Nimesulide inhibits proliferation and induces apoptosis of pancreatic cancer cells by enhancing expression of PTEN

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Abstract. Pancreatic cancer is the fourth leading cause of cancer-associated cases of mortality worldwide. Prostaglandin-endoperoxide synthase 2 (COX-2) is considered a therapeutic target for prevention of pancreatic cancer. Nimesulide, a selective COX-2 inhibitor, can induce cell apoptosis, resulting in an anti-cancer effect. However, the mechanism underlying this effect remains to be elucidated. The present study aimed to evaluate the effects of nimesulide on proliferation of PANC-1 cells using an MTT assay. Apoptosis was evaluated by DNA laddering and Annexin V-fluorescein isothiocyanate/propidium iodide-stained flow cytometry. Furthermore, western blot analysis was used to elucidate the mechanism underlying nimesulide treatment in PANC-1 cells. It was determined that proliferation of PANC-1 cells was inhibited by nimesulide in a dose-dependent manner. Nimesulide promoted apoptosis of PANC-1 cells. Western blot analysis demonstrated that nimesulide increased expression of cleaved caspase-3 and apoptosis regulator Bax (Bcl-2 associated protein X), and decreased the expression of pro-caspase-3 and apoptosis regulator Bcl-2 (B-cell lymphoma 2). Furthermore, nimesulide enhanced expression of phosphatase and tensin homolog (PTEN), and decreased the expression level of COX-2 and vascular endothelial growth factor. In summary, the results of the present study demonstrated that nimesulide could induce apoptosis and inhibit growth of PANC-1 cells by enhancing the expression of PTEN, which indicates the potential of nimesulide to prevent tumor angiogenesis.

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Introduction

Pancreatic cancer is one of the most common human gastrointestinal malignancies and the fourth leading cause of cancer-associated cases of mortality worldwide (1). In China, morbidity of pancreatic cancer ranks seventh among malignancies and pancreatic cancer is the sixth leading cause of mortality among all cancer types (2). Furthermore, the incidence of pancreatic cancer has demonstrated an upward trend in recent years (3). Primary characteristics of pancreatic cancer include late diagnosis, strong local invasion, early metastasis, high mortality rate, poor prognosis and low long-term survival (4). Compared with other common treatments, including chemotherapy, radiotherapy and biological therapy (5-7), surgical excision is considered the most effective option at present, but only 10-15% of patients undergo complete tumor resection (8). Despite the available therapies, the 5-year survival rate is ~5% (8). In addition, within 7 years following surgery of pancreatic cancer the mortality rate among patients is ~100% (9,10). Therefore, it is necessary to develop a more effective treatment for pancreatic cancer.

As previously demonstrated, overexpression of the prostaglandin-endoperoxide synthase 2 (COX-2) gene may be associated with tumorigenesis and progression of breast, prostate and lung cancer (11-13). Furthermore, COX-2 is considered a therapeutic target for prevention of pancreatic cancer (14,15). Nimesulide is a selective COX-2 inhibitor that could delay the progression of pancreatic cancer precursor lesions, inhibit cell proliferation and induce apoptosis (16-18). Phosphatase and tensin homolog (PTEN) is a lipid phosphatase that serves a role in tumor suppression (19). However, the effect of COX-2 inhibitors on PTEN in the context of pancreatic cancer remains to be elucidated.

In the present study, the effects of nimesulide on proliferation and apoptosis of pancreatic cancer cells were investigated with the aim of elucidating the potential PTEN-associated effect of nimesulide on pancreatic cancer.

