

# Metronomic gemcitabine suppresses tumour growth, improves perfusion, and reduces hypoxia in human pancreatic ductal adenocarcinoma

KKY Cham<sup>1</sup>, JHE Baker<sup>2</sup>, KS Takhar<sup>1,3</sup>, JA Flexman<sup>1</sup>, MQ Wong<sup>1</sup>, DA Owen<sup>4</sup>, A Yung<sup>5</sup>, P Kozlowski<sup>5</sup>, SA Reinsberg<sup>5</sup>, EM Chu<sup>1</sup>, C-WA Chang<sup>1</sup>, AK Buczkowski<sup>6</sup>, SW Chung<sup>6</sup>, CH Scudamore<sup>6</sup>, AI Minchinton<sup>2</sup>, DTT Yapp<sup>1,3</sup> and SSW Ng<sup>\*,1,3</sup>

<sup>1</sup>Department of Advanced Therapeutics, British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver, British Columbia, Canada V5Z 1L3;

<sup>2</sup>Department of Medical Biophysics, British Columbia Cancer Agency, Vancouver, British Columbia, Canada; <sup>3</sup>Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada; <sup>4</sup>Department of Pathology & Laboratory Medicine, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada; <sup>5</sup>Magnetic Resonance Imaging Research Centre, University of British Columbia, Vancouver, British Columbia, Canada; <sup>6</sup>Department of Surgery, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada

**BACKGROUND:** The current standard of care for pancreatic cancer is weekly gemcitabine administered for 3 of 4 weeks with a 1-week break between treatment cycles. Maximum tolerated dose (MTD)-driven regimens as such are often associated with toxicities. Recent studies demonstrated that frequent dosing of chemotherapeutic drugs at relatively lower doses in metronomic regimens also confers anti-tumour activity but with fewer side effects.

**METHODS:** Herein, we evaluated the anti-tumour efficacy of metronomic vs MTD gemcitabine, and investigated their effects on the tumour microenvironment in two human pancreatic cancer xenografts established from two different patients.

**RESULTS:** Metronomic and MTD gemcitabine significantly reduced tumour volume in both xenografts. However,  $K^{trans}$  values were higher in metronomic gemcitabine-treated tumours than in their MTD-treated counterparts, suggesting better tissue perfusion in the former. These data were further supported by tumour-mapping studies showing prominent decreases in hypoxia after metronomic gemcitabine treatment. Metronomic gemcitabine also significantly increased apoptosis in cancer-associated fibroblasts and induced greater reductions in the tumour levels of multiple pro-angiogenic factors, including EGF, IL-1 $\alpha$ , IL-8, ICAM-1, and VCAM-1.

**CONCLUSION:** Metronomic dosing of gemcitabine is active in pancreatic cancer and is accompanied by pronounced changes in the tumour microenvironment.

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Pancreatic ductal adenocarcinoma remains a therapeutic challenge in oncology. The 5-year survival rate for unresectable disease is <5% and the median survival is 4–6 months. Current standard treatment consists of weekly gemcitabine administered at 1000 mg m<sup>-2</sup> for 3 weeks, followed by a week of rest before the next cycle begins. Myelosuppression is the dose-limiting toxicity. This pulsatile maximum tolerated dose (MTD)-driven gemcitabine regimen significantly improves clinical benefit response but only provides modest survival benefits (Burriss *et al*, 1997). Previous studies indicated that the efficacy and toxicity of gemcitabine are strongly schedule dependent (Hertel *et al*, 1990; Braakhuis *et al*, 1991, 1995; Boven *et al*, 1993; Veerman *et al*, 1996). In various murine and human tumour models, 120 mg kg<sup>-1</sup> of gemcitabine administered every 3 days was shown to induce greater tumour growth inhibition than 2.5–3.5 mg kg<sup>-1</sup> of gemcitabine injected daily or 240 mg kg<sup>-1</sup> of the drug given weekly (Braakhuis *et al*,

1991, 1995). Furthermore, daily administration of gemcitabine at 1 mg kg<sup>-1</sup> was reported to be more toxic than once-weekly administration at 40 mg kg<sup>-1</sup> in one study (Hertel *et al*, 1990), but was demonstrated to be non-toxic in another (Laquente *et al*, 2008). Although these observations illustrated the importance of dose scheduling in maximising the anti-tumour efficacy and minimising the toxicity of gemcitabine, the physiological changes induced by different gemcitabine treatment schedules in the whole pancreatic tumour *in situ* have not been investigated. Understanding the effect of schedule-dependent drug dosing on tumour physiology will likely lead to the development of more efficacious gemcitabine monotherapy or combination chemotherapy with molecularly targeted agents for pancreatic cancer.

The therapeutic concept of administering cytotoxic agents continuously at lower doses relative to MTDs without drug-free breaks over extended periods, known as ‘metronomic chemotherapy’, is increasingly being recognised as a viable cancer treatment option. In comparison with MTD regimens, metronomic dosing protocols demonstrated reduced toxicity but similar or even increased efficacy in pre-clinical studies (Browder *et al*, 2000;

\*Correspondence: Dr SSW Ng; E-mail: sng@bccrc.ca

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Bertolini *et al*, 2003). More importantly, several Phase II trials have now shown that metronomic chemotherapy regimens using oral cyclophosphamide, methotrexate, etoposide, or capecitabine, in the absence or presence of agents with anti-angiogenic properties (e.g., thalidomide, celecoxib, bevacizumab), are active and minimally toxic in advanced breast cancer (Colleoni *et al*, 2006; Dellapasqua *et al*, 2008), recurrent ovarian cancer (Garcia *et al*, 2008), hormone refractory prostate cancer (Lord *et al*, 2007), and non-Hodgkin's lymphoma (Buckstein *et al*, 2006) among other cancers (Kieran *et al*, 2005; Young *et al*, 2006). The mechanistic basis of metronomic chemotherapy is believed to be primarily anti-angiogenic, either by direct killing of endothelial cells in the tumour vasculature (Browder *et al*, 2000; Klement *et al*, 2000), destruction of bone marrow-derived endothelial progenitors (Bertolini *et al*, 2003; Shaked *et al*, 2005), and/or stimulation of the release of endogenous anti-angiogenic factors such as thrombospondin-1 from perivascular stromal cells (Hamano *et al*, 2004). Moreover, immunostimulation has also been shown to mediate the anti-tumour effects of metronomic cyclophosphamide (Hermans *et al*, 2003; Ghiringhelli *et al*, 2004; Loeffler *et al*, 2005). As the majority of pancreatic cancer patients are elderly and often have poor performance status, metronomic gemcitabine regimens could be advantageous compared with conventional MTD-driven weekly gemcitabine, provided that it is better tolerated and equally or more efficacious. In this study, we sought to determine whether gemcitabine administered at lower doses in a metronomic regimen has anti-tumour activity in pancreatic ductal adenocarcinoma. The first objective was to evaluate the anti-tumour efficacy of low-dose metronomic gemcitabine *vs* MTD-driven weekly gemcitabine, and the second was to examine their effects on the tumour microenvironment in primary pancreatic cancer xenografts grown orthotopically in the pancreas of SCID mice.

