Inhibition of AKT2 Enhances Sensitivity to Gemcitabine via Regulating PUMA and NF-κB Signaling Pathway in Human Pancreatic Ductal Adenocarcinoma

Dong Chen 1,†, Min Niu 2,†, Xuelong Jiao 1, Kejun Zhang 1, Jun Liang 3 and Dianliang Zhang 1,*

1 Department of General Surgery, The Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, China
2 Department of Pharmacy, The Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, China
3 Department of Oncology, The Affiliated Hospital of Qingdao Medical College, Qingdao University, Qingdao 266003, China

† These authors contributed equally to this work.

* Authors to whom correspondence should be addressed; E-Mail: Phdzdl@yahoo.com; Tel./Fax: +86-0532-8291-3405.

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Abstract: Invasion, metastasis and resistance to conventional chemotherapeutic agents are obstacles to successful treatment of pancreatic cancer, and a better understanding of the molecular basis of this malignancy may lead to improved therapeutics. In the present study, we investigated whether AKT2 silencing sensitized pancreatic cancer L3.6pl, BxPC-3, PANC-1 and MIAPaCa-2 cells to gemcitabine via regulating PUMA (p53-upregulated modulator of apoptosis) and nuclear factor (NF)-κB signaling pathway. MTT, TUNEL, EMSA and NF-κB reporter assays were used to detect tumor cell proliferation, apoptosis and NF-κB activity. Western blotting was used to detect different protein levels. Xenograft of established tumors was used to evaluate primary tumor growth and apoptosis after treatment with gemcitabine alone or in combination with AKT2 siRNA. Gemcitabine activated AKT2 and NF-κB in MIAPaCa-2 and L3.6pl cells in vitro or in vivo, and in PANC-1 cells only in vivo. Gemcitabine only activated NF-κB in BxPC-3 cells in vitro. The presence of PUMA was necessary for gemcitabine-induced apoptosis only in BxPC-3 cells in vitro. AKT2 inhibition sensitized gemcitabine-induced apoptosis via PUMA upregulation in MIAPaCa-2 cells in vitro, and via NF-κB activity inhibition in L3.6pl cells in vitro. In
PANC-1 and MIAPaCa-2 cells in vivo, AKT2 inhibition sensitized gemcitabine-induced apoptosis and growth inhibition via both PUMA upregulation and NF-κB inhibition. We suggest that AKT2 inhibition abrogates gemcitabine-induced activation of AKT2 and NF-κB, and promotes gemcitabine-induced PUMA upregulation, resulting in chemosensitization of pancreatic tumors to gemcitabine, which is probably an important strategy for the treatment of pancreatic cancer.

**Keywords:** pancreatic cancer; gemcitabine; AKT; NF-κB; PUMA

### 1. Introduction

Pancreatic cancer has the worst survival rate of all cancers. It is more common in elderly than in younger persons, and <20% of patients present with localized, potentially curable tumors. A much higher percentage of patients present with metastatic disease (40–45%) or locally advanced disease (40%), and have median survival times of 3–6 or 8–12 months, respectively. The overall 5-year survival rate among patients with pancreatic cancer is <5% [1–3]. The frustrating lack of significant clinical advancements in the treatment of metastatic pancreatic cancer remains one of the biggest disappointments in medical oncology. Invasion, metastasis and resistance to conventional chemotherapeutic agents are obstacles to successful treatment of pancreatic cancer, and a better understanding of the molecular basis of this malignancy may lead to improved therapeutics [4–6].

AKT (also known as protein kinase B) is a Ser/Thr kinase that belongs to the AGC family (AMP/GMP kinases and protein kinase C) of kinases [7]. The three AKT isoforms: AKT1, AKT2 and AKT3 are closely related and consist of a conserved N-terminal pleckstrin homology (PH) domain, a central catalytic domain and a C-terminal regulatory hydrophobic motif (HM) [8]. Recent reports have demonstrated that the phosphatidylinositol-3 kinase (PI3K)/AKT pathway is a potent survival signal that may mediate resistance to the apoptotic effects of chemotherapy in different cancer types [9–13].

The current standard care for metastatic pancreatic cancer is gemcitabine, however, the success of this treatment is poor and overall survival has not improved for several decades. Drug resistance (both intrinsic and acquired) is thought to be a major reason for the limited benefit of most pancreatic cancer therapies [2]. It has been reported recently that strong expression levels of AKT2 and phosphorylated AKT (pAKT) are found and p-AKT expression is a significant prognostic indicator for pancreatic cancer [14]. Many reports suggest that inhibition of AKT activation enhances sensitivity to gemcitabine in pancreatic cancer [15–18]. However, the mechanism of AKT activation in pancreatic cancer remains unknown. Relatively little is known about the downstream signaling events that regulate sensitivity to gemcitabine in pancreatic cancer.

Nuclear factor (NF)-κB is a ubiquitous transcription factor that is regulated by a vast array of stimuli, including growth factors, inflammatory mediators, cytotoxic agents such as chemotherapeutic drugs, oxidative stress, and UV light. NF-κB is a dimer composed of various combinations of the five mammalian Rel proteins, namely, p65/RelA, c-Rel, RelB, NF-κB1/p50, and NF-κB2/p52 [19]. The most common form of NF-κB is a dimer of p65/relA and p50, and this dimer is often referred to
simply as NF-κB. In many human cancers, including pancreatic cancer, constitutive activation of NF-κB has been observed and may be associated with chemotherapy resistance, including gemcitabine, and inhibition of NF-κB may be useful for enhancing sensitivity to chemotherapy in cancer therapy [20–23].