Materials and methods

Reagents and cell culture. Nimesulide was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Dimethyl sulfoxide (DMSO), MTT and Annexin V/Dead Cell Apoptosis kit were purchased from Hangzhou Multi

Sciences Biotech Co., Ltd. (Hangzhou, China). Primary antibodies at a dilution of 1:1,000 against cleaved-caspase-3 (cat. no. AC033), pro-caspase-3 (cat. no. AF1261), PTEN (cat. no. AF1426), COX-2 (cat. no. AF1924) and vascular endothelial growth factor (VEGF; cat. no. AF1309), Bcl-2 (cat. no. AB112), Bcl-2 associated protein X (Bax; cat. no. AB026) and β-actin (cat. no. AA128) were utilized in the present study. The following secondary antibodies (Horseradish peroxidase conjugated Goat Anti-Rabbit Immunoglobulin G, 1:5,000; cat. no. A0208; Horseradish peroxidase conjugated Goat Anti-Mouse immunoglobulin G; 1:5,000; cat. no. A0216) were also utilized. All antibodies were supplied by Beyotime Institute of Biotechnology (Haimen, China) Human pancreatic cancer cell line PANC-1 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere at 37˚C with 5% CO_2. DMEM, FBS and 0.25% Trypsin-EDTA were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell proliferation assay. Viability of PANC-1 cells following treatment with nimesulide was evaluated using an MTT assay, as previously described (20). Briefly, $5x10^3$ cells/well (100 μl) were seeded in 96-well plates with different concentrations of nimesulide (0, 25, 50, 100, 200 and 400 μmol/l). DMSO was used as the control treatment. Following incubation at 37˚C for 48 h, 20 μl MTT (5 mg/ml) was added to each well followed by incubation at 37˚C for 4 h. DMSO was utilized to dissolve the purple formazan and the absorbance was measured at a wavelength of 490 nm using a microplate reader (Synergy HTX; BioTek Instruments, Inc., Winooski, VT, USA). The results are expressed as inhibition rates according to the following formula: Inhibition rate (%)=1-(OD treatment-OD blank)/(OD control-OD blank) x100%.

DNA laddering analysis. Cells were collected following treatment with different concentrations of nimesulide at 37˚C for 48 h. The supernatant was discarded following centrifugation at a speed of 1,000 x g for 5 min at room temperature and the pellet was washed with PBS (0.01 M, pH 7.4). Cells were incubated with 500 μl lysis buffer [0.5 M Tris-HCl (pH 8.0), 0.02 mmol/l EDTA and 1% NP-40] in a water bath at 55˚C for 16 h. The solutions were centrifuged at a speed of 12,000 x g for 5 min at 4˚C and treated with RNase A (final concentration, 20 mg/l; cat. no. R6148; Sigma-Aldrich) with 1% SDS and proteinase K (final concentration, 20 mg/l; cat. no. P2308; Sigma-Aldrich). A total of 60 μl 3 M sodium acetate and 600 μl ice-cold absolute ethanol was added, and samples were incubated at -20˚C for at least 1 h, followed by centrifugation at a speed of 12,000 x g for 20 min at 4˚C. Resulting DNA pellets were dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA at pH 7.4) and the DNA ladder was separated by electrophoresis on a 2% agarose gel (21).

Apoptosis assay. Apoptosis of PANC-1 cells were detected using the aforementioned Annexin V/propidium iodide (PI) Apoptosis Detection kit. Briefly, cells were exposed to various concentrations (50, 100, 200 and 400 μmol/l) of nimesulide for 48 h at 37˚C. Control cells were treated with DMSO. Cells were collected and washed twice with PBS. A total of $5x10^5$ cells/ml were re-suspended in 400 μl binding buffer with 5 μl Annexin V-fluorescein isothiocyanate (FITC) and 1 μl PI (100 μg/ml) in the dark. Following incubation at 37˚C for 15 min, cell apoptosis was detected by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) and was analyzed using CellQuest 3.3 software (BD Biosciences).

Table I. Nimesulide inhibits proliferation of PANC-1 cells (n=3).

Nimesulide (μmol/l)	Absorbance	Inhibition rate (%)
0	1.046±0.032	0
25	1.005±0.029	3.5±0.92
50	0.912±0.025[a,b]	12.7±3.29[a,b]
100	0.677±0.036[a-c]	35.2±4.21[a-c]
200	0.532±0.019[a-d]	49.1±3.75[a-d]
400	0.328±0.016[a-e]	68.3±2.87[a-e]

[a]P<0.01 vs. control group, [b]P<0.01 vs. 25 μmol/l, [c]P<0.01 vs. 50 μmol/l, [d]P<0.01 vs. 100 μmol/l, [e]P<0.01 vs. 200 μmol/l.

