(-)-Epigallocatechin-3-gallate induces apoptosis in human pancreatic cancer cells via PTEN

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Abstract. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a cancer suppressor gene and an important negative regulator in the phosphatidylinositide 3-kinase (PI3K)/protein kinase B (Akt)/ mechanistic target of rapamycin (mTOR) signaling pathway. The PI3K/Akt/mTOR pathway can promote cancer cell survival, proliferation and progression. In the present study, the effects of (-)-epigallocatechin-3-gallate (EGCG) on PI3K/Akt/mTOR signaling in pancreatic cancer cells and PTEN knockdown cells were measured, in addition to assessing its therapeutic potential in pancreatic cancer. The apoptosis and proliferation of the cancer cells were examined by flow cytometry and Cell Counting kit-8 assay, respectively. The expression of genes and proteins in the PI3K/Akt/mTOR signaling pathway were investigated by reverse transcription-polymerase chain reaction and western blotting, respectively. The results suggested that the EGCG-induced apoptosis, proliferation inhibition and downregulated expression of phosphorylated (p)-Akt and p-mTOR were partially attenuated in PTEN-knockdown cells. In conclusion, the results indicated that EGCG is able to reduce proliferation and induce the apoptosis of pancreatic cancer cells associated with the expression of PTEN. Additionally, EGCG can suppress the expression of p-Akt and p-mTOR via PTEN to regulate the PI3K/Akt/mTOR pathway. The results suggest that EGCG may represent a potential treatment for pancreatic cancer, based on PTEN activation.

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Introduction

Pancreatic cancer (PC) is one of the most malignant tumors in humans, and its 5-year survival rate is <5% (1). Additionally, the development and metastasis of PC frequently goes undetected, and in China ~80% of patients with PC are inoperable (2). Unfortunately, the incidence of PC is increasing, therefore, effective new medicines and therapeutic targets are required.

Green tea is a popular drink consumed worldwide, and a number of epidemiological studies have indicated an association between tea consumption and reduced incidence of cancer (3-5). (-)-Epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea, and has been shown to inhibit inflammation, oxidation, viruses and oncogenesis (6-9). EGCG acts on numerous molecular targets, and has been demonstrated to have inhibitory ability during the initiation and progression stages of carcinogenesis (10-12). It can inhibit the growth and metastasis of a number of types of cancer (13-15) through a variety of mechanisms (16,17), including modulation of the phosphatidylinositide 3-kinase (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) pathway (18).

PTEN is a potent tumor-suppressor gene and a significant negative regulator of the PI3K/Akt/mTOR pathway. The PI3K/Akt/mTOR pathway modulates cellular functions, including proliferation, differentiation and migration (19). The dysregulation of this pathway has been associated with many types of cancer (20), including PC. PI3K/Akt/mTOR pathway activity promotes cancer cell proliferation, invasion and metastasis and inhibits apoptosis (21).

In a previous study, EGCG was demonstrated to upregulate the expression of PTEN and downregulate the expression of phosphorylated (p)-Akt and p-mTOR in human PC cells (22). In the present study, PC cells with or without PTEN knockdown were treated with EGCG, and the alterations in apoptosis and protein expression of PI3K/Akt/mTOR pathway targets were examined to investigate the therapeutic mechanisms of EGCG in human PC.

Materials and methods

Lentiviral-based RNA interference knockdown of PTEN in PC cells. The human PC cell lines, PANC-1 and BxPC-3,

were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). PTEN knockdown in PANC-1 and BxPC-3 cells was performed as previously described (23). Briefly, lentiviral transduction was used to steadily express short hairpin RNAs (shRNAs) that target PTEN. shRNA constructs were obtained from Sigma-Aldrich (St. Louis, MO, USA). Both the PTEN shRNA construct (TRCN0000219043, including the shRNA for human PTEN) and the luciferase shRNA construct (TRCN0000072247, including the shRNA as a control) were used to produce recombinant lentiviral particles. The PC cells were transfected with the viral particles containing PTEN or luciferase shRNAs for 24 h using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), after which the cells were placed into fresh RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.). The supernatants were collected at 36, 48, 60 and 72 h following transduction, and the supernatants were filtered using 0.45 μm low protein-binding filters (EMD Millipore, Billerica, MA, USA). Subsequently, the viral particles were centrifuged at 20,000 x g at 4˚C for 2 h and then resuspended in fresh RPMI-1640 medium. The lentiviral particles (shPTEN and shLuc) were then introduced to PANC-1 and BxPC-3 cells at a multiplicity of infection of 40. The PTEN knockdown was examined by Western blotting in triplicate experiments.