Recent studies have shown that activation of AKT leads to activation of a series of survival factors, including NF-κB, arming cancer cells to resist induction of apoptosis [24,25]. The apoptosis induced by blocking PI3K/AKT might be ascribed to inhibition of NF-κB activity in pancreatic cancer cell lines [18,26]. However, Arlt et al. have reported that basal AKT activity correlates with sensitivity towards gemcitabine treatment, and that inhibition of PI3K/AKT by LY294002 alters gemcitabine-induced apoptosis, however, it is the constitutive NF-κB activity that confers resistance against gemcitabine [22]. Fahy et al. [18,26] have reported recently that the antiapoptotic effect of AKT activation in pancreatic cancer cells may involve transcriptional induction of NF-κB and Bcl-2 proteins that confer resistance to apoptosis; alteration of this balance allows sensitization to the apoptotic effect of chemotherapy. This was similar to the previous reports. However, Pan et al. [21] have reported that silencing p65/relA induced apoptosis and increased gemcitabine killing of all gemcitabine-sensitive pancreatic cancer cells, and no significant effects were observed on gemcitabine-resistant pancreatic cancer cell lines either in vitro or in vivo. Some studies have recently shown that knockdown of AKT enhances gemcitabine chemosensitivity in pancreatic adenocarcinoma cells [27]. However, there is no evident change in NF-κB activity when AKT activity decreases in PANC-1 cells [18]. We suggested the apoptosis or sensitivity to gemcitabine induced by blocking PI3K/AKT might be ascribed to inhibition of NF-κB activity at least in part, the other signaling pathway may take part in the downstream signaling events of the AKT activity regulation, NF-κB may not be the main mechanisms of apoptosis regulation in some pancreatic cancer cell lines.

PUMA (p53-upregulated modulator of apoptosis) is a Bcl-2 homology 3 (BH3)-only Bcl-2 family member and a critical mediator of p53-dependent and -independent apoptosis induced by a wide variety of stimuli, including genotoxic stress, deregulated oncogene expression, toxins, altered redox status, growth factor/cytokine withdrawal, and infection [28,29]. PUMA ablation or inhibition leads to apoptosis deficiency and increased risk for cancer development and treatment resistance, and inhibition of PUMA expression may be useful for curbing excessive cell death associated with tissue injury and degenerative diseases [30–36]. Therefore, PUMA is a general sensor of cell death stimuli and a promising target for cancer therapy.

De Frias et al. [35] have recently reported that AKT inhibitors may induce apoptosis of chronic lymphocytic leukemia cells irrespective of TP53 status, followed by an increase in PUMA protein levels and decrease in MCL-1 protein level. Fraser and colleagues have found that activation of AKT inhibits cisplatin-induced upregulation of PUMA, and suppresses cisplatin-induced p53 phosphorylation. They have also found that inhibition of AKT increases total and phospho-p53 content and sensitizes p53 wild-type, chemoresistant cells to cisplatin-induced apoptosis [36]. Ishihara et al. [37] have reported that PUMA siRNA inhibits the celecoxib-induced activation and translocation of Bax, release of cytochrome c into the cytosol and induction of apoptosis, suggesting that PUMA plays an important role in celecoxib-induced mitochondrial dysfunction and the resulting apoptosis. Coloff et al. [38] have reported that AKT-mediated cell survival is crucial in normal immunity and cancer, through AKT-dependent stimulation of glycolysis to suppress PUMA expression. Karst et al. [39] have reported a negative relationship between expression of PUMA and pAKT, and boosting PUMA
expression, combined with inhibiting AKT phosphorylation reduces cell survival. PUMA has proapoptotic effects and sensitivity to chemotherapy, thus, it is possible that activated AKT may suppress apoptosis via PUMA downregulation. In the present study, we investigated the hypothesis that inhibition of activated AKT promotes gemcitabine-induced apoptosis and confers gemcitabine sensitivity in cultured pancreatic cancer cells, in part, by PUMA upregulation.

In the present study, we investigated the hypothesis that inhibition of activated AKT promotes gemcitabine-induced apoptosis in cultured pancreatic cancer cells, in part, by PUMA upregulation and/or by NF-κB activity inhibition.

2. Materials and Methods

2.1. Cell Culture

Human PANC-1 and MIAPaCa-2 pancreatic cancer cells, which are resistant to gemcitabine [21,24], were purchased from the American Type Culture Collection (ATCC). The BxPC-3 cell line, which is sensitive to gemcitabine [21], was also purchased from ATCC. PANC-1 and MIAPaCa-2 cells were routinely cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (BxPC-3 were cultured in RPMI 1640) supplemented with 10% fetal bovine serum (FBS) in a 37 °C incubator in a humidified atmosphere of 5% CO₂. Human pancreatic cancer L3.6pl cells, which produce a significantly higher incidence of liver metastasis and number of lymph nodes, were obtained from M.D. Zhang [40,41]. All the cells were maintained in continuous exponential growth by twice-weekly passage in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified incubator containing 5% CO₂ in air at 37 °C.

2.2. Reagents

The antibodies used were AKT2 and wtp53 (DO-1) (Santa Cruz Biotechnology), Phospho-Akt (Ser473), Bcl-2, β-actin (Sigma–Aldrich), Anti-PUMA-α, Anti-NF-κBp65(p65) and caspase-3 (active) (Abgent) and PUMA siRNA (h) (Santa Cruz Biotechnology).

2.3. Construction of Adeno-Associated Virus-Mediated AKT2 siRNA Vector

AKT2 is amplified in human pancreatic cells, and inhibition of AKT2 expression contributes to the pathogenesis and malignant phenotype of this highly aggressive form of human malignancy [42–45]. Although pAKT1 is overexpressed in pancreatic cancer, high pAKT1 expression is a favorable prognostic factor in pancreatic cancer [46]. Therefore, we used AKT2 for the present study. The 21-mer sense and antisense strands of AKT2 RNA oligonucleotides were designed as described previously [47]. AKT2 siRNA duplexes were designed according to AKT2 mRNA sequences obtained from National Center for Biotechnology Information database (accession no: NM_001626.3). RNA oligonucleotides were synthesized by GeneChem (Shanghai, China) as previously described [48]. The sequence was subjected to a Blast search against the human genome sequence to ensure that only the AKT2 gene was targeted. Adeno-associated-virus-mediated transfer of AKT2 siRNA or mock siRNA [rAAV2-AKT2 siRNA or rAAV2-mock siRNA] were generated as described previously [49]. High-titer viruses were produced in 293 cells and purified by CsCl₂ gradient ultracentrifugation.
2.4. Adenoviral Infection

Cells were infected with AKT2 siRNA or mock siRNA at 10, 50, 100 and 500 MOI for 48 h. Expression of various proteins was confirmed by western blotting. EMSA was used to detect NF-κB activity.

