Enhanced FGFR signalling predisposes pancreatic cancer to the effect of a potent FGFR inhibitor in preclinical models

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Background: Fibroblast growth factor receptor (FGFR) signalling has been implicated in pancreas carcinogenesis. We investigated the effect of FGFR inhibition in pancreatic cancer in complementary cancer models derived from cell lines and patient-derived primary tumour explants.

Methods: The effects of FGFR signalling inhibition in pancreatic cancer were evaluated using anti-FRS2 shRNA and dovitinib. Pancreatic cancers with varying sensitivity to dovitinib were evaluated to determine potential predictive biomarkers of efficacy. Primary pancreatic explants with opposite extreme of biomarker expression were selected from 13 tumours for *in vivo* dovitinib treatment.

Results: Treatment with anti-FRS2 shRNA induced significant *in vitro* cell kill in pancreatic cancer cells. Dovitinib treatment achieved similar effects and was mediated by Akt/Mcl-1 signalling in sensitive cells. Dovitinib efficacy correlated with FRS2 phosphorylation status, FGFR2 mRNA level and FGFR2 IIIb expression but not phosphorylation status of VEGFR2 and PDGFRβ. Using FGFR2 mRNA level, a proof-of-concept study using primary pancreatic cancer explants correctly identified the tumours' sensitivity to dovitinib.

Conclusion: Inhibiting FGFR signalling using shRNA and dovitinib achieved significant anti-cancer cancer effects in pancreatic cancer. The effect was more pronounced in FGFR2 IIIb overexpressing pancreatic cancer that may be dependent on aberrant stimulation by stromal-derived FGF ligands.

Pancreatic cancer remains a highly fatal disease despite efforts to improve the treatment over last several decades (American Cancer Society, 2011). Fibroblast growth factor receptors (FGFRs) are transmembrane proteins that, on binding with FGF ligands, trigger the phosphorylation of FGFR substrate 2 (FRS2), a key adaptor protein that is largely specific to FGFRs (Wesche *et al*, 2011). Phosphorylated FRS2 then recruits and activates elements of the Ras/MAPK and PI3K/Akt pathways. Fibroblast growth factor receptor signalling is terminated when the FGF–FGFR complex is endocytosed and ubiquitinatised. Fibroblast growth factor receptor signalling has also been shown to have an important role in

pancreatic ductal and stromal hyperplasia, and cancer progression. Several FGFs including FGF1, 2, 7 and 10 are overexpressed in pancreatic cancer (Kornmann *et al*, 1998; Mahadevan and Hoff, 2007). FGF2 stimulation has been linked to increased pancreatic cancer cell proliferation, motility, invasion and stromal hyperplasia (Escaffit *et al*, 2000; Kuniyasu *et al*, 2001; Nomura *et al*, 2008). The overexpression of FGF7, a soluble stromal factor, was linked to pancreatic cancer progression and increased metastatic potential (Yi *et al*, 1994; Zang *et al*, 2009). Preclinical studies showed that alterations in FGFR1 signalling modulated growth in pancreatic cancer cells (Liu *et al*, 2007; Chen *et al*, 2010). Elevated FGFR2

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expression is associated with more advanced disease and shorter patient survival (Yamanaka *et al*, 1993; Ohta *et al*, 1995; Cho *et al*, 2007), whereas increased FGFR2 phosphorylation has been associated with enhanced pancreatic cancer cell proliferation, migration, invasion, survival and tumour angiogenesis (Nomura *et al*, 2008; Katoh and Katoh, 2009; Wesche *et al*, 2011). In addition, *FGFR2* amplification was detected in a subset of pancreatic cancers during a genome-wide analysis (Nowak *et al*, 2005). As such, FGFR signalling may be a valid therapeutic target in pancreatic cancer.

Our group previously established a primary pancreatic cancer explant model by implanting and propagating surgically resected tumour tissues in SCID mice (Hylander *et al*, 2005; Philip *et al*, 2009). The primary tumours were maintained *in vivo* without passage through cell line phase and the model has been shown to closely mirror the biology of the donor patients' tumours (Philip *et al*, 2009). This platform has been used by us and others (Hylander *et al*, 2005; Rubio-Viqueira *et al*, 2006) in evaluating anti-cancer drugs preclinically.

Dovitinib is a highly potent inhibitor of FGFRs with kinase $IC_{50} < 10 \, nmol \, l^{-1}$; other targets include VEGFR2 and PDGFRβ (kinase $IC_{50} > 10 \, nmol \, l^{-1}$) (Lee, 2005). Preclinically, the small molecule has demonstrated FGFR-dependent anti-tumour effects in a breast cancer model independent of its activity against VEGFR and PDGFRβ (Dey *et al*, 2010). Taeger *et al* (2011) had previously reported the anti-proliferative and -metastatic effects of dovitinib in pancreatic cancer cell line model though the relationship to the underlying FGFR signalling activity was unclear. In this report, we extend this by investigating whether underlying FGFR signalling will affect the effect of a potent FGFR inhibitor such as dovitinib in pancreatic cancer using a complement of cell lines and primary patient-derived explant models. We hypothesise that pancreatic tumour with heighted FGFR signalling is more sensitive to the anti-cancer effects of agents inhibiting FGFR signalling.

MATERIALS AND METHODS

Drug. Dovitinib was obtained from Novartis Institutes for Biomedical Research (Basel, Switzerland). For *in vitro* proliferation assays, dovitinib was prepared as a $10 \, mmol \, l^{-1}$ solution in DMSO. For *in vivo* xenograft studies, dovitinib solution was formulated as $4 \, mg \, ml^{-1}$ in water for oral gavage.

Cell lines and *in vitro* studies. Human pancreatic cancer cell lines L3.6PL, Panc4.30 and Panc2.13 were gifted by Dr Manuel Hidalgo (Johns Hopkins University); and AsPC1, SU86.86 and Panc02.03 were from American Type Culture Collection (ATCC, Manassas, VA, USA). All of the pancreatic cancer cell lines were maintained in DMEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS (Sigma, St Louis, MO, USA) and penicillin–streptomycin and incubated at $37 \,^{\circ}C$ in a fully humidified atmosphere containing 5% CO_2. Pancreatic cell cultures were seeded into 24-well plates and treated with DMSO or indicated agents. Then cells were harvested and the extent of cell death was evaluated by Trypan blue stain counting by TC10 (Bio-Rad, Richmond, CA, USA). Each experiment was performed in triplicate.

RNA interference and gene overexpression studies. A constitutively active form of Akt1 (CA-Akt1) and Mcl-1 cDNA (Upstate, Lake Placid, NY, USA) was generously provided by Dr Shengbing Huang (Mayo Clinic, Rochester, MN, USA) (Rahmani *et al*, 2003) for gene overexpression studies. Briefly, cDNA was cloned into pCDH1-MCS1-EF1-Puro vector (System Bioscience, Mountain View, CA, USA) for lentivirus packaging in 293 TN cells. Pancreatic cells were infected with lentivirus with multiplicity of infection (MOI) of 5 under selective Puromycin $(1 \, \mu g \, ml^{-1})$.