Western blot analysis. Cells were lysed with radioimmunoprecipitation assay lysate (Beyotime Institute of Biotechnology) to extract the total protein. The concentration of total protein was then quantitated using a BCA protein assay kit (Beyotime Institute of Biotechnology). Following this, 40 μg protein was loaded and separated on 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 1 h at 37˚C and probed with specific primary antibodies against COX-2, Bcl-2, Bax, VEGF, cleaved-caspase-3, pro-caspase-3, PTEN and β-actin at 4˚C overnight. Subsequently, the membranes were incubated at 37˚C with their corresponding secondary antibodies for 1 h. Target bands were visualized using an enhanced chemiluminescence solution (Qihai Biotec, Shanghai, China) and the Gel-Pro-Analyzer software (Bethesda, MD, USA) was employed to measure relative band intensities. Each target protein was normalized to the corresponding β-actin band. Protein from untreated cells were loaded onto each gel for comparison.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard deviation (n≥3). One way analysis of variance followed by Tukey's multiple comparisons test was used to compare differences between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Nimesulide inhibits proliferation of PANC-1 cells. The results of MTT assays indicated that the inhibitory effect of nimesulide on the proliferation of PANC-1 cells could be observed from a dose of 50-400 μmol/l (Table I). The inhibitory effect occurred in a concentration-dependent manner.