## MATERIALS AND METHODS

### Establishment of orthotopic primary pancreatic cancer xenografts

Fresh pancreatic ductal adenocarcinoma tissues were obtained, with institutional ethics review board approval, from patients undergoing Whipple resection for pancreatic cancer at Vancouver General Hospital between 2006 and 2008. Resected, non-diagnostic specimens were washed twice in antibiotic-containing RPMI 1640. Necrotic tissues were removed, and viable tumour tissues were cut into small pieces of  $\sim 2 \times 2 \times 2$  mm in size and implanted subcutaneously into male C.B-17 SCID mice (Taconic, Germantown, NY, USA). When the subcutaneous tumours reached 600–800 mm<sup>3</sup>, they were excised and cut into small pieces of  $\sim 2 \times 2 \times 2$  mm in size for surgical implantation into the pancreas of groups of SCID mice as previously described (Ng *et al*, 2001). Two primary pancreatic ductal adenocarcinoma xenograft lines (PaCa8 and PaCa13) established from resected tumour tissues of two different patients were used in this study. All animal studies were conducted in accordance with the guidelines of the Canadian Council for Animal Care.

### Treatment regimens

Gemcitabine hydrochloride (Gemzar; Eli Lilly Canada Inc., Toronto, ON, Canada) was purchased from the British Columbia Cancer Agency pharmacy and dissolved in 0.9% saline (Hospira Healthcare Corp., Montreal, QC, Canada). When the orthotopic xenografts reached a palpable size of  $\sim 200$ – $400$  mm<sup>3</sup>, tumour-bearing mice were randomised into three groups ( $n = 5$  each) and treated with vehicle control (0.9% saline, i.p.), one cycle of MTD gemcitabine (240 mg kg<sup>-1</sup> on days 1, 8, and 15, i.p., followed by

a 1-week break), or metronomic gemcitabine (30 mg kg<sup>-1</sup> on days 1, 4, 7, 10, 13, 16, 19, 22, 25, and 28, i.p.) for 4 weeks. The dose and schedule used in the MTD gemcitabine regimen were chosen to emulate clinical treatment protocols. When administered on a weekly basis, 240 mg kg<sup>-1</sup> ( $\sim 724$  mg m<sup>-2</sup> for a 20-g mouse) was previously reported to be the MTD of gemcitabine in mice (Veerman *et al*, 1996). Animal weight was monitored twice a week during the course of treatment.

### Dynamic contrast-enhanced magnetic resonance imaging

All studies were conducted using a 7.0 T magnetic resonance scanner (Bruker, Ettlingen, Germany), with a quadrature birdcage coil for transmission and a  $1.7 \times 1.4$  cm rectangular surface coil for reception. At the end of the treatment cycle (day 29), the three groups of tumour-bearing mice were anaesthetised with isoflurane (Baxter Corporation, Mississauga, ON, Canada) and administered an i.v. bolus injection of the magnetic resonance contrast agent gadolinium (Gd)-DTPA (Omniscan; Nycomed, Oslo, Norway) at 0.3 mmol kg<sup>-1</sup>. Serial images were acquired to monitor changes in the concentration of the contrast agent in each pixel during the initial uptake and subsequent washout in the tumour. Magnetic resonance imaging scans followed the technique previously described by Lyng *et al* (1998). Briefly, a proton-density-weighted spin-echo MSME scan (TE/TR = 11.9/6000 ms) was first acquired to serve as a baseline for conversion of pixel intensity to absolute concentration values of the contrast agent. Gd-DTPA was injected using a tail-vein catheter over  $\sim 15$  s. Starting at the time of injection, a series of 41 consecutive T<sub>1</sub>-weighted scans were acquired, with each scan lasting 64 s (spin-echo MSME, TR/TE = 11.9/500 ms). In-plane spatial resolution was 312  $\mu$ m with 10–14 slices at a thickness of 1 mm or 1.5 mm, depending on the size of the tumour. The Gd-DTPA concentration–time curve for each pixel was fitted to a two-compartment Kety model (Kety, 1960; Tofts *et al*, 1999) that describes the pharmacokinetics of the contrast agent using two parameters:  $K^{\text{trans}}$ , volume transfer constant between vascular space and extravascular extracellular space; and  $v_e$ , fractional volume of extravascular extracellular space.  $K^{\text{trans}}$ , which is often a mixed measure of blood flow and vascular permeability (Leach *et al*, 2003), was determined using in-house software and expressed as median values of  $K^{\text{trans}}$  in viable tumour tissues.