RNA interference was based on pGreenPuro system (System Bioscience) expressing small hairpin RNA (shRNA). pGreen-FRS2α, pGreen-Mcl-1 and pGreenPuro-vec constructs, encoding shRNA for FRS2α (sh-FRS2), Mcl-1 (shMcl-1) or a negative control (vector) respectively, were prepared by inserting the target sequence for human FRS2α (shRNA1: 5′-CCGTGATAGACATC-GAGAGAA-3′ or shRNA2: 5′-CCGTGCAGAAGAATTATTT-3′) or Mcl-1 (5′-GGACTTTTATACCTGTTAT-3′) into pGreenPuro. 293 TN cell was stably transfected with the constructs and three packaging plasmids using Lipofectamine 2000 reagent (Invitrogen) to package lentivirus; and then pancreatic cells were infected with lentivirus with multiplicity of infection of 5. Clones with stable downregulated FRS2α or Mcl-1 expression were selected with puromycin $(1 \, \mu g \, ml^{-1})$.

Immunoblotting. For immunoblot analysis, the cells were treated with the indicated agents and then collected in lysis buffer (Cell Signaling, Danvers, MA, USA). Total protein was quantified using Coomassie protein assay reagent (Bio-Rad). An equal amount of protein $(60 \, \mu g)$ was separated by SDS–PAGE and electrotransferred onto nitrocellulose membrane. The following primary antibodies were used: FGFR2, VEGFR1, p-VEGFR2 (Y1214) and VEGFR2, p-PDGFRβ (Y751) and PDGFRβ (1 : 1000, R&D Systems, Minneapolis, MN, USA); Mcl-1 (1 : 1000, BD PharMingen, Sparks, MD, USA); p-Akt(S473), Akt, p-Erk1/2(T202/T204), Erk, p-GSK3β(S9), GSK-3β, Bid, tBid, cyclin D1, cleaved caspase 3, cleaved poly(ADP-ribose) polymerase (PARP), human Bcl-2 and Bcl-xL (1 : 1000–1 : 5000, Cell Signaling); p-FRS2α(Y196) and FRS2α (1 : 200, Santa Cruz Biotechnology, Santa Cruz, CA, USA). β-actin (1 : 500,000, Sigma) was measured as control for equal loading. Blots were exposed to HRP-conjugated goat anti-mouse or goat anti-rabbit IgG secondary antibodies (1 : 5000, KPL, Gaithersburg, MD, USA) and then developed by enhanced chemiluminescence (Pierce, Rockford, IL, USA). For semi-quantitative analysis, protein expression was quantified by densitometric analysis using Quantity One 4.6.5 (Bio-Rad). FRS2 phosphorylation ratio is calculated by the equation (p-FRS2α/FRS2α). In Figure 2F, FGFR2 expression, and the phosphorylation status of FRS2, VEGFR2 and PDGFRβ were compared ('normalised') to β-actin of L3.6PL and expressed as ratio.

RNA extraction and RT–PCR for FGFRs and subtypes. RNA from pancreatic cells or tumours was extracted using TRIzol reagent (Invitrogen) according to the manufacturers' protocol. cDNA was obtained from $5 \, \mu g$ of total RNA, using the SuperScript III Reverse Transcriptase kit (Invitrogen) with oligos-dT primers. Semi-quantitative PCR was performed as follows: $2 \, \mu l$ of $10 \times$ Buffer (Roche, Indianapolis, IN, USA), $0.2 \, \mu l$ of Taq polymerase $(5 \, U \, \mu l^{-1}$ Roche), $0.4 \, \mu l$ of $10 \, mM$ dNTP mix (Roche), $0.1 \, \mu l$ of each primer $(100 \, \mu M)$, $1 \, \mu l$ of cDNA, filled to a final volume of $20 \, \mu l$ with sterile H_2O. Thermal cycling reaction using an Icycler device (Bio-Rad) was: $94 \,^{\circ}C$ for 2 min; followed by 25–35 cycles of $95 \,^{\circ}C$ for 30 s, $60 \,^{\circ}C$ for 30 s, $72 \,^{\circ}C$ for 45 s for detection of FGFR2. The amplified products were further extended by additional incubation at $72 \,^{\circ}C$ for 10 min. PCR products were then loaded on a 1% agarose gel containing ethidium bromide. All quantitations were normalised to GAPDH. FGFR2 and GAPDH primers were as follows: FGFR1(IIIb) forward 5′-ACCAGTCTGCGTGGCTCACT-3′, reverse 5′-TGCCGGCCTCTCTTCCA-3′; FGFR1(IIIc) forward, 5′-GGACTCTCCCATCACTCTGCAT-3′, reverse 5′-CCCCTGTGCAATAGATGATGATC-3′; FGFR2 forward, 5′-TGACATTAACCGTGTTCCTGAG-3′, reverse 5′-TGGCGAGTCCAAAGTCTGCTAT-3′; FGFR2(IIIb) forward, 5′-GATAAATAGTTCCAATGCAGAAGTGCT-3′, reverse 5′-TGCCCTATATAATTGGAGACCTTCA-3′; FGFR2 (IIIc) forward, 5′-GGATATCCTTTCACTCTGCATGGT-3′, reverse, 5′-TGGAGTAAATGGCTATCTCCAGGTA-3′; GAPDH forward 5′-GAAGGCTGGGGCTCATTTG-3′, reverse 5′-AGGGGCCATCCACAG-TCTTC-3′.

Immunohistochemistry. Tumour tissue was fixed overnight in 10% neutral-buffered formalin at room temperature, transferred to 70% ethanol and processed for paraffin embedding using a Thermo Electron Excelsior tissue processor (Pittsburgh, PA, USA). Paraffin blocks were sectioned to $4\,\mu m$ thickness and placed on positively charged glass slides. Tissues were stained using a Discovery automated slide machine (Ventana Medical Systems, Tucson, AZ, USA). The primary antibodies used were Ki67 (1:750 dilution, Novocastra Laboratories, Newcastle upon Tyne, UK), and CD34 (EK-MP.12, 1:100 dilution, Accurate Chemical & Scientific Corp, Westbury, NY, USA). Secondary antibody was a goat anti-rabbit F(ab′)$_2$ biotinylated antibody, 1:100 dilution (Jackson Immuno-Research, West Grove, PA, USA). Sections were counter-stained with hematoxylin to enhance visualisation of tissue morphology. General tissue morphology was evaluated using H&E staining. For TUNEL assay, tissue samples were embedded in paraffin and cut into 4-μm-thick consecutive sections. After deparaffinised in three changes of xylene and rehydrated in descending concentrations of ethanol, the sections were treated with $20\,\mu g\,ml^{-1}$ proteinase K at $37\,^{\circ}C$ for 15 min and then incubated with TDT buffer containing $12.5\,\mu m$ biotinylated dUTP (Boehrinnger Mannheim, Mannheim, Germany) and 0.15 units per μl TDT (Takara, Kyoto, Japan) at $37\,^{\circ}C$ for 70 min. After terminated in terminating buffer (300 mm sodium chloride and 30 mm sodium citrate), the sections were incubated in streptavidin–peroxidase complex for 30 min and then developed with diaminobenzidine-tetra-hydrochloride for 1–5 min as a substrate. A pathologist, blinded to the treatments applied, analysed the staining semi-quantitatively and five representative high power fields per slide were evaluated for each marker. The proportion of tumour nuclei-stained positive were scored for Ki67 and TUNEL and expressed as percentage; and the number of tumour microvessels was scored per high power field to determine the microvessel density. The five representative high power fields were then averaged to determine the score for the slide.