2.5. Transient Transfection

Cells or AKT2 siRNA (mock siRNA)-transfected cells were cultured overnight in six-well plates and then transfected with 2, 10 or 20 µg PUMA siRNA (and negative control) using Lipofectamine Plus (Invitrogen) in 1 mL serum-free medium according to the manufacturer’s instructions. Four hours post-transfection, each well was supplemented with 1 mL medium containing 20% FBS. Twenty-four hours post-transfection, medium was removed and the cells were harvested or treated with gemcitabine for a further 72 h.

2.6. Drug Treatments

L3.6pl, BxPC-3, PANC-1 and MIAPaCa-2 cells were plated at a density of 5 × 10⁴ cells/cm² on six-well plates 18 h before initiation of treatment. At the time of treatment, cell density was >70%. The cells were treated with (1) 1 µM gemcitabine (MIAPaCa-2, BxPC-3 and PANC-1) or 0.5 µM gemcitabine (L3.6pl) for 72 h; (2) 10, 50 and 100 MOI AKT2 siRNA (mock siRNA) transfection for 48 h, followed by the same concentration of gemcitabine for 72 h; (3) 0.1, 0.5 or 1 µM gemcitabine for 72 h; (4) MIAPaCa-2 and L3.6pl cells were treated with 100 MOI AKT2 siRNA (mock siRNA) transfection for 48 h, followed by 1 µM gemcitabine and 5, 10 or 20 U tumor necrosis factor (TNF)-α for 72 h; or (5) MIAPaCa-2 and L3.6pl cells were treated with 100 MOI AKT2 siRNA (mock siRNA) transfection for 48 h followed by 2, 10 or 20 µg PUMA siRNA transfection for 24 h, then the cells were treated with 1 µM gemcitabine for an additional 72 h.

2.7. MTT Assay

Cell viability was examined by the MTT assay method. At various times, 20 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) was added to each well and incubated for a further 2 h. Upon termination, the supernatant was aspirated and the MTT formazan formed by metabolically viable cells was dissolved in 100 µL isopropanol. The plates were mixed for 30 min on a gyratory shaker, and absorbance was measured at 595 nm using a plate reader.

2.8. TUNEL Assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed to detect apoptotic cells according to the manufacturer’s instructions. The in vivo TUNEL assay was performed as described previously [50]. Stained sections of tumors of each group were reviewed, and the apoptosis index (AI) was determined by TUNEL staining, by counting at least 1000 cells in five randomly selected high-power fields (magnification, ×200).
2.9. Preparation of Nuclear and Cytoplasmic Extracts

Nuclear and cytoplasmic soluble extracts were prepared from the cells described above in various groups in various time points using a rapid version of the method as previously described [51,52]. Cytoplasmic extracts were obtained by diluting the supernatant obtained after the first centrifugation with three volumes of buffer D [52].

2.10. Western Blotting

Total cellular proteins were isolated and the protein concentration of the sample was determined by Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). NF-κB p65/relA, AKT phosphorylated at Ser473 and β-actin were detected as described previously [23,27]. For wtp53 (DO-1), Bcl-2, phospho-p21Cip/Waf1, phospho-IκBα, PUMA and caspase-3 analysis, aliquots of 1–10 µg of proteins were resolved by SDS-PAGE, transferred to membranes, and probed with the above mentioned primary antibodies. The targeted protein was revealed by enhanced chemiluminescence (ECL). The membrane was incubated with an ECL solution (Biological Industries) and exposed to ECL film (Eastman Kodak, Rochester, NY, USA) to visualize specifically labeled proteins. The resulting exposed films were then analyzed by densitometry. All experiments were performed at least three times.

2.11. NF-κB Activity Assay

The cells (1 × 10^5) described above in various groups in various time points were seeded in 60-mm dishes and simultaneously transfected with Lentivector-NF-κB-luc and Lentivector-Ubiquitin-Renilla-Luc (25 µL of each viral supernatant/mL medium) mixed with polybrene (4 µg/mL medium) to develop stable cells expressing the NF-κB reporter and the renilla luciferase control [27]. Reporter activities were measured using the Dual Luciferase reporter assay system (Promega) at 24 h after transfection, according to the manufacturer’s instructions. Reporter activities were normalized using Renilla luciferase values.

2.12. Detection of NF-κB Binding Activity by EMSA

Nuclear protein extracts were prepared as described previously [51–55]. The sequence of the NF-κB oligonucleotide probe was 5′-AGTTGAGGGACTTTCCCAGGC-3′. EMSA was performed as described previously [51,52].

2.13. Tumor Xenografts and Tissue Staining

All animal experiments were approved by Qingdao Medical College, Qingdao University. Tumor xenografts were established by s.c. injection of 5 × 10^6 L3.6pl, PANC-1 or MIAPaCa-2 cells into both flanks of 5–6-week-old female athymic nude mice. When the tumor was 50–100 mm^3, the mice were randomized into the following treatment groups (n = 6 each): (1) untreated control; (2) gemcitabine (80 mg/kg) twice weekly (i.v. injection); (3) each tumor was injected with rAAV2-AKT2 siRNA or rAAV2-mock siRNA at 10^9 pfu in 100 µL PBS. Each treatment was repeated three times (from day 0,
once every other day); and (4) gemcitabine and rAAV2-AKT2 siRNA or rAAV2-mock siRNA, following the schedule for the individual treatments. Tumor growth was monitored three times weekly by calipers to calculate tumor volumes according to the formula (length × width²)/2. TUNEL staining on frozen sections was done according to the manufacturer’s instructions. The protein of AKT2, pAkt (Ser473), NF-κB, bcl-2, PUMA and β-actin in frozen sections was determined by western blotting. Frozen sections were also analyzed by hematoxylin and eosin staining.