### Immunofluorescence staining and determination of tumour hypoxia, cell proliferation, and microvessel density

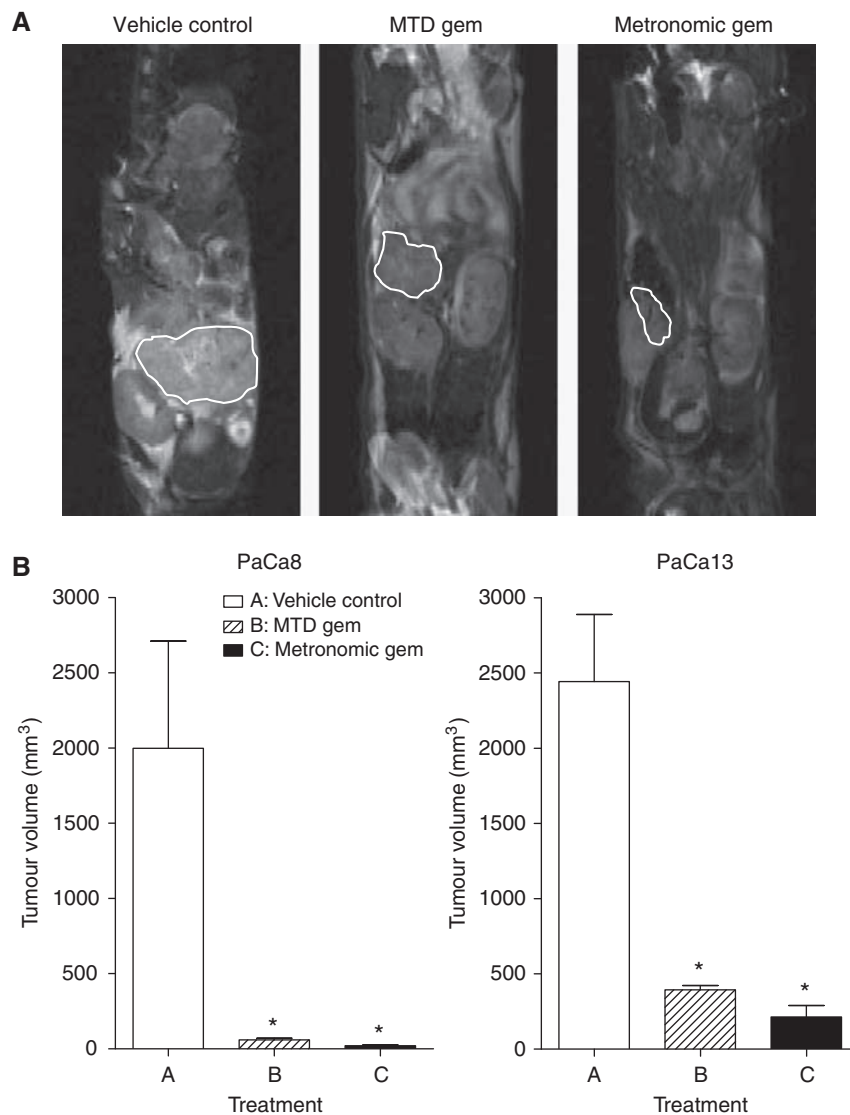
At the end of the treatment cycle (day 29), the three groups of tumour-bearing mice were injected with hypoxia marker EF5 (Dr Cameron Koch, University of Pennsylvania, PA, USA) at 30 mg kg<sup>-1</sup> i.v. and with proliferation marker BrdU (Sigma, St Louis, MO, USA) at 1500 mg kg<sup>-1</sup> i.p. at 3 h and 2 h before euthanasia, respectively. The orthotopic tumours were excised immediately after euthanasia. Perpendicular tumour diameters were measured with a caliper, and tumour volumes were calculated using the formula  $\pi/6 \times a \times b^2$ , where  $a$  is the longest dimension of the tumour and  $b$  is the width. Tumours were then snap frozen in OCT (Sakura Finetek, Torrance, CA, USA) in liquid nitrogen. Cryosections of 10  $\mu$ m were cut from each tumour and fixed in 50% (v/v) acetone/methanol for 10 min at room temperature. Endothelial cells were stained with a monoclonal rat anti-mouse CD31/PECAM-1 antibody (1:100; BD PharMingen, San Diego, CA, USA), followed by an Alexa 647-conjugated goat anti-rat secondary antibody (1:200; Molecular Probes, Eugene, OR, USA). Reduced EF5 adducts in viable hypoxic cells were stained on the same section using a Cy3-conjugated monoclonal antibody to ELK3-51 (1:200) (Lord *et al*, 1993). Staining for incorporated BrdU was performed as previously reported (Kyle *et al*, 2003). Sections were counterstained with haematoxylin, dehydrated, and mounted. At each stage of staining,

whole tumour sections were imaged ( $\times 10$  objective) using a robotic fluorescence microscope (Zeiss Imager Z1, Oberkochen, Germany), a cooled monochrome CCD camera (Retiga 4000R, QImaging, Surrey, BC, Canada), a motorised slide loader and x-y stage (Ludl Electronic Products, Hawthorne, NY, USA), and customised NIH ImageJ software (<http://rsb.info.nih.gov/ij/>) running on a G5 Macintosh computer (Apple, Cupertino, CA, USA). All parameters stained on the same section were imaged separately, and subsequently overlaid and aligned with the removal of imaging artefacts. Tumour hypoxia and cell proliferation were visualised by EF5+ and BrdU+ staining, respectively. False colour images were generated using Adobe Photoshop CS3. Using NIH software applications and user-supplied algorithms, images were cropped to tumour tissue boundaries with necrosis removed; fluorescent images were inverted and a threshold was applied to the CD31 image. Neighbouring CD31+ pixels were grouped as CD31+ objects (i.e., vessels). All image pixels were sorted to determine their distance from the nearest vessels and the average distance between vessels, which reflects microvessel density, was reported. A short average distance between vessels indicates high microvessel

density. The extent of tumour hypoxia or cell proliferation was reflected by the percentage of EF5+ or BrdU+ pixels, respectively, which was calculated as the number of EF5+ or BrdU+ pixels divided by the total number of tumour tissue pixels, and multiplied by 100%.

#### Assessment of stromal cell apoptosis in primary xenografts

Cryosections of  $10\ \mu\text{m}$  were cut, air dried, and fixed in 50% (v/v) acetone/methanol for 10 min at room temperature. Sections were stained with H&E for histological examination. To detect apoptosis in cancer-associated fibroblasts, sections were double stained with a FITC-conjugated monoclonal anti- $\alpha\text{SMA}$  antibody (1:200; Sigma) and an anti-cleaved-caspase 3 antibody (1:200; Cell Signaling Technology, Danvers, MA, USA), followed by an Alexa 546-conjugated secondary antibody (1:200; Molecular Probes). To determine apoptosis in endothelial cells, sections were incubated with anti-CD31/PECAM-1 antibody (1:100), followed by a FITC-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and with