***In vivo* xenograft studies.** The patient-derived primary pancreatic tumours #12424 and #10978 were previously established and maintained by the laboratory of Dr Elizabeth Repasky at Roswell Park Cancer Institute (RPCI, Buffalo, NY, USA) (Hylander *et al*, 2005). These primary tumours were maintained in mice and have never been passaged through cell lines. Tumours used here are generated from third and fourth passage generation for #12424 and #10978, respectively. Donor tumours were resected, minced into

small pieces, resuspended in PBS and implanted s.c. into the right hind flanks of female immunodeficient nu/nu mice (6–8 weeks old, 18–22 g, Charles River Laboratories, Wilmington, MA, USA). L3.6PL or Su8686 cells (5×10^6) were injected s.c. into the flank of SCID mice (RPCI). Tumours were monitored until they reached a mean tumour volume of 100 or 250 mm^3. Mice were assigned randomly to different groups (five mice per treatment group) before starting dovitinib dosing. Dovitinib was administered by oral gavage once daily at $40\,mg\,kg^{-1}$. Tumour volume was measured in two dimensions (length and width) twice weekly using Ultra Cal-IV calipers and was analysed using studylog software (Studylog Systems, San Francisco, CA, USA). Tumour volume (mm^3) = (length $\times$ width^2)/2. Per cent tumour growth inhibition (TGI) was determined as [1 − (change in mean tumour volume after 28 days of dovitinib treatment)/(change in mean tumour volume after 28 days of vehicle treatment)] $\times$ 100. Mouse body weights were also recorded twice weekly and the mice were observed daily. Mice with tumour volumes $\geqslant$ 2,000 mm^3 or with losses in body weight $\geqslant$ 20% from their initial body weight were promptly euthanised per Institutional Animal Care and Use Committee guidelines. All animal studies using primary pancreatic tumours and cell lines were approved by the Institutional Animal Care and Use Committee of RPCI (Workman *et al*, 2010). The oversight also included the handling of the human primary pancreatic tumours.

Statistical analysis. The reported values represent the means ± s.d. for at least three independent experiments performed in triplicate. The significance of differences between experimental variables was determined using Student's *t*-test.

RESULTS

Inhibition of FGFR signalling by FRS2α knockdown exerted potent pro-apoptotic effects in pancreatic cancer cell lines. FRS2α, a downstream adaptor protein for FGFR1–4, has a key role in mediating FGF signalling. We evaluated the effects of FRS2 knockdown using shRNA in pancreatic cancer cell lines to determine the dependency of cell viability on FGF signalling. Compared with the empty vector counterpart, FRS2α expression was mostly abrogated by shRNA1 or shRNA2 in L3.6PL, Panc4.30 and AsPC1 cells (Figure 1A). FRS2α-targeting shRNAs induced
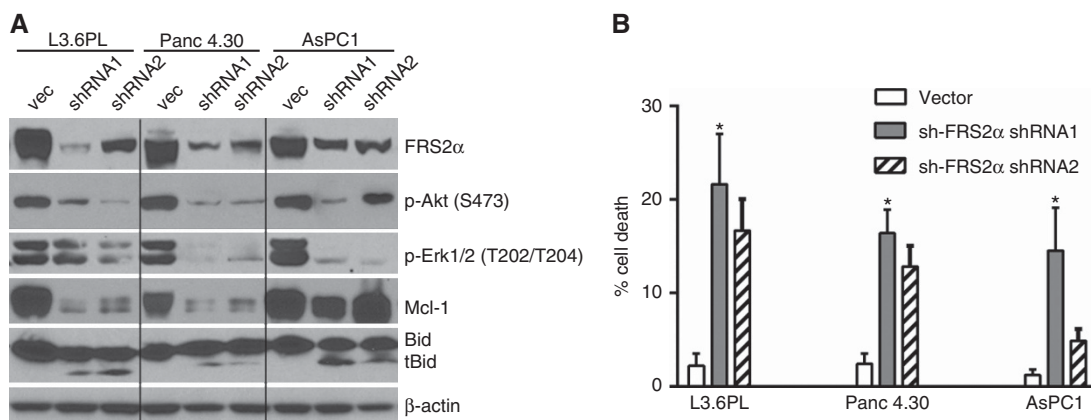

Figure 1. Inhibition of FGFR signalling by FRS2α knockdown exerted pro-apoptotic effects in pancreatic cancer cell lines, and was mediated via Akt/Mcl-1 axis. (**A**) Effect of FRS2α-targeting shRNAs on FGFR downstream signalling pathways in L3.6PL, Panc4.30 and AsPC1. Cells were infected with lentivirus-targeting shRNA1 (5′-CCGTGATAGACATCGAGAGAA-3′) or shRNA2 (5′-CCGTGCAGAAGAATTATTT-3′). Knockdown of FRS2α was confirmed and downstream signalling molecules were detected by immunoblotting. shRNA1 had higher efficiency in the knockdown. (**B**) The cell-killing effects of FRS2α-targeting shRNA1 and 2. The extent of cell death following infection with sh-FRS2α (shRNA1) lentivirus or vector was determined by Trypan blue stain according to Materials and Methods. In each case, values represent the means ± s.d. for three experiments (*$P < 0.05$, compared with negative control).

marked decrease of phosphorylated AKT or ERK, with a decrease in Mcl-1 and cleaved Bid expression. These changes were accompanied by increased cell death compared with empty vector counterparts (Figure 1B; $P < 0.05$), suggesting the dependence of L3.6PL, Panc4.30 and AsPC1 on FGFR signalling, and that the AKT and ERK pathways may have a functional role in FRS2α shRNA-induced cell death.

Dovitinib treatment exerted significant pro-apoptotic effect in pancreatic cancer cell lines with heightened FGFR signalling activation. We next evaluated the feasibility of targeting FGFR signalling in pancreatic cancer using dovitinib, a potent pan-FGFR small molecule inhibitor. Dovitinib is also a potent inhibitor of