2.14. Statistical Analysis

All experiments were independently performed at least three times. Unless otherwise stated, a representative experiment is displayed. Error bars denote SDs. P values were calculated by Student’s t test or ANOVA. Differences were considered to be statistically significant at P < 0.05.

3. Results

3.1. Knockdown of AKT2 Reduces NF-κB Activity in Pancreatic Cancer Cell Lines

Western blotting was used to measure wt-p53, NF-κBp65 and AKT2 (pAKT) (Figure 1A). NF-κB activity was measured using the reporter assay and nuclear NF-κB DNA binding was observed by EMSA (Figure 1B,C). Dose-dependent inhibition of pAKT and NF-κB activation was found in L3.6pl, PANC-1 and MIAPaCa-2 cells treated with AKT2 siRNA, but not in BxPC-3 cells because of their low basal AKT2 level (Figure 1A–C). There was no obvious change in wt-p53 expression in the four cell lines (Figure 1A). Mock siRNA (10, 50, 100 or 500 MOI) did not have any effect on protein expression and activity in the four cell lines (data not shown).

3.2. Differential Response to Chemotherapy in Pancreatic Cancer Cell Lines with Varying Levels of AKT2 Inhibition

Fahy et al. [21,26] have reported that AKT inhibition is associated with gemcitabine chemosensitization in MIA-PaCa-2 cells via inhibition of NF-κB activity. We evaluated whether NF-κB activity suppression via AKT2 specific inhibition chemosensitized to gemcitabine in the other pancreatic cancer cells. Two gemcitabine-sensitive (L3.6pl and BxPC-3) and two resistant (PANC-1 and MIA-PaCa-2) cell lines [21] were exposed to a series of concentrations of AKT2 siRNA or mock siRNA (10, 50, 100 or 500 MOI) for 48 h to knock down AKT2, followed by 0.5 µmol/L gemcitabine for L3.6pl cells and 1 µmol/L for PANC-1, BxPC-3 and MIA PaCa-2 cells for 72 h, as described previously [21,54]. MTT analysis was conducted to measure cell viability and proliferation in response to gemcitabine treatment in AKT2 siRNA-transfected-L3.6pl, BxPC-3, PANC-1 and MIAPaCa-2 cells (Figure 2A). In all the four cell lines, depletion of AKT2 alone did not induce significant proliferation inhibition compared to the controls (P > 0.05) (Figure 2A). However, gemcitabine treatment in AKT2-silenced cells resulted in significant cell proliferation inhibition in L3.6pl cells (P < 0.01) and MIA PaCa-2 cells (P < 0.05) compared to cells treated with gemcitabine alone (Figure 2A). Treatment with gemcitabine for 72 h led to cell proliferation inhibition that was similar to that with combined treatment of BxPC-3 and PANC-1 cells (P > 0.05) (Figure 2A). The same sensitivities were obtained when the effects of gemcitabine (0.5 or 1 µM) combined with AKT2 inhibition (MOI = 100) on
apoptosis were analyzed using TUNEL assay (Figure 2B), and when the four cells transfected with 100 MOI rAAV2-AKT2 siRNA were treated with 0.1–1 µM gemcitabine (data not shown).

**Figure 1.** Effects of AKT2 inhibition on NF-κB activity. (A) L3.6pl, BxPC-3, PANC-1 and MIAPaCa-2 cells were treated with 10, 50, 100 or 500 MOI rAAV2-AKT2 siRNA for 48 h. AKT2, pAKT (pAKTscr473), wt-p53 and NF-κBp65 levels were detected by western blotting; (B) Nuclear extracts prepared from L3.6pl, BxPC-3, PANC-1 and MIAPaCa-2 cells after 48 h incubation with 10, 50, 100 and 500 MOI rAAV2-AKT2 siRNA, and were assayed by EMSA; (C) NF-κB transcriptional activity was estimated using a reporter assay.
Figure 2. MTT and TUNEL assay showing cytotoxic effect of gemcitabine on AKT2 siRNA-transfected pancreatic cancer cell lines. (A) Cell viability assay was performed at 72 h after treatment with gemcitabine. AKT2 siRNA-transfected L3.6pl and MIA PaCa-2 cells showed significantly higher susceptibility to gemcitabine compared to gemcitabine alone (* P < 0.05, ** P < 0.01). AKT2 siRNA-transfected BxPC-3 and PANC-1 cells showed similar susceptibility to gemcitabine compared to gemcitabine alone (P > 0.05); (B) TUNEL analysis for measuring apoptosis induced by AKT2 inhibition, gemcitabine, and AKT2 inhibition combined with gemcitabine treatment. AKT2 siRNA transfection alone failed to induce apoptosis and inhibited proliferation in the four cell lines. In both assays, three separate experiments were performed and data from one representative experiment (mean ± SD of four replicates) are shown.