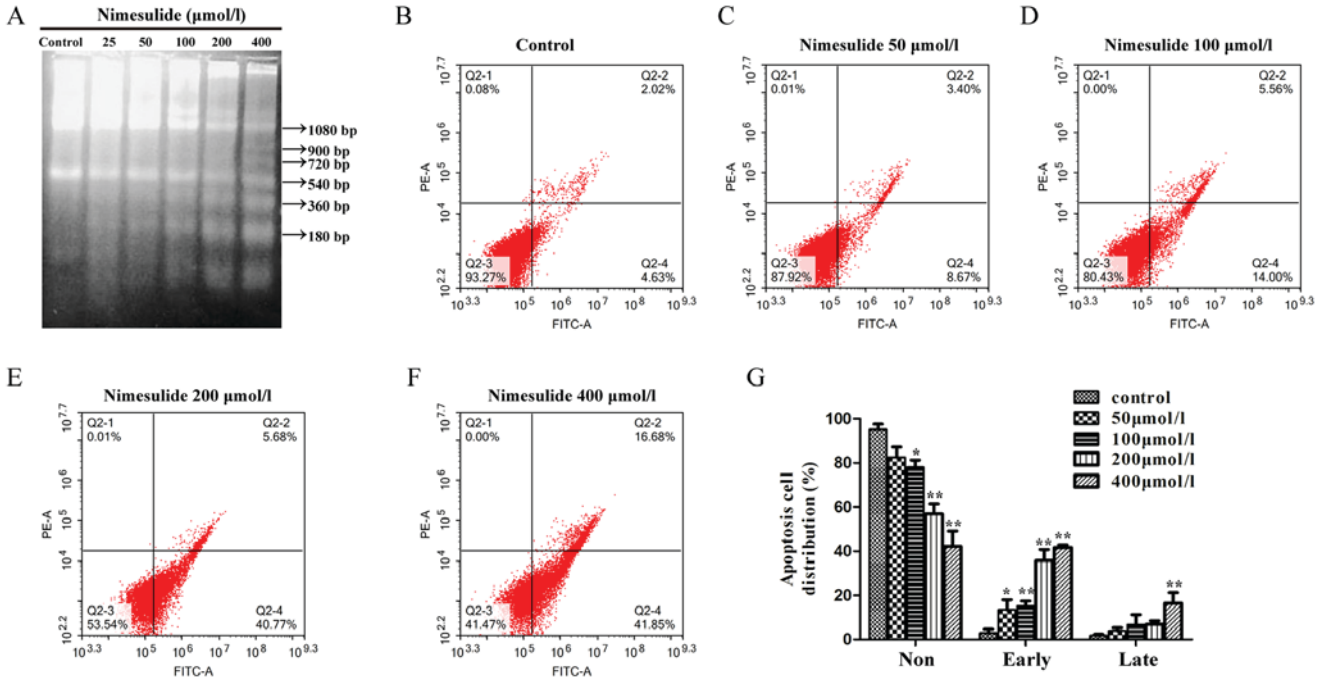

Figure 1. Apoptosis analysis of PANC-1 cells following treatment with nimesulide. (A) DNA laddering revealed the presence of apoptotic DNA fragmentation following treatment with different concentrations of nimesulide (0, 25, 50, 100, 200 and 400 μmol/l) for 48 h. PANC-1 cells were treated with nimesulide at a concentration of (B) 0, (C) 50, (D) 100, (E) 200 and (F) 400 μmol/l for 48 h and the apoptosis rate was determined by flow cytometry with Annexin V-fluorescein isothiocyanate/propidium iodide double dye assay. (G) Flow cytometry results were analyzed quantitatively. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01 vs. the control group.

Nimesulide induces apoptosis of PANC-1 cells. DNA laddering demonstrated that characteristics of apoptosis occurred following a 48 h treatment with nimesulide from the concentration of 50-400 μmol/l (Fig. 1A). The results of flow cytometry demonstrated that treatment with 200 and 400 μmol/l nimesulide for 48 h significantly increased early apoptosis of PANC-1 cells, compared with control cells (Fig. 1B-G). The above results indicated that nimesulide could induce early and late apoptosis of PANC-1 cells. To further investigate the mechanisms underlying nimesulide-induced apoptosis in PANC-1 cells, downstream mediators in the apoptotic cascade were analyzed by western blotting (Fig. 2). Following treatment with 100 and 200 μmol/l nimesulide for 48 h, increased expressions of Bax and cleaved caspase-3 was observed, respectively. Expression of pro-caspase-3 and Bcl-2 decreased following treatment with 100 and 50-200 μmol/l nimesulide, respectively.

Nimesulide decreases expression of COX-2 in PANC-1 cells. Protein expression of COX-2 in PANC cells was downregulated following treatment with nimesulide. Following treatment with 100, 200 or 400 μmol/l nimesulide for 48 h, cells demonstrated significantly lower expression of COX-2 protein compared with the untreated control cells (Fig. 3). Therefore, nimesulide suppressed expression of COX-2 in PANC-1 cells. The above results suggest the nimesulide may function as a COX-2 inhibitor in pancreatic cancer cells.

Nimesulide enhances expression of PTEN and downregulates expression of VEGF in PANC-1 cells. To elucidate the mechanism underlying the anti-proliferative and pro-apoptotic effects of nimesulide, protein expression of PTEN and VEGF were determined (Fig. 4). Following treatment with 400 μmol/l nimesulide for 48 h, expression of PTEN increased, compared with the control group. Expression of VEGF decreased significantly following treatment with 100 and 400 μmol/l nimesulide. These results indicated that PTEN and VEGF may be involved in the anti-proliferative and pro-apoptotic effects of nimesulide in PANC cells. However, the expression of VEGF was not significant following treatment with 200 μmol/l nimesulide. This may have been due to experimental error, but further study is required for clarification.

Discussion

Pancreatic cancer is an aggressive malignant disease and is one of the tumor types that is intrinsically resistant to chemotherapy (22,23). Apoptosis, also known as programmed cell death, serves a role in maintaining homeostasis of both normal and neoplastic cells (24). Suppression of cancer cell apoptosis is considered to contribute to development and progression of carcinomas by triggering gene mutations and promoting resistance to immune-based cytotoxicity (25). Previous studies have demonstrated that nimesulide promotes apoptosis of KOSC-2 oral squamous carcinoma cells (26). Nimesulide can also induce apoptosis by inactivating the Janus kinase 2/signal transducer and activator of transcription 3 pathway in Eca-109 cells (21). Consistent with the aforementioned studies, the present study demonstrated that nimesulide can induce apoptosis of PANC-1 cells as demonstrated by DNA laddering and Annexin V-FITC/PI experiments. Bax is a pro-apoptotic protein, whereas Bcl-2 is an anti-apoptotic protein (27). It has been previously demonstrated that downregulation of Bcl-2