PDGFRβ and VEGFR2, though Dey *et al* (2010) previously demonstrated that the major effects of dovitinib were primarily related to FGFR blockade. The dose–response effect of dovitinib was evaluated in a panel of six human pancreatic cancer cell lines (L3.6PL, Panc4.30, AsPC1, Panc2.13, SU86.86 and Panc02.03). In Figure 2A, pancreatic cancer cells were treated with increasing concentrations of dovitinib (0–10 μM) for 3 days. Using 10 μM as a cutoff, Panc2.13, SU86.86 and Panc02.03 were considered as resistant (IC_{50} not identified), and L3.6PL, Panc4.30 and AsPC1 sensitive to dovitinib treatment ($IC_{50} < 10$ μM). The expression of FGFR1–4 was determined in Figure 2B and were not significantly different between dovitinib-sensitive and –resistant cell lines (Supplementary Figure S1). We evaluated the status of apoptotic
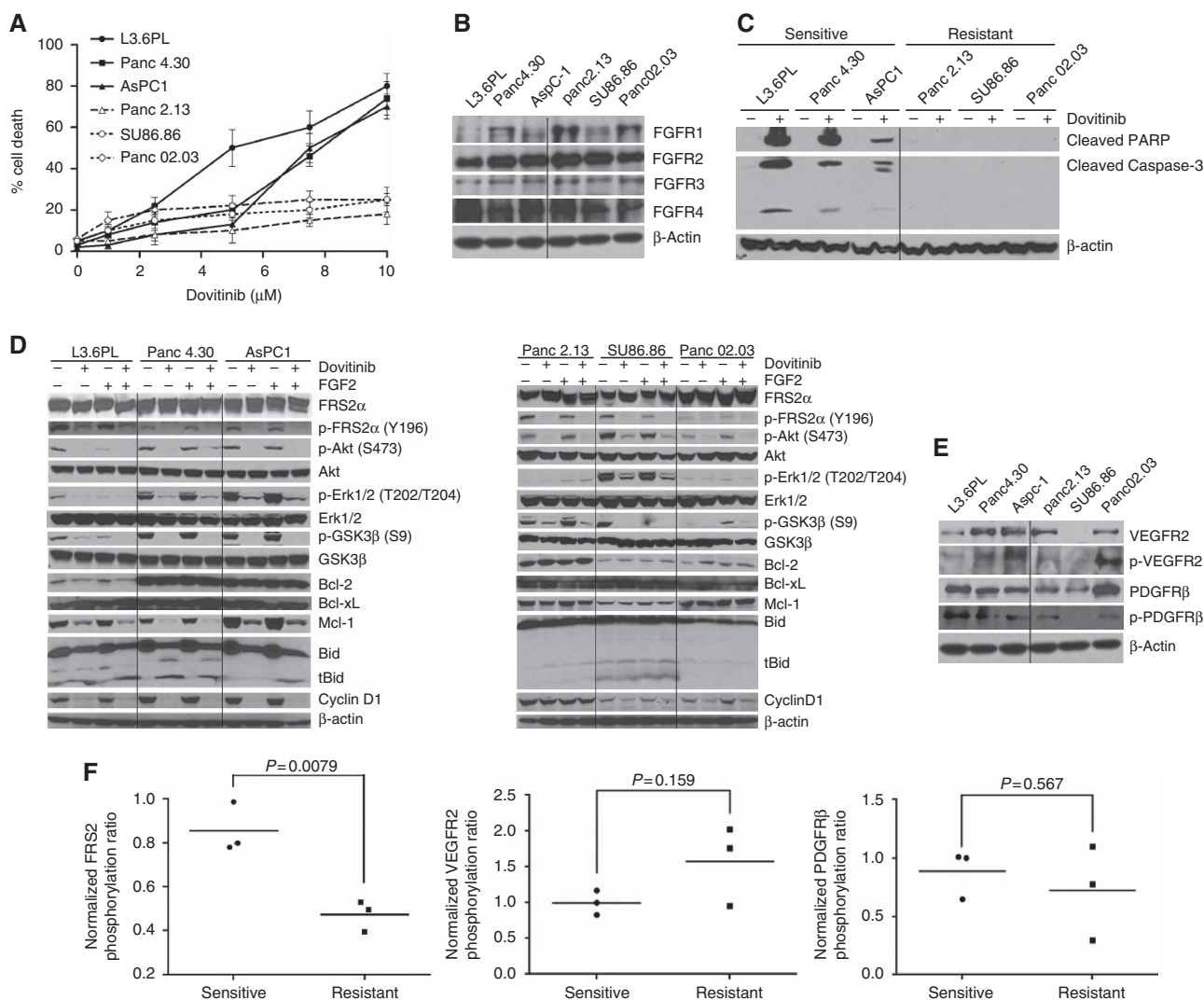

Figure 2. Dovitinib's pro-apoptotic effect was related to FGFR signalling inhibition and more pronounced in pancreatic cancers with elevated p-FRS2/FRS2 ratio. (**A**) Dose–response of the *in vitro* anti-cancer effects of dovitinib in six pancreatic cancer cell lines. Pancreatic cancer cells were exposed to 0–10 μM dovitinib for 3 days. The extent of cell death was monitored by Trypan blue stain according to Materials and Methods. In each case, values represent the means ± s.d. for three experiments. (**B**) Expression of FGFR1, 2, 3 and 4 in untreated cell lines determined by immunobloting. (**C**) Serum growth-sensitive (L3.6PL, Panc4.30, AsPC1) and -resistant (Panc2.13, SU86.86, Panc02.03) cells were treated with 10 μM dovitinib for 72 h and whole-cell lysates were immunoblotted with cleaved caspase 3 and cleaved PARP antibodies. (**D**) Overnight serum-starved cells were pretreated with 10 μM dovitinib for 1 h with (+) or without (−) FGF2 (10 ng ml^{-1}) stimulation. Lysates from sensitive (**D**, left panel) and resistant (**D**, right panel) cells were subject to SDS–PAGE and immunoblotted with the indicated antibodies. (**E**) Expression of phosphorylated and total VEGFR2 and PDGFRβ in untreated cell lines determined by immunoblotting. (**F**) Expression of p-/t-FRS2, p-/t-VEGFR2 and p-/t-PDGFRβ in untreated pancreas cancer cell lines from panels **D** and **E** were measured and normalised to β-actin for each cell line according to Materials and Methods. Phosphorylation status was calculated using the formula (phosphorylated protein/total protein). The relative phosphorylation status of FRS2, VEGFR2 and PDGFRβ of each cell line were compared with L3.6PL in the scatterplots.
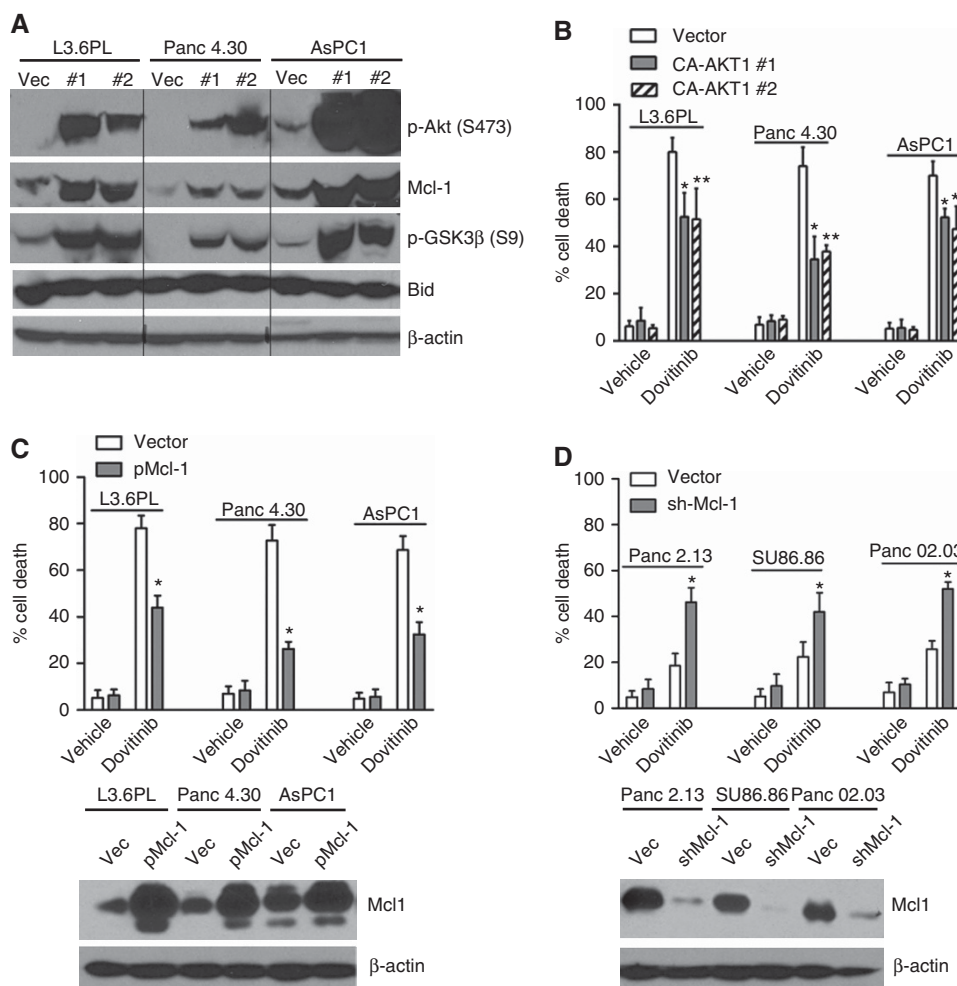
Figure 3. Akt and Mcl mediate dovitinib's anti-cancer effects in pancreatic cancer cells. (**A**) Dovitinib-sensitive pancreas cancer cell lines, L3.6PL, Panc4.30 and AsPC1, were stably transfected with a CA-AKT1 and two single clones, #1 and #2, were selected for analysis. Ectopic expression of CA-AKT1 dramatically increased the expression of Mcl-1 and phosphorylated GSK-3β. (**B**) Sensitive cells ectopically expressing CA-AKT1 were treated with 10 μM dovitinib for 3 days, after which the extent of cell death was determined by Trypan blue stain according to Materials and Methods. Pancreatic cancer cells were stably transfected with Mcl-1 in sensitive cell lines (**C**) or infected with lentivirus expressing shRNA-targeting Mcl-1 in resistant cell lines (**D**). Cells were harvested and efficiency of overexpression or knockdown of Mcl-1 was confirmed by western blotting. Treated cells were harvested for cell death rate analysis. In each case, values represent the means ± s.d. for three experiments (*$P < 0.05$, compared with negative control).