Cell culture and treatment. PC cells were incubated at 37˚C in a 95% air and 5% CO_2 atmosphere in Roswell Park Memorial Institute 1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, Logan, UT, USA). The cells with or without PTEN knockdown were treated with 40 μg/ml EGCG (Sigma-Aldrich) for 48 h, with control cells treated with deionized water. Subsequently, cell proliferation was examined using a Cell Counting kit-8 (CCK-8) assay. Apoptosis was detected by flow cytometry. The expression of genes and proteins in the PI3K/Akt/mTOR signaling pathway were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and western blotting.

Cell proliferation assays. PC cell proliferation was measured by CCK-8 assays as previously described (24). Briefly, the cells (with/without PTEN knockdown) were plated in 96-well plates of 5,000 cells/well. Following culture at 37˚C for 24 h, the cells were treated with 40 μg/ml EGCG for 24, 48 and 72 h. The cells were incubated with CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) solution (10 μl/well) for 2 h, and then the absorbance was measured at 450 nm using a microplate reader (Model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The alterations in cell growth were calculated as the Inhibition Ratio (%) = (1-treated group OD values/control group OD values) x 100. The experiments were performed in triplicate.

Apoptosis assays. The apoptosis rates of PANC-1 and BxPC-3 cells were examined using an annexin V-fluorescein isothiocyante (FITC) apoptosis detection kit (BioVision, Inc., Milpitas, CA, USA) as described previously (22). Briefly, the cells were dissociated using trypsin, and 10 μl annexin V-FITC and 10 μl propidium iodide were then added to the cells in the dark for 10 min. Stained cells were analysed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences, Franklin, NJ, USA). Cells in the lower right quadrant of the dot plot were considered to be in early apoptosis, and those in the upper right quadrant were in late apoptosis. The experiments were performed in triplicate.

RT-PCR analysis. PI3K, Akt, PTEN, and mTOR mRNA expression was analyzed by RT-PCR as previously described (22). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. Total RNA was extracted from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was treated with DNase (Promega Corporation, Madison, WI, USA), prior to reverse transcription into cDNA using the RETROscript™ kit (cat. no. AM1710; Thermo Fisher Scientific, Inc.), which contained dNTPs, a RNase inhibitor, M-MLV reverse transcriptase and RT buffer (Tris-HCl, KCl, $MgCl_2$ and DTT). RT-PCR was conducted using an AccessQuick RT-PCR System (Promega Corporation). A total of 30 cycles of amplification were performed using the following conditions: Denaturation at 94˚C for 30 sec, annealing at 58˚C for 30 sec, and extension at 72˚C for 1 min. The primer sequences were as follows: PI3K forward, 5'-AGGAGCGGTACAGCAAAGAA-3' and reverse, 5'-GCCGAACACCTTTTTGAGTC-3'; AKT forward, 5'-TGAAAACCTTCTGTGGGACC-3' and reverse, 5'-TGGTCCTGGTTGTAGAAGGG-3'; PTEN forward, 5'-CAGAAAGACTTGAAGGCGTAT-3' and reverse, 5'-CGTCGTGTGGGTCCTGAGTGA-3'; mTOR forward, 5'-CTGGGACTCAAATGTGTGCAGTTC-3' and reverse, 5'-GAACAATAGGGTGAATGATCCGGG-3'; and GAPDH forward, 5'-GGAAGGTGAAGGTCGGAGT-3' and reverse, 5'-CCTGGAAGATGGTGATGGG-3'. The PCR products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide, and the results analyzed using NIH Image 1.60 software (National Institutes of Health, Bethesda, MD, USA). The experiments were performed in triplicate.