**Figure 1** (A) Representative anatomical magnetic resonance scans of PaCa13 tumours after treatment with vehicle control (0.9% saline), maximum tolerated dose gemcitabine (MTD gem;  $240\ \text{mg}\ \text{kg}^{-1}$  on days 1, 8, and 15), or metronomic gemcitabine ( $30\ \text{mg}\ \text{kg}^{-1}$ , q3d) for 4 weeks. (B) Effects of different treatments on PaCa8 and PaCa13 tumour growth. Columns, mean of 4–5 tumours; bars, s.e. \* $P < 0.05$ , significantly different from that of vehicle control.

anti-cleaved-caspase 3 antibody, followed by the Alexa 546-conjugated secondary antibody (1:200). Images of whole tumour sections were captured ( $\times 20$  objective) using a Leica fluorescence microscopic imaging system (Leica Microsystems Inc, Richmond Hill, ON, Canada) equipped with both cooled 350FX monochrome and 300FX colour cameras for fluorescence and bright field imaging, respectively, an automated scanning stage (DM6000B), and Surveyor software (Objective Imaging, Kansasville, WI, USA). After removal of artefacts, composite tumour images for  $\alpha$ SMA, CD31, and cleaved-caspase 3 staining were overlaid, aligned, thresholded, and analysed using in-house software programs developed in MATLAB (The Mathworks, Natick, MA, USA). The percentage of apoptotic cancer-associated fibroblasts (CAFs) or endothelial cells was reported as the number of  $\alpha$ SMA + cleaved-caspase 3 + or CD31 + cleaved-caspase 3 + pixels divided by the total number of  $\alpha$ SMA + or CD31 + pixels, respectively, and multiplied by 100%.

### Evaluation of tumour levels of growth factors and cytokines

Harvested tumours from all treatment groups were homogenised in lysis buffer (50 mM HEPES, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl<sub>2</sub>, 100 mM NaF, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 100  $\mu$ g ml<sup>-1</sup> PMSF, 5  $\mu$ g ml<sup>-1</sup> leupeptin, and 5  $\mu$ g ml<sup>-1</sup> aprotinin) using a Polytron PT10-35 homogeniser (Kinematica AG, Lucerne, Switzerland) and stored at  $-80^{\circ}\text{C}$ . Total protein concentration in each tumour lysate was determined using a Micro BCA proteins assay kit (Pierce, Rockford, IL, USA). The protein levels of 89 human antigens that include numerous growth factors and cytokines were quantified in the tumour lysates using HumanMAP Antigen version 1.6 (Rules Based Medicine, Austin, TX, USA).

### Statistics

All results were presented as mean  $\pm$  s.e. Comparisons were made with one-way analysis of variance, followed by the Newman-Keuls test or the Dunnett test, with  $P < 0.05$  as the criterion for statistical significance.

## RESULTS

### Anti-tumour activity of metronomic gemcitabine

Representative anatomical magnetic resonance scans of PaCa13 tumours after treatment are shown in Figure 1A. The effects of treatments on tumour volume in the two primary tumour xenograft lines are summarised in Figure 1B. Metronomic and MTD gemcitabine significantly decreased the volume of PaCa8 tumours by 99 and 97%, respectively, and that of PaCa13 tumours by 91 and 84%, respectively, compared with vehicle control. There was a trend towards greater tumour volume reductions in the metronomic gemcitabine-treated group compared with the MTD gemcitabine-treated group. Neither of the treatments caused any significant weight loss in tumour-bearing mice relative to the vehicle control, indicating a lack of toxicity (data not shown).

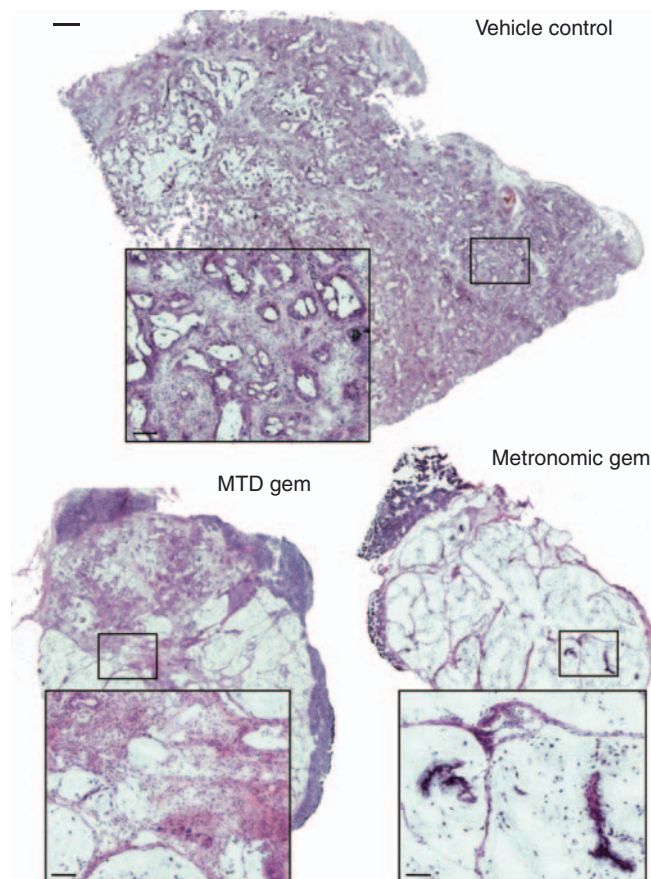
### Morphological changes induced by metronomic gemcitabine in primary pancreatic cancer xenografts

H&E staining of vehicle control-treated PaCa8 xenografts showed that they are moderately differentiated ductal adenocarcinomas (Figure 2), whereas PaCa13 xenografts are poorly differentiated ductal adenocarcinoma (data not shown). PaCa8 tumours were debulked and seemed to have undergone mucinous differentiation, with few viable cancer cells remaining after metronomic gemcitabine treatment. Mucinous differentiation was also evident, although to a much lesser extent, with more viable cancer cells