markers in Figure 2C and observed marked mitochondrial-mediated apoptosis with cleavage of caspase 3 and PARP in sensitive cell lines compared with resistant cell lines.

The expression of signalling proteins downstream to FGFRs of sensitive cell lines were then compared with resistant cells to elucidate the underlying mechanisms of dovitinib's pro-apoptotic effect. FGF2 stimulation following serum starvation, to eliminate signal by other growth factors, was used to better characterise dovitinib's effect on FGFR signalling. Western blot analysis validated the presence of FGFR2 and FRS2α in all cell lines tested (Figure 2D). The expression of p-FRS2α (Y196), a docking site for Grb2-Sos complexes, was decreased with dovitinib treatment in both sensitive and resistant cell lines, indicating inhibition of FGFRs by dovitinib. Decreased p-FRS2α expression by dovitinib treatment was associated with marked decrease in the phosphorylation (activation) of AKT, GSK-3β and Erk in both sensitive and resistant cell lines, suggesting that Akt and Erk signalling inhibition were pharmacodynamics downstream effects by dovitinib but did not predict anti-cancer effect.

The expression of Bcl-2 family members were analysed and no major changes in expression of Bcl-2 and Bcl-xL proteins were observed following treatment. Interestingly, Mcl-1 was downregulated with dovitinib treatment in sensitive cell lines but no significant changes in Mcl-1 level was observed in resistant cell lines. In the sensitive but not resistant cell lines, dovitinib treatment decreased Bid expression, a key BH3 domain-only protein, with associated increase in cleaved Bid (tBid). No changes were observed in other BH3 domain-only proteins (Bim, PUMA and Bad, data not shown). This suggests that dovitinib treatment induced Bid cleavage by caspase 8 to tBid, which translocated to mitochondria and induced apoptosis via cytochrome c release. Cyclin D1, a cell proliferation marker, was decreased more significantly following dovitinib treatment in sensitive cells and not the resistant cells.