3.3. Effect of Gemcitabine on Activation of AKT and NF-κB in Pancreatic Cancer Cells

Following exposure to apoptotic stimuli, cells may engage survival mechanisms to subvert the induction of cell death. NF-κB and AKT activity is such signaling, which may increase survival and protect cancer cells from chemotherapy [11–14,20–23]. Colin et al. [15] have reported that gemcitabine induces a rapid increase in pSer$^{473}\text{Akt}$ levels over a period of 15 min to 6 h. No significant activation of AKT in PANC-1 or AsPC-1 cells was observed following gemcitabine treatment. L3.6pl, BxPC-3, PANC-1 and MIAPaCa-2 cells were treated with 1 µM gemcitabine for 24–72. Significant activation of AKT2 was shown in MIAPaCa-2 and L3.6pl cells over 48 h, but AKT2 activity began to
decline after 48 h (Figure 3A,B). No significant activation of AKT2 in PANC-1 and BxPC-3 cells was observed following gemcitabine treatment for 72 h, which was consistent with the study of Banerjee et al. [54] (Figure 3A,B). Pan et al. [21] have reported that gemcitabine treatment for 24 h does not influence NF-κB activity in pancreatic cancer cells in vitro. However, Amit Verma et al. [53] have reported that NF-κB activity was significantly increased by 0.1 µM gemcitabine for 48 h in L3.6pl cells. There was significant NF-κB activity in MIAPaCa-2 and L3.6pl cells after 48 h treatment with gemcitabine, and after 72 h treatment in BxPC-3 cells (Figure 3A,B) (P < 0.05). No significant activation of NF-κB in PANC-1 cells was observed following gemcitabine treatment for 72 h (P > 0.05) (Figure 3A,B). Gemcitabine treatment did not induce NF-κB and AKT2 activity in PANC-1 cells. Although NF-κB activity was induced in BxPC-3 cells after gemcitabine treatment, no significant activation of AKT2 was observed. However, significant activation of AKT2 in L3.6pl and MIAPaCa-2 cells was observed, followed by activation of NF-κB after gemcitabine treatment. To evaluate whether activation of NF-κB after gemcitabine treatment was AKT2 dependent, L3.6pl and MIAPaCa-2 cell lines were transfected with AKT2 siRNA or mock siRNA (MOI 100) for 48 h, and then the cells were treated with 1 µM gemcitabine for 72 h. As shown in Figure 3C, NF-κB activity was reduced significantly in L3.6pl and MIAPaCa-2 cells after combined treatment. We therefore confirmed that gemcitabine-induced activation of NF-κB is AKT2 dependent in L3.6pl and MIAPaCa-2 cells.

**Figure 3.** Western blotting and EMSA for AKT2 (pSer473), pAKT and NF-κBp65 following treatment with gemcitabine and/or AKT2 siRNA. (A) Western blotting for AKT2 (pSer473), pAKT and NF-κBp65 in four pancreatic cancer cell lines following treatment with gemcitabine (1 µM) for 72 h in L3.6pl, MiaPaCa-2, BxPC-3 and PANC-1 cells; (B) Relative Akt2 (pSer473), pAKT and NF-κBp65 levels are shown (vs. control, * P < 0.05); (C) Nuclear extracts prepared from L3.6pl and MIAPaCa-2 cells after 48 h incubation with 1 µM gemcitabine combined with 100 MOI AKT2 siRNA were assayed by EMSA.
3.4. PUMA Is Required for Gemcitabine-Induced Apoptosis in Pancreatic Cancer Cells

We first investigated the effect of gemcitabine on p53, PUMA and PUMA-responsive gene product cytochrome C and caspase-3 in L3.6pl, MiaPaCa-2, BxPC-3 and PANC-1 cells growing in normal media. All these experiments were done three times. The cells exhibited significantly increased p53, PUMA, cytochrome C and caspase-3 expression after treatment with gemcitabine in BxPC-3 cells as compared with MiaPaCa-2, L3.6pl and PANC-1 cells (Figure 4A). Furthermore, BxPC-3 cells exhibited significantly decreased PUMA, cytochrome C and caspase-3 expression when gemcitabine-treated cells were transfected with PUMA siRNA (20 µg) to knock down PUMA content, and significantly decreased AI without affecting p53 (Figure 4B). Although gemcitabine induced apoptosis in MiaPaCa-2 and L3.6pl cells, followed by upregulation of p53, PUMA, cytochrome C and caspase-3, no significant difference was found compared with the PUMA siRNA-transfected groups (data not shown). The data suggest that PUMA is required for gemcitabine-induced apoptosis mainly in gemcitabine-sensitive BxPC-3 in vitro.

Figure 4. Gemcitabine modulates p53 downstream target protein expression and induces apoptosis. L3.6pl, MiaPaCa-2, BxPC-3 and PANC-1 cells were treated with gemcitabine (0.1, 0.5 and 1 µM) or combination of gemcitabine and PUMA siRNA. Levels of p53, PUMA, cytochrome C (cyt. C) and caspase-3 were determined by western blotting; (A) Basal PUMA protein was detectable in PANC-1 cells, but was undetectable in L3.6pl, MiaPaCa-2 and BxPC-3 cells. Expression of p53, PUMA, cyt C and caspase-3 proteins was significantly increased in the BxPC-3 cell line treated with gemcitabine. There was no significant increase in proteins in L3.6pl, MiaPaCa-2 and PANC-1 cells treated with gemcitabine; (B) BxPC-3 cells were transfected with PUMA siRNA in the presence or absence of 0.1–1 µM gemcitabine. PUMA and p53-responsive gene product cyt C and caspase-3 were significantly decreased, however, p53 was not affected; (C) BxPC-3 cells transfected with PUMA siRNA had a significantly decreased AI (* P < 0.05, ** P < 0.01).
3.5. Induction of PUMA-Dependent Sensitivity to Gemcitabine by Inhibition of AKT2 Activity, as a Mechanism of Apoptosis Promotion in Pancreatic Cancer Cells

As shown above, inhibition of AKT2 enhanced sensitivity to gemcitabine in MiaPaCa-2 and L3.6pl cells. Fahy et al. [18,26] have reported that AKT inhibition is associated with chemosensitization in MIA-PaCa-2 pancreatic cancer cells via inhibition of NF-κB activity. We investigated how inhibition of AKT2 enhanced sensitivity to gemcitabine, by inducing the PUMA or NF-κB signaling pathway. MiaPaCa-2 and L3.6pl cells were treated with 100 MOI AKT2 siRNA or mock siRNA for 48 h, and then the transfected cells were exposed to 1 μM gemcitabine for 4 h. TNF-α (5–20 U) was added to the cells treated with rAAV2 and gemcitabine for 72 h. TNF-α significantly increased NF-κB activity in both cell lines in a concentration-dependent manner (Figure 5A, E). PUMA and PUMA-responsive gene product cytochrome C and active caspase-3 were significantly increased in MiaPaCa-2 cells treated with AKT2 siRNA and gemcitabine (Figure 5C). There was no significant increase in L3.6pl cells (Figure 5G). Enhancement of apoptosis induced by combined gemcitabine and AKT2 siRNA was not decreased significantly when NF-κB activity was activated and its downstream bcl-2 protein was increased in MiaPaCa-2 cells (Figure 5B). However, enhancement of apoptosis induced by combined gemcitabine and AKT2 siRNA was decreased significantly when NF-κB activity and its downstream bcl-2 protein were increased in L3.6pl cells (Figure 5F).