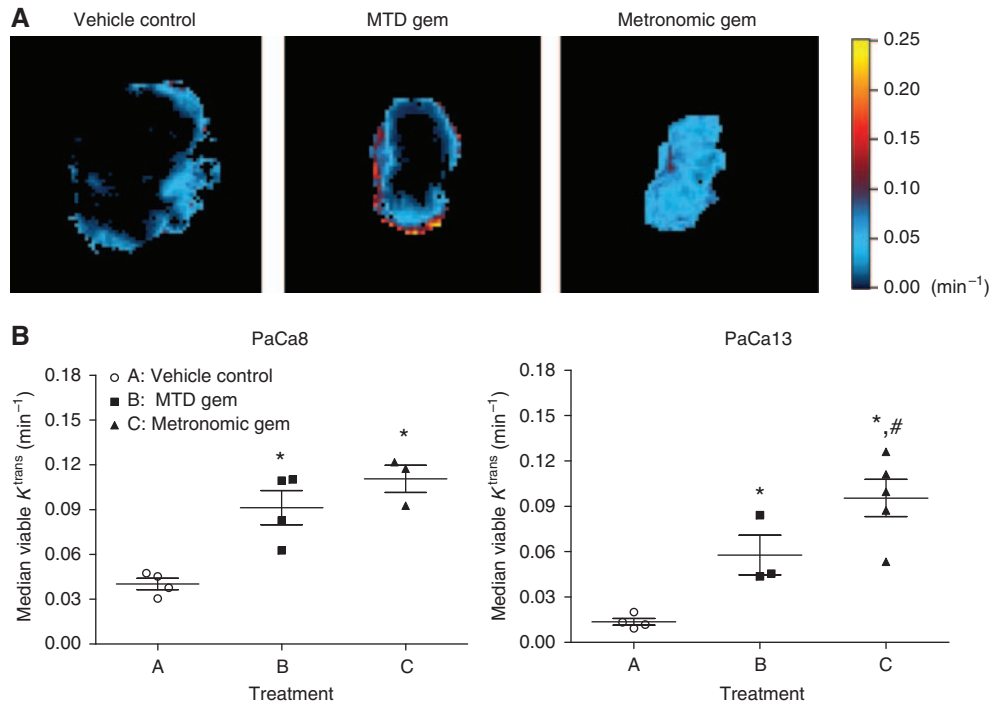
remaining in MTD gemcitabine-treated PaCa8 tumours (Figure 2). Similar morphological changes, but of lesser extent, were noted in PaCa13 tumours after metronomic and MTD gemcitabine treatment, with the former inducing more pronounced effects than the latter (data not shown). Overall, metronomic gemcitabine exerted greater histological effect than MTD gemcitabine in the two xenografts.

### Effects of metronomic gemcitabine on $K^{\text{trans}}$ in primary xenografts

Representative  $K^{\text{trans}}$  maps of PaCa13 tumours after injection of the contrast agent Gd-DTPA are shown in Figure 3A. Contrast enhancement was observed predominantly in the tumour periphery in the vehicle control and MTD gemcitabine groups. Interestingly, treatment with metronomic gemcitabine resulted in a uniformly strong contrast enhancement throughout the entire tumour.  $K^{\text{trans}}$  values were subsequently derived from the maps. In PaCa8 tumours, the average values for median viable  $K^{\text{trans}}$  were significantly higher in the metronomic and MTD gemcitabine groups (by 2.8- and 2.3-fold, respectively) compared with the vehicle control group (Figure 3B). In PaCa13 tumours, metronomic and MTD gemcitabine also significantly increased  $K^{\text{trans}}$  values by 6.8- and 4.1-fold, respectively, relative to the vehicle control. Moreover, average values for median viable  $K^{\text{trans}}$  were significantly higher in metronomic gemcitabine-treated PaCa13 tumours compared with their MTD-treated counterparts (Figure 3B).



**Figure 2** Representative composite haematoxylin and eosin (H&E) images (scale bar: 500  $\mu$ m) of PaCa8 tumours after treatment with vehicle control (0.9% saline), maximum tolerated dose gemcitabine (MTD gem; 240 mg kg<sup>-1</sup> on days 1, 8, and 15), or metronomic gemcitabine (30 mg kg<sup>-1</sup>, q3d) for 4 weeks. Squares, magnified images (scale bar: 83  $\mu$ m) of the corresponding areas in the tumour sections.



**Figure 3** (A) Representative  $K^{trans}$  maps of PaCa13 tumours after treatment with vehicle control (0.9% saline), maximum tolerated dose gemcitabine (MTD Gem; 240 mg kg<sup>-1</sup> on days 1, 8, and 15), or metronomic gemcitabine (30 mg kg<sup>-1</sup>, q3d) for 4 weeks. (B) Effects of different treatments on median values of  $K^{trans}$  in viable tumour tissues in PaCa8 and PaCa13 tumours. Each symbol represents an evaluable tumour from a mouse in the corresponding group. —, mean; bars, s.e. \* $P < 0.05$ , significantly different from that of vehicle control. # $P < 0.05$ , significantly different from that of MTD gemcitabine.

### Tumour hypoxia, cell proliferation, and microvessel density after metronomic gemcitabine treatment

Figure 4 shows representative composite images of PaCa13 tumour sections from different treatment groups. Large areas of EF5 + staining (red), indicative of hypoxia, were evident in vehicle control-treated tumours. After MTD gemcitabine treatment, hypoxia remained prominent in the tumours despite their reduction in size. In sharp contrast, metronomic gemcitabine-treated tumours had little or no EF5 + staining. Quantitatively, metronomic gemcitabine decreased the percentage of EF5 + pixels by 2.2-fold in PaCa13 tumours compared with the vehicle control; MTD gemcitabine had the opposite effect, significantly increasing the percentage of EF5 + pixels by 2.2-fold (Figure 5A). In PaCa8 tumours, metronomic and MTD gemcitabine significantly and similarly reduced hypoxia (Figure 5A). Levels of tumour cell proliferation, as determined from BrdU + staining, were similar in all treated PaCa13 tumours compared with the vehicle control. Similar observations were also evident in PaCa8 tumours (data not shown).

Microvessel density was reported as the average distance between CD31 + objects (blue staining in Figure 4) on whole tumour sections. Short and long average distances between vessels indicate high and low microvessel density, respectively. In PaCa13 tumours, only metronomic gemcitabine significantly decreased the average distance between blood vessels and therefore increased microvessel density (Figure 5A). In PaCa8 tumours, the average distance between vessels was not significantly altered by metronomic or MTD gemcitabine (Figure 5A).