To investigate whether the activity of FGFR, VEGFR2 and PDGFRβ signalling affect dovitinib's pro-apoptotic effect, we contrasted FGFR1–4 expression (Figure 2B), and phosphorylation ratio of FRS2α (Figure 2D), VEGFR2 and PDGFRβ (Figure 2E) between untreated sensitive and resistant cells. There was significantly elevated FGFR signalling activity in untreated dovitinib-sensitive cells, as measured by higher FRS2 phosphorylation ratio, than resistant cells ($P = 0.0079$) but not that of VEGFR2
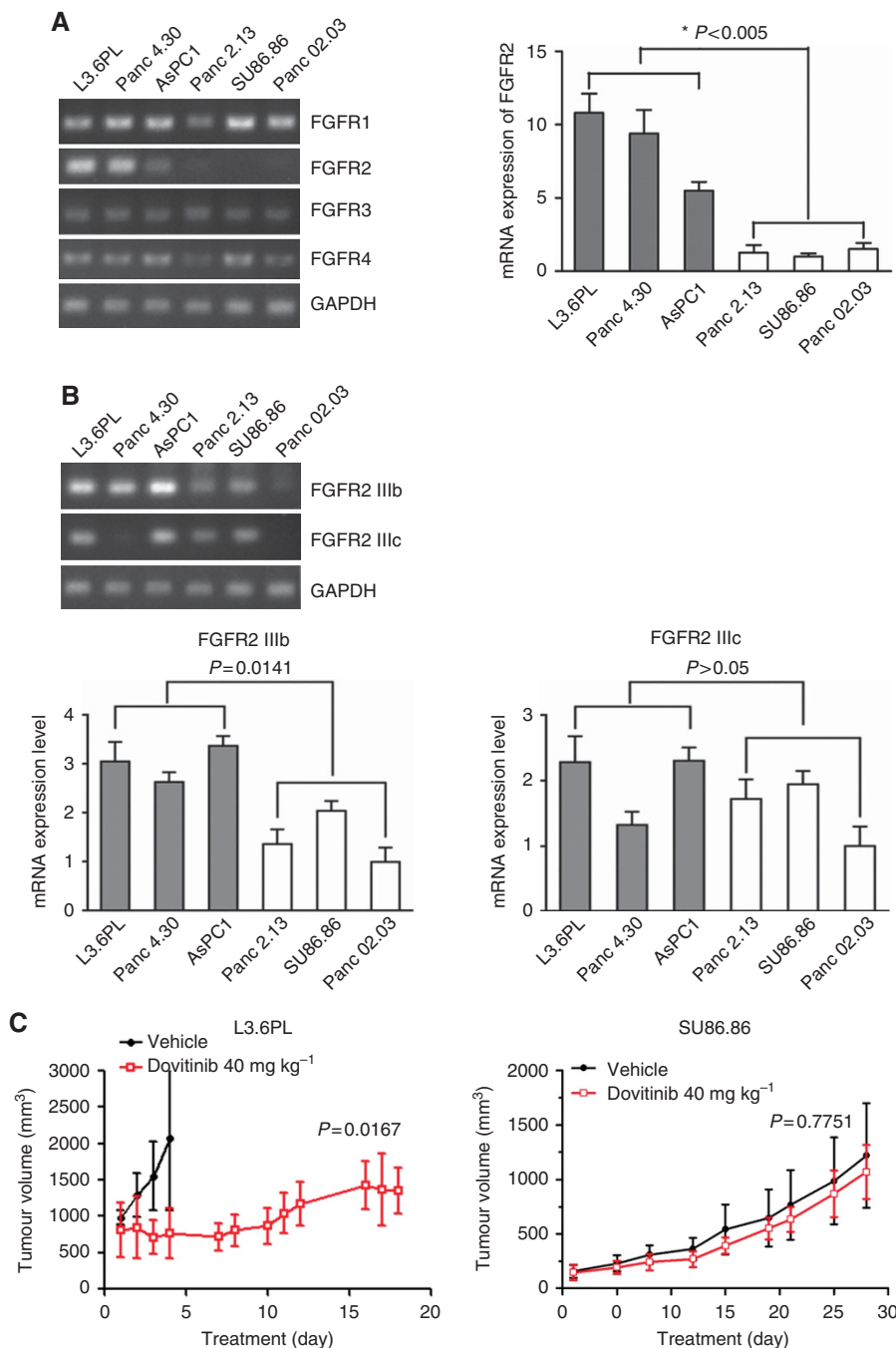
Figure 4. *In vivo* growth inhibition by dovitinib in pancreatic cancer cell line xenografts is related to FGFR2 IIIb level. (**A**) Expression of FGFR1, 2, 3 and 4 mRNAs in six pancreatic cell lines. Transcript levels were measured by semi-quantitative RT–PCR analysis with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control. The FGFR2 mRNA level was normalised to GAPDH for each cell line and presented in the bar chart. (**B**) Expression levels of IIIb and IIIc subtypes of FGFR2 were measured semi-quantitatively by RT–PCR, normalised to GAPDH for each cell line and presented in bar charts. (**C**) Mice bearing L3.6PL (high FGFR2 mRNA level) or SU86.86 (low FGFR2 mRNA level) tumour received orally administered dovitinib at $40\,mg\,kg^{-1}$ or vehicle (control) once daily. Tumour volume was measured twice weekly over the course of the study and values represent the means ± s.d. There were five mice per treatment arm.

and PDGFRβ (Figure 2F); and, there was no correlation between dovitinib sensitivity and FGFR1–4 expression (Supplementary Figure S1).

AKT/Mcl-1 axis mediates dovitinib's pro-apoptotic effect in sensitive but not resistant cells. The PI3K/Akt and MAPK pathways are key mediators of FGF signalling with the former being a primary transmitter of anti-apoptotic signals in cancer cells (Beenken and Mohammadi, 2009; Wesche *et al*, 2011).

To investigate whether Akt signalling had a functional role in mediating dovitinib-induced apoptosis, sensitive cell lines were stably transfected with a constitutively active AKT1 (CA-AKT1) and two single clones for each were selected for analysis (Figure 3A). Overexpression of CA-AKT1 dramatically increased the expression of Mcl-1 and phosphorylated GSK-3β, indicating the AKT-dependent regulation of Mcl-1. Notably, no cleaved Bid was detected in CA-AKT1-treated sensitive cells. Compared with empty vector counterparts, cell death after treatment with dovitinib

was substantially reduced in CA-AKT1-treated cells ($P < 0.01$ in Figure 3B). We next investigated the role of Mcl-1 by performing studies to overexpress and knockdown Mcl-1 in sensitive and resistant cell lines, respectively. Compared with empty vector counterparts, ectopic expression of Mcl-1 in sensitive cell lines dramatically reduced cell deaths by dovitinib (Figure 3C; $P < 0.01$).

Conversely, Mcl-1 abrogation using shRNA significantly increased cell death in dovitinib-resistant cell lines (Figure 3D; $P < 0.05$), suggesting Mcl-1 had a functional role in mediating dovitnib's anti-cancer effect.

Taken together, these results indicate that, in sensitive cells, the AKT/Mcl-1 is a key mediator of dovitinib's pro-apoptotic effect.
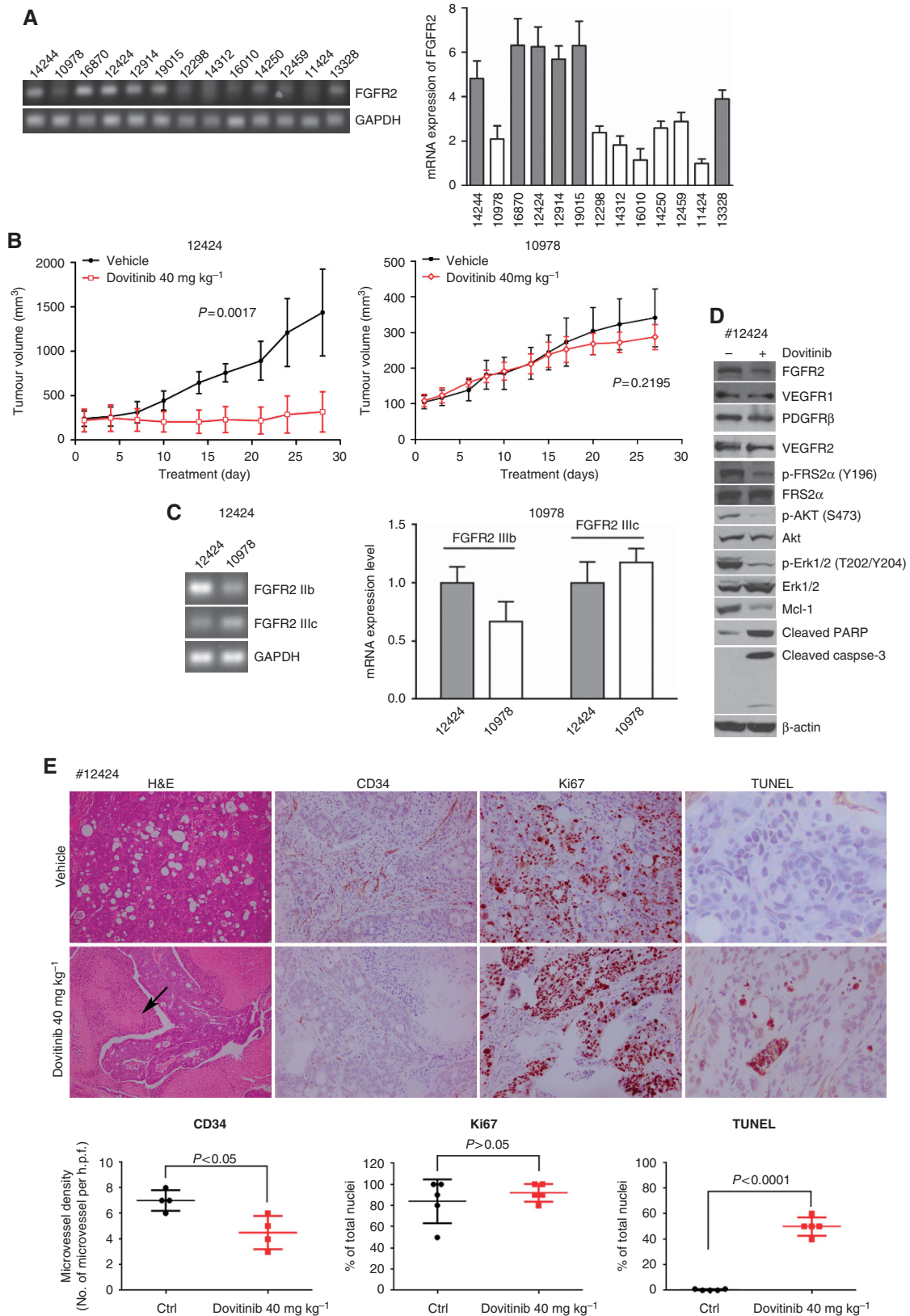
However, the signalling cascades linking Akt to Mcl-1 remained to be elucidated. Previous studies indicated that GSK-3β, inactivated by Akt, phosphorylates Mcl-1 on Serine 159, an event that promoted Mcl-1 degradation (Maurer *et al*, 2006; Ding *et al*, 2007). Here, overexpression of CA-AKT1 potentiated phosphorylated GSK-3β (inactivation), supporting GSK-3β as an intermediate regulator of Mcl-1.