To determine whether inhibition of AKT2 enhances sensitivity to gemcitabine via PUMA upregulation, MiaPaCa-2 cells treated with AKT2 siRNA and gemcitabine were transfected with PUMA siRNA to knock down PUMA (Figure 5C). Enhancement of apoptosis induced by combined gemcitabine and AKT2 siRNA was decreased significantly (Figure 5D). However, apoptosis was not decreased significantly when L3.6pl cells treated with AKT2 siRNA and gemcitabine were transfected with PUMA siRNA (Figure 5H).

AKT2/pAKT expression was not found in basal level and gemcitabine-treated BxPC-3 cells, therefore, we could conclude that there was no relationship between AKT2/pAKT expression and sensitivity to gemcitabine. Although the AKT2 basal level was high in PANC-1 cells, there was no relationship between AKT2/pAKT expression and sensitivity to gemcitabine.

These results indicate that inhibition of AKT2 enhanced sensitivity to gemcitabine in MiaPaCa-2 cells via a PUMA-dependent, but not NF-κB pathway. This was contrary to previous studies [18,26]. In L3.6pl cells, inhibition of AKT2 enhanced sensitivity to gemcitabine via an NF-κB-dependent, but not PUMA pathway. In PANC-1 cells, resistance to gemcitabine is not AKT2 or NF-κB-dependent. However, BxPC-3 cells were sensitized to gemcitabine via an AKT2/pAKT-independent NF-κB and PUMA pathway.
3.6. Effect of Gemcitabine Alone and in Combination with AKT2 siRNA on Primary Tumor Growth in Pancreatic Cancer In Vivo

To determine whether AKT2 confers tumor resistance in vivo, tumor xenografts were established by s.c. injection of $5 \times 10^6$ L3.6pl, PANC-1, BxPC-3 or MIAPaCa-2 cells into both flanks of 5–6-week-old female athymic nude mice. When the tumor was 50–100 mm$^3$, it was injected with rAAV2-AKT2 siRNA or rAAV2-mock siRNA at $10^9$ pfu in 100 µL PBS, and the treatment was repeated three times (once every other day, from day 0). To avoid potential systemic effects of different viruses, rAAV2-AKT2 siRNA and rAAV2-mock siRNA were injected into separate tumors in the same...
animals. rAAV2-AKT2 siRNA did not have any effect on tumor growth compared with PBS alone or rAAV2-mock siRNA alone after 35 days treatment (data not shown).

To determine whether gemcitabine can effectively inhibit growth of established tumors in vivo, gemcitabine alone (80 mg/kg), twice weekly (i.v. injection) was used to treat established tumors for 35 days. In contrast to gemcitabine sensitivity levels in vitro, gemcitabine significantly reduced tumor weight in PANC-1 and L3.6pl xenografts ($P < 0.05$), whereas BxPC-3 and MIAPaCa-2 became drug resistant ($P > 0.05$) (Figure 6A).

**Figure 6.** Effect of rAAV2-AKT2 siRNA combined with gemcitabine on tumor growth. (A) Tumor weight in mice treated with vehicle control, gemcitabine (gem.; 80 mg/kg twice weekly, i.v.), rAAV2-AKT2 siRNA combined with gemcitabine, Columns, mean (six samples per group); bars, SD. All statistical tests were two sided (* $P < 0.05$, ** $P < 0.01$, *** $P > 0.05$); (B) Growth curve of L3.6pl, PANC-1, BxPC-3 and MIAPaCa-2 tumors ($n = 6$ per group) treated with gemcitabine (80 mg/kg twice weekly, i.v.) or rAAV2-AKT2 siRNA combined with gemcitabine. Student’s $t$ test versus control group; * $P < 0.05$, ** $P < 0.01$.

To determine whether knockdown of AKT2 could sensitize to gemcitabine, we treated established tumors of PANC-1, BxPC-3, L3.6pl and MIAPaCa-2 with gemcitabine combined with rAAV2-AKT2 siRNA or rAAV2-mock siRNA. Established MIAPaCa-2 and PANC-1 tumors subjected to combined treatment grew much slower and reached less than twice the initial volume, with at least 60% growth suppression compared with gemcitabine alone ($P < 0.05$; Figure 6A,B). Combined treatment significantly reduced tumor weight in PANC-1 and MIAPaCa-2 xenografts compared with gemcitabine alone (Figure 6A) ($P < 0.05$). In established BxPC-3 and L3.6pl tumors, AAV2-AKT2 siRNA combined with gemcitabine did not affect tumor growth compared with gemcitabine alone ($P > 0.05$; Figure 6A,B).
3.7. Effect of Gemcitabine Alone and in Combination with AKT2 siRNA on Primary Tumor Apoptosis in Pancreatic Cancer In Vivo

TUNEL assay revealed many apoptotic cells in the established L3.6pl and PANC-1 tumors treated with gemcitabine alone compared with control tumors ($P < 0.05$) (Figure 7). However, treatment with gemcitabine alone did not increase the number of apoptotic cells in MIAPaCa-2 and BxPC-3 established tumors (Figure 7). In PANC-1 and MIAPaCa-2 established tumors, rAAV2-AKT2 siRNA combined with gemcitabine treatment increased the number of apoptotic cells compared with gemcitabine alone (Figure 7). In the L3.6pl and BxPC-3 established tumors, rAAV2-AKT2 siRNA combined with gemcitabine did not affect the number of apoptotic cells compared with gemcitabine treatment alone ($P > 0.05$) (Figure 7). These data show that knockdown of AKT2 can effectively sensitize to gemcitabine treatment and inhibit growth of MIAPaCa-2 and PANC-1 established tumors in vivo, at least partially through induction of apoptosis.