### Effects of metronomic gemcitabine on endothelial cell and CAF apoptosis

None of the treatments significantly increased endothelial cell apoptosis in the two primary xenografts, as the percentages of CD31 and cleaved-caspase 3 double-positive pixels in the

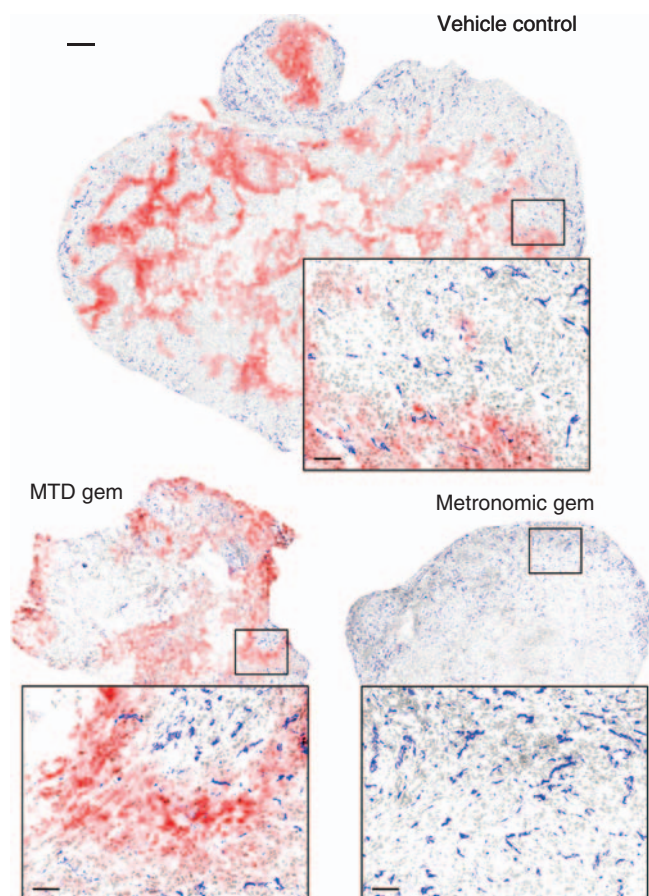
treated tumours were similar to those in vehicle control tumours (Figure 5B). In PaCa8 tumours, metronomic and MTD gemcitabine significantly increased CAF apoptosis by 34-fold and 29-fold, respectively, as reflected by the increased percentages of  $\alpha$ SMA and cleaved-caspase 3 double-positive pixels (Figure 5B). In PaCa13 tumours, there was a trend towards increased CAF apoptosis after either gemcitabine regimen, but such increases did not reach statistical significance (Figure 5B).

### Tumour levels of growth factors and cytokines in response to metronomic gemcitabine

Metronomic gemcitabine induced significant changes in the levels of 37 and 14 out of 89 proteins in the PaCa8 and PaCa13 tumours, respectively, compared with vehicle control (Supplementary Table S1). The proteins, the levels of which were significantly reduced following metronomic gemcitabine therapy, were more numerous in PaCa8 tumours than in PaCa13 tumours (29 vs 13, respectively). Many of the downregulated proteins are pro-angiogenic growth factors and cytokines. In general, the decreases in the levels of these proteins caused by metronomic gemcitabine were more pronounced than those induced by MTD gemcitabine (e.g., 21-fold vs 6-fold for CD40 ligand, 12-fold vs 4.1-fold for EGF, 34-fold vs 20-fold for GM-CSF, 57-fold vs 15-fold for ICAM-1, 397-fold vs 75-fold for IL-1 $\alpha$ , and 189-fold vs 18-fold for IL-8, respectively, in PaCa8 tumours; 8.9-fold vs 1.9-fold for VCAM-1, 2.4-fold vs 1.7-fold for von Willebrand factor, respectively, in PaCa13 tumours).

## DISCUSSION

This study demonstrated that metronomic dosing of gemcitabine is active against pancreatic ductal adenocarcinoma. It is noteworthy that, although both metronomic and MTD gemcitabine



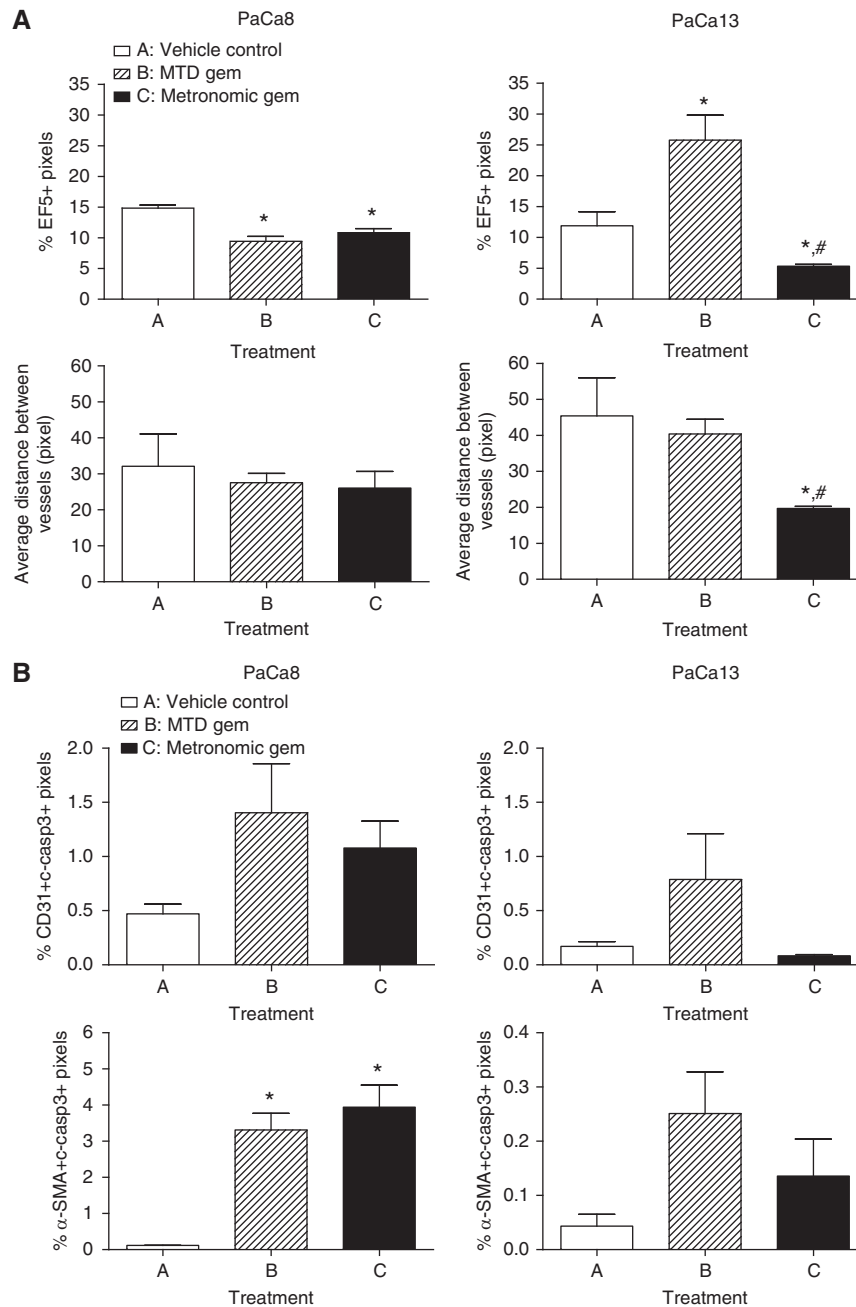
**Figure 4** Representative composite images (scale bar: 500  $\mu\text{m}$ ) of PaCa13 tumours showing EF5 (red), CD31 (blue), and nuclear (grey) staining after treatment with vehicle control (0.9% saline), maximum tolerated dose gemcitabine (MTD gem; 240  $\text{mg kg}^{-1}$  on days 1, 8, and 15), or metronomic gemcitabine (30  $\text{mg kg}^{-1}$ , q3d) for 4 weeks. Squares, magnified images (scale bar: 83  $\mu\text{m}$ ) of the corresponding areas in the tumour sections.