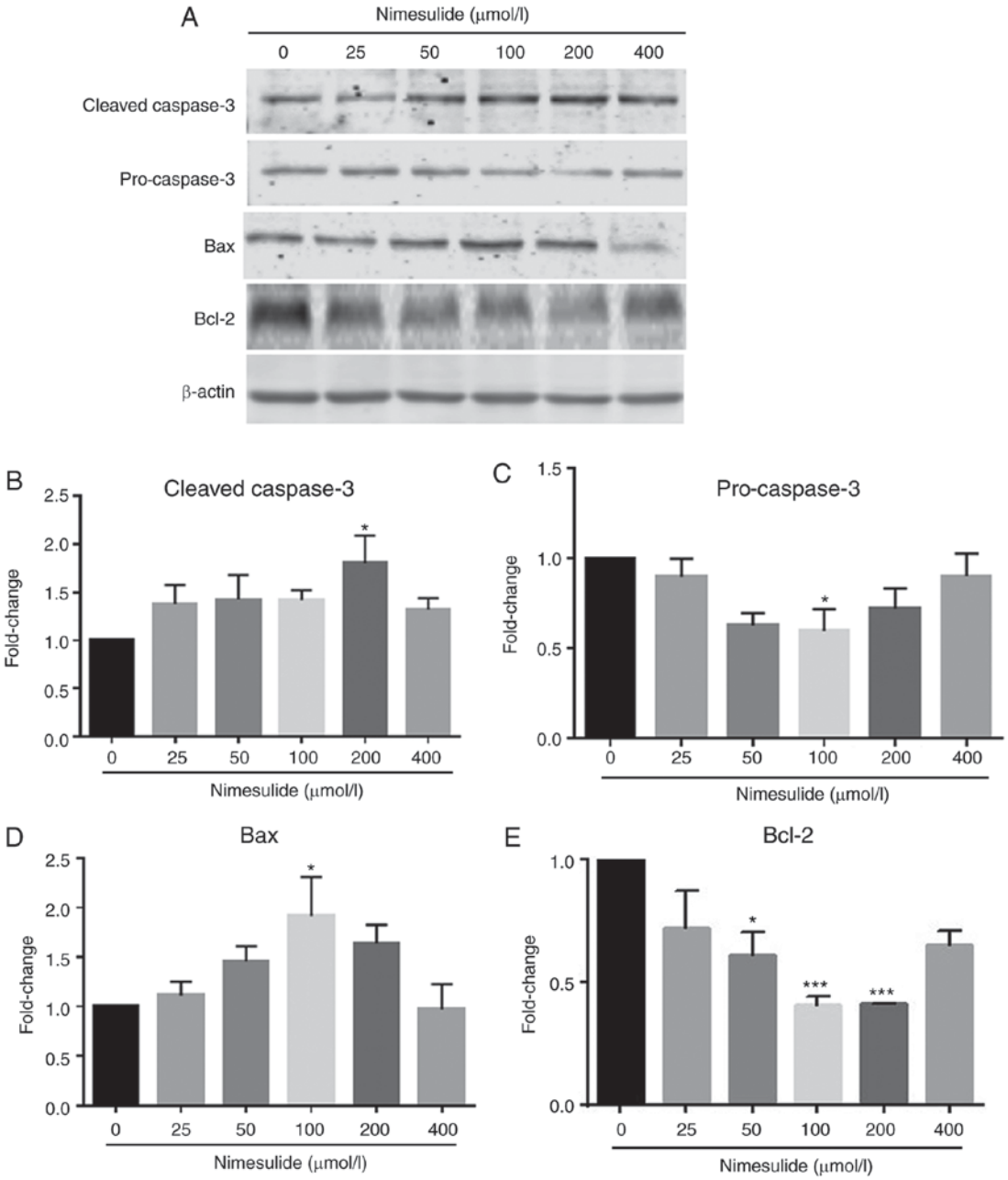

Figure 2. Effects of nimesulide on expression of cleaved-caspase-3, pro-caspase-3, Bax and Bcl-2 in PANC-1 cells. (A) PANC-1 cells were treated with different concentrations of nimesulide (0, 25, 50, 100, 200 and 400 μmol/l) for 48 h and protein expression was determined by western blotting. Expression of (B) cleaved caspase-3, (C) pro-caspase-3, (D) Bax and (E) Bcl-2 was analyzed. *P<0.05 and ***P<0.001 vs. the control group. Bax, Bcl-2 associated protein X; Bcl-2, B-cell lymphoma 2.

enables oligomerized Bax to insert into the outer mitochondrial membrane and promote apoptosis (28). The present study demonstrated that nimesulide could decrease expression levels of Bcl-2 and increase expression levels of Bax in PANC-1 cells, which suggested that apoptosis induced by nimesulide treatment of PANC-1 cells may be due to the activation of mitochondrial apoptotic pathways.

It has been previously reported that PTEN can regulate angiogenesis of human pancreatic cancer cells and that it is a suppressor of pancreatic ductal adenocarcinoma (29,30). Therefore, enhanced expression of PTEN in PANC-1 cells following treatment with nimesulide