**Figure 7.** Effect of rAAV2-AKT2 siRNA in combination with gemcitabine on tumor growth. TUNEL analysis of apoptotic cells. Results shown as mean ± SEM from two independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P > 0.05$ by Student’s t test.

3.8. Effect of Gemcitabine Alone and in Combination with AKT2 siRNA on PUMA and NF-κB in Pancreatic Cancer In Vivo

We measured the levels of PUMA and its downstream targets, NF-κBp65 and its downstream targets bcl-2, AKT2 and pAKT in established tumors by western blotting. After 6 weeks treatment, there was a dramatic increase in the levels of PUMA and its downstream targets in MIAPaCa-2 and PANC-1 established tumors treated with rAAV2-AKT2 siRNA combined with gemcitabine, and in control tumors (Figure 8A,B). In PANC-1 established tumors, gemcitabine significantly increased PUMA level and its downstream targets compared with control tumors, which was contrary to the in vitro study (Figure 4). However, in MIAPaCa-2 established tumors, gemcitabine did not increase PUMA level, which agreed with the in vitro study (Figure 4). We also observed that gemcitabine increased NF-κBp65 level in PANC-1 established tumors, which was contrary to the in vitro study (Figure 8C). In MIAPaCa-2 and PANC-1 established tumors, rAAV2-AKT2 siRNA combined with gemcitabine significantly decreased NF-κB expression and increased expression of PUMA and its
downstream targets compared with control tumors (Figure 8A,B). Pan et al. [21] have reported that silencing NF-κB does not sensitize to gemcitabine-induced apoptosis in vivo and in vitro, therefore, we suggest that rAAV2-AKT2 siRNA combined with gemcitabine inhibits growth of MIAPaCa-2 and PANC-1 established tumors by PUMA upregulation, at least in part. Whether NF-κB downregulation plays an important role needs further investigation.

**Figure 8.** Western blotting analysis of the effect of rAAV2-AKT2 siRNA in combination with gemcitabine treatment on levels of PUMA and its downstream targets, NF-κBp65 and its downstream targets bcl-2, AKT2 and pAKT in established tumors after 6 weeks treatment. (A,B) In MIAPaCa-2 and PANC-1 established tumors, rAAV2-AKT2 siRNA combined with gemcitabine significantly decreased NF-κB expression and increased expression of PUMA and its downstream targets compared with control tumors; (C) Treatment with rAAV2-AKT2 siRNA combined with gemcitabine did not increase the levels of PUMA and its downstream targets compared with gemcitabine treatment alone in L3.6pl cells. However, there was a decrease in NF-κB expression in L3.6pl established tumors treated with rAAV2-AKT2 siRNA combined with gemcitabine.

L3.6pl established tumors treated with gemcitabine alone showed a significant increase in expression of nuclear p65 expression and PUMA and its downstream targets compared with control tumors, which agreed with the in vitro study (Figure 3). However, treatment with rAAV2-AKT2 siRNA combined with gemcitabine did not increase the levels of PUMA and its downstream targets compared with gemcitabine treatment alone (Figure 8C). We observed a significant decrease in NF-κB expression in L3.6pl established tumors treated with rAAV2-AKT2 siRNA combined with gemcitabine (Figure 8C). In BxPC-3 established tumors, gemcitabine or rAAV2-AKT2 siRNA combined with gemcitabine treatment did not affect expression of NF-κB and PUMA (data not shown).

4. Discussion

Studies have established AKT as an important regulator of cell proliferation and survival [56,57]. Furthermore, AKT also plays an important role in cancer therapy by promoting resistance to the
apoptosis-inducing effects of chemotherapy [56–59]. In some pancreatic cancer cells, inhibition of AKT has repeatedly and consistently been shown to sensitize to the apoptotic effect of chemotherapy [16,18,26]. The mechanism by which AKT activation in these cancer cells confers chemoresistance is not clear. However, in some pancreatic cancer cells, basal AKT activity does not correlate with sensitivity towards gemcitabine treatment, nor does inhibition of PI3K/AKT by LY294002 alter gemcitabine-induced apoptosis [22].

Our results that demonstrated that inhibition of AKT2 activity itself did not inhibit growth and promote apoptosis in PANC-1, L3.6pl, BxPC-3 and MIAPaCa-2 cells in vitro and in vivo. The results of the present study also illustrate the variable expression and activity of AKT across a panel of pancreatic cancer cell lines, although basal level of activation could not be used to predict sensitivity to gemcitabine treatment. Therefore, we investigated the mechanism by which pancreatic cancer cells are sensitized or become resistant to gemcitabine treatment.

In the present study, the cell lines L3.6pl and BxPC-3 were sensitive to gemcitabine, whereas MIAPaCa-2 and PANC-1 cells were resistant in vitro, which is consistent with the recent study by Pan et al. [21]. In contrast to gemcitabine sensitivity levels in vitro, the growth of PANC-1 xenografts was inhibited by gemcitabine treatment, whereas BxPC-3 cells became resistant, consistent with the recent study by Pham et al. [60], which suggests that the tumor microenvironment has an important role in determining drug sensitivity.

The mechanism of L3.6pl and BxPC-3 cell sensitivity to gemcitabine in vitro is somewhat different. In BxPC-3 cells, gemcitabine induces the direct targeting of p53-dependent PUMA upregulation, followed by significant cell death and induction of apoptosis. However, inhibition of PUMA activity using an siRNA directed at PUMA could reduce chemosensitivity to gemcitabine. We showed that p53 was required for gemcitabine-induced apoptosis in BxPC-3 cells in vitro, and that this was dependent upon induction of PUMA. In L3.6pl cells, though sensitive to gemcitabine, no obvious PUMA upregulation was shown after gemcitabine treatment. The mechanism for this is unknown and could be explained by increased translation of other (BH3)-only proteins. Although gemcitabine induces the direct targeting of p53-dependent PUMA upregulation in MIAPaCa-2 cells, PUMA was not sufficient to induce apoptosis in vitro. In PANC-1 and L3.6pl cells, gemcitabine did not induce changes in the PUMA profile in vitro, however, gemcitabine induced an obvious increase in the PUMA profile in established tumors. Therefore, we suggest that gemcitabine sensitizes tumors in vivo by inducing PUMA upregulation. BxPC-3 cells became drug resistant in vivo, opposite to its mechanism of inducing PUMA profile in vitro. These observations may be attributed to the mechanism of PUMA upregulation to sensitize gemcitabine in pancreatic cancer.