induced significant tumour volume reductions in PaCa8 and PaCa13 primary xenografts, the total dose of gemcitabine administered over 4 weeks in the metronomic group was less than half of that given in the MTD group (300 vs 720  $\text{mg kg}^{-1}$ , respectively). Metronomic gemcitabine did not induce significant weight loss in tumour-bearing mice, indicating a lack of toxicity. These findings suggest that the use of metronomic gemcitabine in the clinic would be advantageous because such a regimen is more effective and better tolerated in terms of total drug dose than MTD weekly gemcitabine.

We then examined what might have contributed to the anti-tumour effects of metronomic gemcitabine. H&E staining of tumour sections revealed more prominent morphological changes that were characterised by more extensive mucinous differentiation, lower overall cell density, and fewer cancer cells in metronomic gemcitabine-treated tumours compared with their MTD-treated counterparts. These observations point to a direct and more pronounced effect of the metronomic regimen on pancreatic cancer cells. Indeed, it has been suggested that metronomic chemotherapy might directly affect tumour cells by inducing their differentiation (Kerbel and Kamen, 2004). Furthermore, tumour cell re-population, which often occurs during drug-free breaks between cycles of MTD chemotherapy, may also be prevented using metronomic regimens (Kerbel and Kamen, 2004).

At the functional level, our dynamic contrast-enhanced magnetic resonance imaging data demonstrated that metronomic gemcitabine treatment improves global tumour perfusion. The strong homogeneous contrast enhancement and the significant increases in  $K^{\text{trans}}$  values observed in metronomic gemcitabine-treated PaCa8 and PaCa13 tumours *in situ* were indicative of better tissue perfusion throughout the tumour. On the other hand, contrast enhancement was confined to the tumour periphery, and  $K^{\text{trans}}$  values were increased to a lesser extent in MTD gemcitabine-treated tumours. Improved tissue perfusion after metronomic gemcitabine treatment was also consistent with the substantial decreases in hypoxia observed in these tumours. This is reminiscent of the effects after vessel 'normalisation' as reported by Jain (2001). It is noteworthy that better tissue perfusion did not result in increased tumour cell proliferation, as BrdU staining in metronomic gemcitabine-treated tumours was not significantly different from that in vehicle control- or MTD gemcitabine-treated tumours. Improved vascular function is likely to promote the delivery of subsequent doses of gemcitabine itself to the tumour; thus, the previously reported poor tumour penetration of gemcitabine, even when administered at MTD (Huxham *et al*, 2004), might be alleviated with metronomic dosing regimens. As significant hypoxia has been detected in clinical pancreatic cancer (Koong *et al*, 2000), and hypoxia contributes to radioresistance and chemoresistance, metronomic gemcitabine-induced decreases in hypoxia could potentially enhance the efficacy of subsequently administered radiotherapy or MTD-based chemotherapy. The increases in  $K^{\text{trans}}$  values observed in metronomic gemcitabine-treated PaCa8 and PaCa13 tumours might seem to contradict the reductions in  $K^{\text{trans}}$  values that have often been reported after treatment with 'dedicated' anti-angiogenic agents (Morgan *et al*, 2003; Batchelor *et al*, 2007), and more recently with metronomic capecitabine plus celecoxib (Steinbild *et al*, 2007). However, the post-treatment decreases in  $K^{\text{trans}}$  values in these studies were not always accompanied by tumour volume reductions. It is possible that tumour volume reductions induced by metronomic gemcitabine in PaCa8 and PaCa13 tumours might lead to more substantial decreases in interstitial fluid pressure, thereby markedly improving the patency of tumour blood vessels and, in turn, tumour blood flow. As  $K^{\text{trans}}$  is a mixed measure of tumour blood flow and vascular permeability (Strecker *et al*, 2003; Yankeelov *et al*, 2006), an overall increase in  $K^{\text{trans}}$  might be detected if the increase in tumour blood flow is greater than the decrease in vascular permeability. It is also important to highlight the fact that, although metronomic and MTD regimens of gemcitabine induced similar tumour volume reductions in our studies, the two treatments resulted in different patterns of perfusion.