indicated a possible novel role of nimesulide in the treatment of pancreatic cancer in addition to inhibition of COX-2. The major substrate, with which PTEN interacts, is phosphatidylinositol (3,4,5)-trisphosphate, which is produced by the action of phosphoinositide-3-kinases (PI3Ks) (19). The PI3K/RAC-alpha serine/threonine-protein kinase (Akt) signaling pathway serves a role in the development of resistance to carcinoma therapy, and inhibition of the PI3K/Akt signaling pathway may suppress cancer cell growth and induce apoptosis in various cancer types (31-33). However, effects of upregulation of PTEN by nimesulide on PI3K/Akt signaling-mediated apoptosis of PANC-1 cells remains to be elucidated. Activation of peroxisome proliferator-activated receptor γ in human pancreatic cancer cells has been

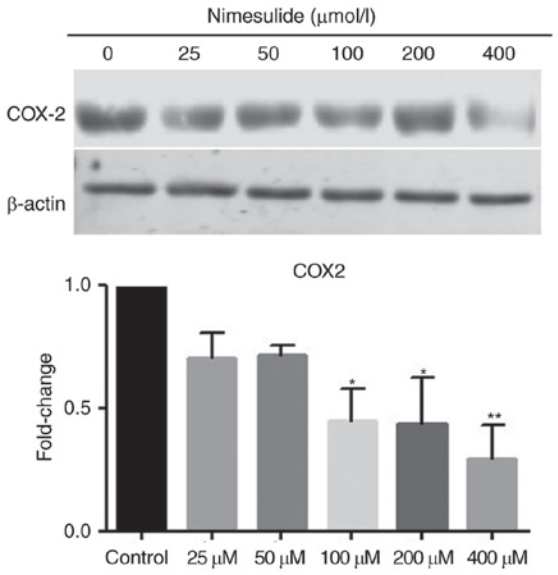

Figure 3. Effects of nimesulide on expression of COX-2 in PANC-1 cells. PANC-1 cells were treated with different concentrations of nimesulide (0, 25, 50, 100, 200 and 400 μmol/l) for 48 h and COX-2 expression was evaluated using western blot analysis. *P<0.05 and **P<0.01 vs. the control group. COX-2, prostaglandin-endoperoxide synthase 2.