Dovitinib's anti-cancer effects correlated with FGFR2 IIIb mRNA level in pancreatic cancer. Our *in vitro* studies in Figure 2F showed that dovitinib's pro-apoptotic effect was most pronounced in pancreatic cells with heightened FGFR signalling as indicated by increased FRS2 phosphorylation ratio. As we did not detect significant difference in the expression level of FGFR1–4 between dovitinib-sensitive and -resistant cells, we investigated their mRNA expression level and found a significantly higher FGFR2 mRNA level in the sensitive cells (L3.6PL, Panc4.30 and AsPC1) than the resistant (Panc2.13, SU8686 and Panc02.03) (Figure 4A) but not FGFR1, 3, 4 (Supplementary Figure S2A).

The importance of FGFR1 and 2 in pancreas carcinogenesis had previously been reported and the phenotype may be altered by the variation in the splicing in the Ig-like domain III of the receptor (IIIb and IIIc isoforms) (Nomura *et al*, 2008; Chen *et al*, 2010). Relationship between dovitinib's pro-apoptotic and expression of IIIb and IIIc isoforms of FGFR1 and 2 was then investigated. We observed significantly higher FGFR2 IIIb mRNA level in dovitinib-sensitive pancreatic cells than resistant cells (Figure 4B) but the same was not observed for FGFR2 IIIc and FGFR1 isoforms (Supplementary Figure S2B).

Next, we investigated if the above *in vitro* observation could be similarly observed *in vivo*, and SCID mice bearing tumours derived from high (L3.6PL) and low (SU86.86) FGFR2 mRNA expressing cell lines in SCID mice were treated with dovitinib (Figure 4C). Significant tumour growth inhibition was observed in L3.6PL following dovitinib treatment but not SU86.86, consistent with observations from *in vitro* studies.

FGFR2 mRNA expression predicted for dovitinib efficacy in patient-derived primary pancreatic cancer explant model. Based on studies above, we hypothesised that dovitinib exerts significant tumour growth inhibition in primary pancreas tumours with a high FGFR2 mRNA level but not in low-expressing tumours. A panel of 13 patient-derived primary pancreas cancer explants was evaluated for FGFR2 mRNA expression by RT–PCR (Figure 5A), and primary tumours #12424 (high FGFR2 mRNA level) and #10978 (low FGFR2 mRNA level) were selected for *in vivo* efficacy studies. Following 28 days of dovitinib treatment, compared with control, significant tumour growth inhibition was observed in #12424 (TGI 91.9%) and not in #10978 (TGI 15.8%) (Figure 5B). There was no significant difference in body weights and side effects between the vehicle and dovitinib-treated animals at the dose evaluated (Supplementary Figure S3).

Representative tumours were harvested at the end of 28 days of treatment and analysed for changes in the FGFR pathway signalling proteins. The FGFR2 IIIb mRNA level was higher in the untreated tumours of #12424 (dovitinib-sensitive) than the resistant #10978 (Figure 5C). In the dovitinib-sensitive #12424, dovitinib-treated tumour had decreased expression of p-FRS2α, p-AKT, p-ERK and Mcl-1 than control (Figure 5D). The expression of VEGFR2 and PDGFRβ were not significantly different following treatment. Hematoxylin and eosin staining showed broad necrosis of core tumour tissue in dovitinib-treated tumours (Figure 5E, arrow). The microvessel density, evaluated by CD34, was significantly less in dovitinib-treated tumour (4.5 ± 0.6) than control (7.0 ± 0.4, $P < 0.05$). TUNEL expression was significantly higher in the dovitinib-treated (50.0 ± 3.2) than control (0.4 ± 0.2, $P < 0.0001$), whereas the proliferative index Ki67 was not significantly different between dovitinib-treated (92 ± 4) and control (84 ± 9, $P > 0.05$).

DISCUSSION

The clinical development of molecularly targeted drugs had largely failed in pancreatic cancer so far despite encouraging preclinical rationales. The failure may be due to the highly heterogeneous nature of the disease (Jones *et al*, 2008). FGF/FGFR signalling has been implicated in pancreatic carcinogenesis and an understanding of the susceptible molecular characteristics may facilitate the development of FGFR inhibitors. A main aim of this report was to determine whether FGFR signalling activity influenced the efficacy of a potent FGFR inhibitor in pancreatic cancer. Using gene manipulation techniques, we confirmed that FGFR signalling inhibition by *FRS2*α gene knockdown did exert pro-apoptotic effects in pancreatic cancer cell lines. We then showed that treatment with dovitinib, a potent FGFR multikinase inhibitor, achieved effects similar to *FRS2*α gene knockdown in two complementary preclinical models derived from cell lines and patient-derived primary tumour explants. The next aim was to identify the molecular features associated with dovitinib efficacy. In contrast to report of Taeger *et al* (2011) that focused primarily on the pharmacodynamics effects of dovitinib , we evaluated dovitinib in a panel of pancreas cancer cell lines and primary tumour explants with varying degree of dovitinib sensitivity, and showed that pancreatic cancers with heightened FGFR signalling were predisposed to dovitinib's anti-cancer effect. In addition, we found that FGFR2 mRNA level, particularly FGFR2 IIIb isoform, may be predictive of dovitinib sensitivity.

