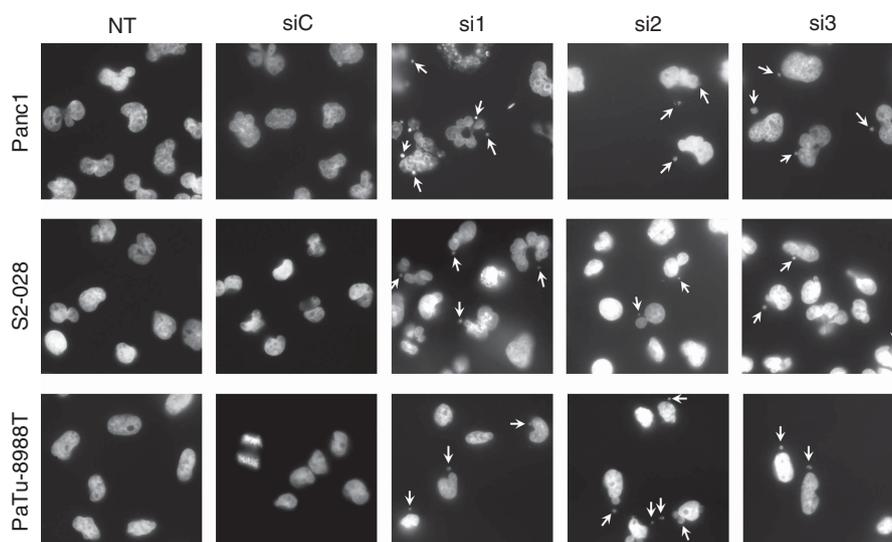
Figure 5. Loss of *TTK* function leads to genomic instability. Fluorescence microscopy was done using DAPI after transient knockdown of *TTK* expression. Microscopic analysis revealed formation of micronuclei (white arrows) in all the three cell lines tested, indicating genomic instability and chromosomal missegregation.

the functional significance of *TTK* overexpression in pancreatic cancer has previously not been analysed.

TTK is an essential component of the SAC, which in normal cells functions to ensure that anaphase is not initiated until each sister chromatid pair is correctly attached to the mitotic spindle (Stucke *et al*, 2002; Liu and Winey, 2012; Morin *et al*, 2012). The SAC thus serves as a safeguard against chromosome missegregation and subsequent aneuploidy. In an apparent contradiction to its role as a checkpoint protein, *TTK* has been shown to be overexpressed in breast cancer and other solid cancers (Yuan *et al*, 2006; Salvatore *et al*, 2007; Daniel *et al*, 2011; Tao *et al*, 2012), and RNAi-mediated or pharmacological inhibition of *TTK* function efficiently attenuated growth of a variety of cancer cell lines (Colombo *et al*, 2010; Tardif *et al*, 2011). Our own results demonstrate that this also holds true for pancreatic cancer. RNAi-mediated silencing of *TTK* gene expression significantly inhibited proliferation and viability of three different pancreatic cancer cell lines as measured by BrdU and MTT assays, strongly induced cell death by both, apoptotic as well as necrotic pathways, and

significantly attenuated anchorage-independent growth, a hallmark feature of malignantly transformed epithelial cells. Intriguingly, *TTK* inhibition seems to affect non-transformed cell types to a much lesser degree. In a report by Daniel *et al* (2011), transient silencing of *TTK* decreased viability of the breast cancer cells, but did not inhibit growth of the non-malignant MCF10A cell line. Similarly, Colombo *et al* (2010) describe that pharmacological inhibition of *TTK* function reduced viability of a variety of cancer cell lines, but not of normal cells. Our own experiments confirm this observation, as the immortalised but non-transformed hTERT-HPNE cell line was practically unaffected by transient silencing of *TTK*. Similar results have also been shown for other SAC components such as *Mad2* and *BubR1* (Kops *et al*, 2004; Michel *et al*, 2004; Janssen *et al*, 2009; Li *et al*, 2010).

As varying degrees of chromosomal aberrations are commonly observed in most cancers, it can thus be assumed that cancer cells require SAC functions to sustain growth and proliferation in the presence of low levels of aneuploidy. In addition, defects in other checkpoint functions are likely to contribute to the selective

sensitivity of transformed cells towards SAC inhibition. In normal cells, aneuploidy induces an ATR/p53 response, which results in cell cycle arrest at both, G1/S and G2/M boundaries, and thus prevents propagation of cells with improper chromosomal content (Toledo *et al*, 2008; Thompson and Compton, 2010). However, the p53 tumour suppressor is mutationally inactivated or deleted in many human cancers, including 50–75% of pancreatic tumours (Buchholz and Gress, 2009). Accordingly, RNAi-mediated *TTK* downregulation was not associated with phosphorylation of typical ATR/p53 targets such as H2AX and Chk1 in our cells, and flow cytometric analyses produced no evidence of a common cell cycle arrest phenotype across the individual siRNAs used in the experiments.

In contrast, H2AX phosphorylation was readily observed following exposure of the cells to UV light, demonstrating that part of the DDR machinery is still intact in the pancreatic cancer cells. At the same time, this implies that, contrary to what has been observed for MCF7 breast cancer and SW480 colon cancer cells (Janssen *et al*, 2011), SAC inhibition in pancreatic cancer cells does not result in appreciable levels of DNA damage. Instead, it appears that complete undamaged chromosomes were missegregated, as evidenced by the frequent formation of micronuclei after mitosis, which were readily detectable in *TTK* knockdown cells. This is in agreement with observations in A2780 ovarian cancer and HCT colon cancer cells, where *TTK* inhibition caused an overall increase in cellular γH2AX levels, but did not result in DNA damage and formation of γH2AX foci (Colombo *et al*, 2010; Tardif *et al*, 2011). As a final consequence, chromosomal segregation errors ultimately lead to massive aneuploidy, which is incompatible with cellular survival, inducing cell death by either apoptotic mechanisms or mitotic catastrophe.

Taken together, our results demonstrate that pancreatic cancer cells critically depend on the presence of functionally intact SAC machinery. As a kinase that is overexpressed in pancreatic tumours and represents an integral component of the SAC in pancreatic cancer cells, *TTK* thus appears to be an attractive target for the development of novel therapeutic strategies against this deadly disease. Additional antitumour potency may be gained by a combination with spindle poison-based chemotherapeutics such as nab-Paclitaxel, which is an emerging new standard in the treatment of pancreatic cancer patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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