Given that metronomic chemotherapy has been previously associated with anti-angiogenesis (Browder *et al*, 2000; Klement *et al*, 2000; Bertolini *et al*, 2003; Hamano *et al*, 2004; Shaked *et al*, 2005), the effects of metronomic vs MTD gemcitabine on pancreatic tumour vasculature were investigated. PaCa13, but not PaCa8, tumours showed significant increases in microvessel density after metronomic gemcitabine treatment. MTD gemcitabine had no effect on microvessel density in both xenograft lines. Neither metronomic nor MTD gemcitabine significantly increased endothelial cell apoptosis in any of the xenografts. However, it should be emphasised that microvessel density and possibly endothelial cell apoptosis are time-dependent measures during anti-angiogenesis-based therapies (Hlatky *et al*, 2002). Any significant endothelial cell dropout might have occurred shortly after the first or second dose of drug, similar to that previously reported after cyclophosphamide treatment (Browder *et al*, 2000), and therefore might not have been captured at the end of the 4-week treatment period in our study. The anti-angiogenic and anti-tumour efficacy of metronomic gemcitabine in our primary xenograft studies could be further attributed to its ability to induce marked decreases, more so than MTD gemcitabine, in a multitude



**Figure 5** (A) Effects of treatment with vehicle control (0.9% saline), maximum tolerated dose gemcitabine (MTD gem;  $240 \text{ mg kg}^{-1}$  on days 1, 8, and 15), or metronomic gemcitabine ( $30 \text{ mg kg}^{-1}$ , q3d) for 4 weeks on hypoxia and average distance between vessels in PaCa8 and PaCa13 tumours. Columns, mean of 3–5 tumours; bars, s.e. \* $P < 0.05$ , significantly different from that of vehicle control. # $P < 0.05$ , significantly different from that of MTD gemcitabine. (B) Effects of the three treatments on apoptosis of endothelial cells (CD31 and c-casp3 double-positive pixels) and cancer-associated fibroblasts ( $\alpha$ -SMA and c-casp3 double-positive pixels) in PaCa8 and PaCa13 tumours. Columns, mean of 2–5 tumours; bars, s.e. c-casp3, cleaved-caspase 3. \* $P < 0.05$ , significantly different from that of vehicle control.

of pro-angiogenic growth factors and cytokines including EGF, IL-1 $\alpha$ , IL-8, MCP-1, ICAM-1, VCAM-1, and others (Supplementary Table S1). Anti-cancer treatment regimens that cause broad-spectrum disruption of the tumour growth factor/cytokine network to a greater degree are likely to be more efficacious than those that only affect a small number of these molecules to a lesser extent. It is conceivable that the more prominent decreases in pro-angiogenic growth factors and cytokines after metronomic gemcitabine therapy ultimately improved tumour vascular function and tissue perfusion, and led to a corresponding reduction in tumour hypoxia.

Furthermore, metronomic gemcitabine significantly increased apoptosis of CAFs in PaCa8 tumours, suggesting that other stromal components could also be targets of metronomic chemotherapy regimens. In pancreatic ductal adenocarcinoma, this could be therapeutically significant, because one of the hallmarks of the malignancy is the presence of a strong desmoplastic reaction that is associated with extensive fibroblast proliferation and modified extracellular matrix deposition. A myriad of pro-angiogenic molecules, such as VEGF and EGF among others, are known to be upregulated in CAFs (Bhowmick *et al*, 2004). Increasing apoptosis in this cell population would thus deplete the supply

of these molecules and, in turn, restrict angiogenesis and inhibit tumour growth more effectively. The lack of significant increases in CAF apoptosis in metronomic gemcitabine-treated PaCa13 tumours might be explained by the differential sensitivity of individual patient's CAFs to such treatment. It should be emphasised that PaCa8 and PaCa13 are primary xenografts established from surgically resected human pancreatic tumour tissues. Given the inherent heterogeneity of individual patient's tumours and their differential responses to treatments in the clinical situation, it is not surprising that metronomic and MTD gemcitabine resulted in different profiles of change of pro-angiogenic growth factors and cytokines, and, similarly, in differences in the levels of CAF apoptosis in PaCa8 and PaCa13 tumours. This highlights the value of primary human tumour models in the pre-clinical evaluation of experimental treatment modalities (Rubio-Viqueira *et al*, 2006). In addition to anti-angiogenesis, immunostimulation has also been shown to mediate the anti-tumour effects of metronomic cyclophosphamide (Hermans *et al*, 2003; Ghiringhelli *et al*, 2004, 2007; Loeffler *et al*, 2005). The effect of metronomic gemcitabine on the immune system, however, has not been reported in the literature, although a recent study showed that conventional MTD gemcitabine regimen (3 weeks on, 1 week off) reduces CD8<sup>+</sup> T cells, increases CD11c<sup>+</sup> dendritic cells, but does not significantly alter the number of CD4<sup>+</sup> T cells in pancreatic cancer patients after one cycle of treatment when compared with pre-treatment baseline (Soeda *et al*, 2009). The possibility that immunomodulation may have a role in mediating the anti-tumour effects of metronomic gemcitabine cannot be excluded.

In conclusion, our results showed that metronomic gemcitabine is well tolerated and more effective than MTD gemcitabine in pancreatic cancer. It markedly reduces tumour levels of various pro-angiogenic molecules, and improves tumour vascular function and tissue perfusion (as reflected by the increase in  $K^{\text{trans}}$  and the decrease in tumour hypoxia). Pancreatic ductal adenocarcinoma is characterised by a dense fibrovascular stroma consisting of

different cell types (e.g., cancer-associated fibroblasts, endothelial cells, immune cells, and so on) (Bardeesy and DePinho, 2002). These assorted populations of stromal cells and pancreatic cancer cells are unlikely to all be in a susceptible growth phase at the time when a single MTD of gemcitabine is administered once a week; it follows that more frequent dosing at a lower but still effective concentration of the drug would increase the probability of eliminating or inducing differentiation in more cells of any cell type (Kamen *et al*, 2006). Considering that suboptimal blood flow (Komar *et al*, 2009) and hypoxia (Koong *et al*, 2000) contribute to chemotherapy or radiotherapy failure in pancreatic cancer, it would be interesting to evaluate whether the tumour physiological changes (i.e., increased perfusion and decreased hypoxia) induced by metronomic gemcitabine treatment as described in this study may be exploited for the design of more effective combination therapies. Oral formulations of an increasing number of chemotherapeutic drugs are now available or are being developed. The anti-tumour efficacy of oral gemcitabine (Veltkamp *et al*, 2008), used in a metronomic regimen similar to the one described herein, warrants clinical investigation in pancreatic cancer.

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