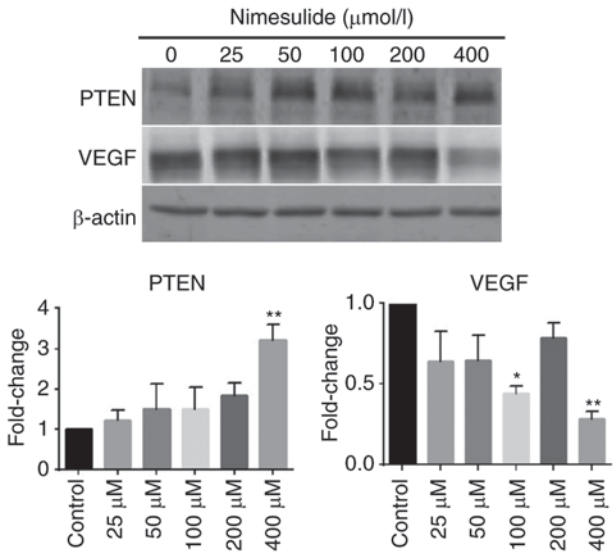

Figure 4. Effects of nimesulide on the expression of PTEN and VEGF in PANC-1 cells. PANC-1 cells were treated with different concentrations of nimesulide (0, 25, 50, 100, 200 and 400 μmol/l) for 48 h and expression of PTEN and VEGF was evaluated using western blot analysis. Treatment with nimesulide increased the expression of PTEN and decreased the expression of VEGF. *P<0.05 and **P<0.01 vs. the control group. PTEN, phosphatase and tensin homolog; VEGF, vascular endothelial growth factor.

demonstrated to be associated with enhanced expression of PTEN and apoptosis (34), which suggests that there may be a PI3K/Akt-independent mechanism underlying the anti-apoptotic and PTEN-enhancing effect of nimesulide in PANC-1 cells.

VEGF, a selective mitogen of vascular endothelial cells, serves a role in angiogenesis (35). In endothelial cells, PTEN antagonizes PI3K signalling, which mediates VEGF expression and angiogenesis (36). Overexpression of PI3K and Akt could induce transcription of VEGF and promote the formation of new blood vessels (37). Furthermore, following inhibition of PTEN, PI3K/Akt is activated, resulting in cell division, increased cell volume, apoptosis and tumor angiogenesis (38,39). In the present study, the results indicated that nimesulide increased PTEN expression but decreased expression levels of VEGF, which suggested that nimesulide may inhibit angiogenesis of PANC-1 cells.

The carcinogenic role of COX-2 overexpression has been demonstrated in a number of human malignancies, including pancreatic cancer (40). Overexpression of COX-2 is associated with tumor aggressiveness and growth in cancer biology (41,42). However, a previous study reported that nimesulide induces apoptosis in MIA PaCa-2 cells (no COX-2 protein expression) and BxPC-3 cells (high COX-2 protein expression), which suggested that the effect of nimesulide may be independent of COX-2 protein expression (16). In the present study, the results demonstrated that nimesulide decreases the expression of COX-2 and increases the expression of PTEN, but also results in inhibition of proliferation of PANC-1 cells. Furthermore, COX-2 positively regulates Akt signalling by suppressing the activity of PTEN (43) and prostaglandin E2 (44). In a previous study, In cells transformed with erb-b2 receptor tyrosine kinase 2, the activation or inhibition of mitogen-activated protein kinase and PI3K/Akt cascades resulted in the up- and downregulation of COX-2, respectively (45). In summary, the anti-cancer effect of nimesulide in PANC-1 cells may be associated with the interaction between PTEN and COX-2.

In conclusion, the results of the present study demonstrated that nimesulide induced an anti-cancer effect on PANC-1 cells. Specifically, nimesulide inhibited proliferation and promoted apoptosis of PANC-1 cells via enhancement of expression of PTEN. Furthermore, the results of the present study suggest that nimesulide may prevent tumor angiogenesis by inhibiting expression of VEGF. The regulatory effects of nimesulide on PANC-1 cells may be associated with interactions between PTEN and COX-2 through the PI3K/Akt signalling pathway. However, this hypothesis requires further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YC and AS conceived and designed the study. MC, TW and YC performed the experiments. YC wrote the paper. YC, MC and TW reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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