PI3K/AKT is a fundamental signaling pathway that mediates several cellular processes, including cell proliferation, growth, survival and motility [61–63]. Increased activation, deregulation and mutation of the components in the PI3K/AKT pathway have been implicated in driving tumorigenesis and conferring resistance to chemotherapy [64,65].

Previous studies have shown [18,26] that inhibition of PI3K or AKT decreases the level of the antiapoptotic protein Bcl-2 and increases the level of the proapoptotic protein BAX. Furthermore, inhibition of AKT decreased the function of NF-κB, which is capable of transcriptional regulation of the Bcl-2 gene in MIAPaCa-2 cells. Inhibition of this pathway increased the apoptotic effect of chemotherapy. However, we found that inhibition of AKT2 enhanced sensitivity to gemcitabine in
MiaPaCa-2 cells, followed by decreased NF-κB activity in vitro. When NF-κB activity was recovered in MiaPaCa-2 cells, enhancement of apoptosis induced by gemcitabine combined with AKT2 siRNA was not decreased significantly when NF-κB activity was activated and its downstream bcl-2 protein was increased. On the contrary, enhancement of apoptosis induced by gemcitabine combined with AKT2 siRNA was decreased significantly when PUMA was inhibited. These results indicate that inhibition of AKT2 enhances sensitivity to gemcitabine in MiaPaCa-2 cells via a PUMA-dependent, but not the NF-κB pathway in vitro. In MIAPaCa-2 established tumors, rAAV2-AKT2 siRNA combined with gemcitabine significantly inhibited tumor growth, followed by PUMA upregulation after 5 weeks treatment, contrary to the study in vitro. Pan et al. [27] have reported that silencing of p65/relA is effective alone and in combination with gemcitabine in gemcitabine-sensitive but not gemcitabine-resistant pancreatic cancer cells. In the present study, although combined treatment decreased NF-κB activity, we suggest that knockdown of AKT2 combined with gemcitabine inhibits the in vivo growth of MIAPaCa-2 established tumors by PUMA upregulation, and not NF-κB downregulation.

PANC-1 would have been resistant to the tested agent gemcitabine in vitro, although inhibition of AKT2 decreased the NF-κB activity and its downstream bcl-2 protein, it did not induce apoptosis. Furthermore, gemcitabine did not induce PUMA upregulation. The results indicated that resistance to gemcitabine in PANC-1 cells in vitro was not AKT2-, PUMA- or NF-κB-dependent. However, in PANC-1 established tumors, gemcitabine or combined treatment significantly promoted apoptosis and inhibited tumor growth, followed by increased PUMA upregulation and decreased NF-κB activity. Pan et al. [21] have reported that silencing of p65/relA does not sensitize PANC-1 cells to gemcitabine in vitro and in vivo. We therefore suggest that gemcitabine alone or in combination with AKT2 inhibition inhibits in vivo tumor growth by PUMA upregulation, but not by NF-κB downregulation in PANC-1 established tumors.

In L3.6pl cells in vitro, inhibition of AKT2 enhances sensitivity to gemcitabine, followed by decreased NF-κB activity. When the NF-κB activity was recovered, enhancement of apoptosis induced by gemcitabine combined with AKT2 siRNA was decreased significantly when activity of NF-κB activity and its downstream bcl-2 protein was increased in L3.6pl cells. However, PUMA did not undergo obvious changes. These results indicated that inhibition of AKT2 enhanced sensitivity to gemcitabine via an NF-κB-dependent, but not the PUMA pathway in L3.6pl cells in vitro. In L3.6pl established tumors, gemcitabine significantly inhibited tumor growth, followed by upregulation of PUMA and NF-κB. When rAAV2-AKT2 siRNA combined with gemcitabine treatment inhibited NF-κB activity, tumor growth was not inhibited, compared with gemcitabine treatment alone. We therefore suggest that AKT2 inhibition did not sensitize L3.6pl cells to gemcitabine in vivo. Silencing of p65/relA by AKT2 inhibition did not sensitize gemcitabine to L3.6pl cells in vivo, contrary to the study of Pan et al. [21].

BxPC-3 would have been sensitive to the tested agent gemcitabine, and gemcitabine treatment increased activity of NF-κB and its downstream bcl-2 protein significantly. PUMA and its downstream were also increased significantly. A previous study has shown that knockdown of NF-κB sensitizes BxPC-3 cells to gemcitabine [21]. Our present study found that PUMA was also required for gemcitabine-induced apoptosis in pancreatic cancer cell line BxPC-3. We therefore conclude that BxPC-3 cells were sensitized to gemcitabine via both an NF-κB- and PUMA-dependent pathway, but
not the AKT2/pAKT pathway in vitro. In BxPC-3 established tumors, gemcitabine and combined treatment did not have an obvious effect on tumor growth and apoptosis, in contrast to the study of Pan et al. [21], however, it was consistent with the recent study by Pham et al. [60].

5. Conclusions

The level of AKT activation is not likely to be useful in selecting individual pancreatic tumors for AKT inhibition in combination with gemcitabine. The sensitivity levels of pancreatic cancer cells to gemcitabine are different. AKT inhibition sensitizes pancreatic cancer cells to gemcitabine via PUMA upregulation and/or decreased NF-κB activity. Our findings suggest that AKT inhibitors may have therapeutic potential when used in combination with gemcitabine in reversing drug resistance in some pancreatic cancer patients.

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Conflict of Interest

The authors promised there were not any possible conflicts of interest in this research.

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