Dovitinib, in addition to FGFRs, also abrogates VEGFR2 and PDGFRβ signalling in the low nanomolar concentration range (Lee, 2005). Taeger *et al* (2011) showed that dovitinib's anti-cancer effects in pancreatic cancer were related to co-inhibition of these kinases and the relative contribution of

Figure 5. FGFR2 mRNA expression level predicts for dovitinib sensitivity in patient-derived primary pancreatic cancer explant model.
(**A**) Expression levels of FGFR2 mRNA were determined in 13 patient-derived primary pancreatic tumours. Transcript levels were measured by semi-quantitative RT–PCR analysis with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control. The FGFR2 mRNA level was normalised to GAPDH for each tumour and presented in the bar chart. (**B**) Primary pancreatic tumour #12424 (high FGFR2 mRNA level) and #10978 (low level) were implanted s.c. into the right hind flanks of female immunodeficient nu/nu mice, and treated with vehicle ($n = 5$) or dovitinib ($n = 5$) orally once daily at $40\,mg\,kg^{-1}$ when the tumours grew to pre-determined size. Tumour volume and mice weight were monitored twice weekly over the course of the study. (**C**) mRNA level of FGFR2 IIIb and IIIc subtypes by RT–PCR in untreated #12424 and #10978 were normalised to GAPDH, and presented in bar charts. (**D**) Lysates from #12424 tumours treated with $40\,mg\,kg^{-1}$ dovitinib or vehicle were subject to immunoblotting as indicated. (**E**) Immunohistochemical staining of pancreatic tumours treated with dovitinib and vehicle. The harvested tumours from primary tumour #12424 were processed and the slides were stained with antibodies to H&E (magnification, $\times 100$), CD34 (magnification, $\times 100$), Ki67 (magnification, $\times 100$) or TUNEL (magnification, $\times 200$ in #12424 group and $\times 100$ in SU86.86 group). Representative fields are shown. Arrow indicates necrosis. The expression level of CD34, Ki67 and TUNEL of representative fields were scored by a pathologist blinded to treatment applied, and presented in scatterplots.

respective receptor signalling was difficult to determine. Dey *et al* (2010) reported that, in breast cancer model, dovitinib's anti-cancer effects were mediated primarily by FGFR inhibition and not VEGFR2 and PDGFRβ. Here, we found that dovitinib's anti-cancer effects were related directly to elevated FGFR pathway activation/phosphorylation status in untreated/control pancreatic cancer cells but not to that of VEGFR2 and PDGFRβ signalling. Next, using FGFR2 mRNA expression level as a surrogate for FGFR activity, we correctly identified one dovitinib-sensitive and one -resistant tumour from a panel of 13 patient-derived primary pancreatic cancer explants. Furthermore, dovitinib's efficacy seemed to be related to the expression level of FGFR2 IIIb isoform and not of FGFR2 IIIc. As such, evidence so far seemed to suggest that FGFR pathway inhibition is more likely the predominant contributor to dovitnib's anti-cancer effects in pancreatic cancer.

The intracellular kinase domain of FGFRs is structurally similar to VEGFR2 and PDGFR. The extracellular domains II and III of FGFRs constitute the binding site for FGF ligands (Wesche *et al*, 2011). Alternative splicing in domain III in FGFR1–3, not FGFR4, creates isoforms (IIIb and IIIc) with varying binding affinity to various FGF ligands (Katoh and Katoh, 2009). Physiologically, FGFR IIIb and IIIc isoforms are differentially expressed in epithelial and mesenchymal cell types, respectively, and are regulated by distinct groups of FGF ligands. Altered splicing in FGFRs switches ligand-binding affinity and can allow tumour cells to be stimulated by a broader range of FGFs than under physiological conditions, leading to aberrant paracrine signalling loop (Brooks *et al*, 2012). In prostate cancer, gain in FGFR2 IIIc and loss of FGFR2 IIIb expression was linked to progression from androgen dependence to independence; and in rat bladder cancer, gain in FGFR2 IIIc expression was associated with epithelial-to-mesenchymal transition (Yan *et al*, 1993; Baum *et al*, 2008). FGFR2 IIIc overexpression was linked to increased pancreatic cancer cell proliferation and conferred stem cell-like phenotype, and correlated with earlier liver recurrence in pancreatic cancer patients following surgical resection (Ishiwata *et al*, 2012).

Our study is the first to report on the relationship between FGFR2 IIIb expression and susceptibility to a potent FGFR inhibitor. Dovitinib inhibits FGFR signalling by interrupting the intracellular kinase activity and as such, splice variations in the FGF ligand-binding domain III should not significantly affect dovitinib's effect on these isoforms. Moreover, dovitinib successfully abrogated the phosphorylation of FRS2 that is a downstream signalling adaptor to FGFR1–4. The differential impact of dovitinib is thus more likely due to preferential targeting of pancreatic cancers overexpressing FGFR2 IIIb that are dependent on paracrine regulation by mesenchymal-derived FGF ligands. FGF7, FGF10 and FGF22 are subfamily of FGFs secreted by mesenchymal cells such as fibroblasts, endothelial and inflammatory cells that specifically bind to FGFR2 IIIb on epithelial cells to regulate embryogenesis and adult tissue homoeostasis (Katoh, 2008). FGFR2 IIIb overexpression was linked to poorer prognosis in pancreatic cancer patients and interactions between stromal-derived FGF10 and FGFR2 IIIb enhanced pancreatic cancer cell migration and invasion in *in vitro* studies (Nomura *et al*, 2008). FGF7 overexpression had also been linked to pancreatic cancer aggressiveness (Yi *et al*, 1994; Zang *et al*, 2009).

Interestingly, even though we observed a differential expression of FGFR2 mRNA level between dovitinib-sensitive and -resistant cells, the same was not true for FGFR2 protein by immunobloting. However, it was clear that there was heightened FGFR signalling indicated by increased FRS2 phosphorylation in the sensitive cells. According to current understanding of receptor tyrosine kinase physiology (Wesche *et al*, 2011), a potential explanation is that, in sensitive cells, FGFR2 degradation and recycling was accelerated following increased FGFR2 activation that led to a compensatory increase in *FGFR2* gene transcription to maintain a steady supply of FGFR2 ready for ligand binding. The end result is thus no significant difference in receptor expression between cells with and without heighted FGFR2 signalling activity.

CONCLUSION

In summary, we showed that dovitinib's anti-cancer effect in pancreatic cancer correlated with the underlying FGFR signalling activity, and the efficacy may be most pronounced in cancer cells overexpressing FGFR2. We propose that FGFR2 IIIb overexpression enhances cancer cells' ability to interact with and become dependent on paracrine stimulation by mesenchymal-derived FGF ligands. Such hypothesis will need further investigation and be validated in other cancer types. We plan to investigate the relevance of these potential predictive biomarkers in ongoing pancreatic cancer clinical trials using dovitinib at our institution (ClinicalTrials.gov ID NCT01497392).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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