Western blotting. Protein extraction and western blotting were conducted as previously described (22). In brief, the cells were rinsed with phosphate-buffered saline, and lysed with lysis buffer for 30 min. Subsequently, the lysates were centrifuged at 12,000 x g for 10 min, and the protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Following this, the proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 80 V for 1.5 h, and then transferred onto polyvinylidene fluoride membranes (EMD Millipore) at 100 V for 2.5 h. Following incubation in bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) at 4˚C for 1 h, the membranes were incubated with the following primary antibodies: Rabbit anti-PTEN (1:1,000; cat. no. 9188), rabbit anti-PI3K (1:1,000; cat. no. 4249), rabbit anti-Akt (1:1,000; cat. no. 4685), mouse anti-p-Akt (1:500; cat. no. 12694), rabbit anti-mTOR (1:1,000; cat. no. 2983), rabbit anti-p-mTOR (1:1,000; cat. no. 5536) and mouse anti-β-actin (1:500; cat. no. 3700) monoclonal antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) in Tris-buffered saline-Tween-20; (Sigma-Aldrich) overnight. Membranes were then incubated with horseradish peroxidase-conjugated

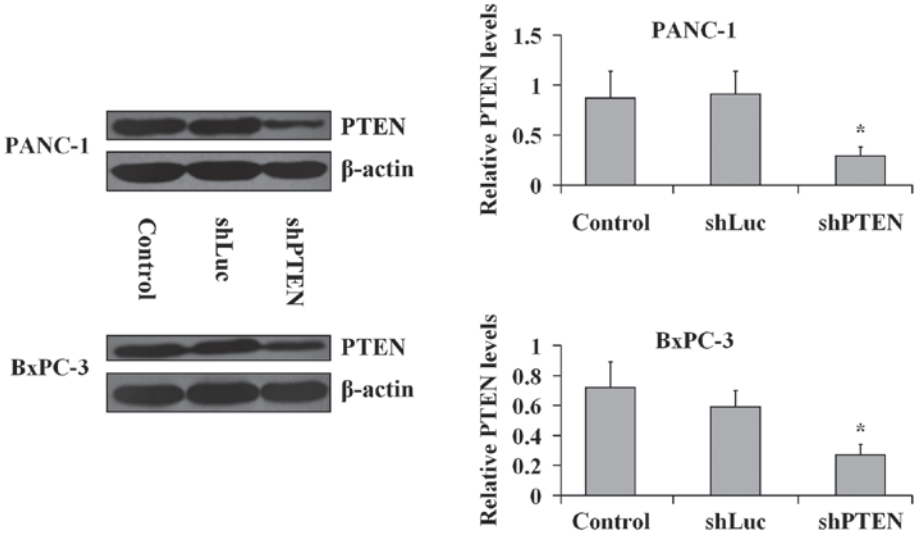

Figure 1. PTEN knockdown by RNA interference in prostate cancer cells. PANC-1 and BxPC-3 cells were transfected with viral particles containing PTEN or Luc shRNAs for 24 h and the knockdown of PTEN confirmed by western blotting analysis. *P<0.05 vs. control group (untreated cells). PTEN, phosphatase and tensin homolog deleted on chromosome 10; Luc, luciferase; shRNA, short hairpin RNA.

goat anti-rabbit (1:1,000; cat. no. 7074) and goat anti-mouse (1:2,500; cat. no. 7076) secondary antibodies (Cell Signaling Technology, Inc.). Following rinsing, the bands were detected using an enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Chalfont, UK). Relative protein levels were normalized to β-actin as the internal control. The experiments were performed in triplicate.

Statistical analysis. Statistical analysis was performed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). The data are presented as the mean ± standard deviation. Differences between groups were examined using one-way analysis of variance followed by Fisher's least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

PTEN knockdown by RNA interference in PC cells. PANC-1 and BxPC-3 cells were transfected with the viral particles including PTEN or luciferase shRNAs for 24 h and the knockdown of PTEN was confirmed by western blotting analysis (Fig. 1). The β-actin expression levels in the treated group did not differ with the levels in the control (untreated) group (P>0.05). PTEN expression levels were significantly lower in shPTEN groups compared with the control group (P<0.05), Furthermore, the expression levels of PTEN in the shLuc groups were not different compared with the controls (P>0.05).

EGCG inhibits PC cell proliferation via PTEN. PANC-1 and BxPC-3 cells with or without PTEN knockdown were cultured in medium with or without 40 μg/ml EGCG for 24, 48 and 72 h, and proliferation was examined by CCK-8 assays (Fig. 2). In PANC-1 cells, the inhibition ratio in the EGCG group at 48 and 72 h was significantly higher compared with the shPTEN group (P<0.05 and P<0.01, respectively). Furthermore, the inhibition ratio in the shPTEN+EGCG group was not significantly different compared with shPTEN group (P>0.05). In BxPC-3 cells, the inhibition ratios in the EGCG group were significantly greater compared with the shPTEN group (P<0.01). Additionally, the inhibition ratios in the shPTEN+EGCG group was significantly higher than the shPTEN group (P<0.05).

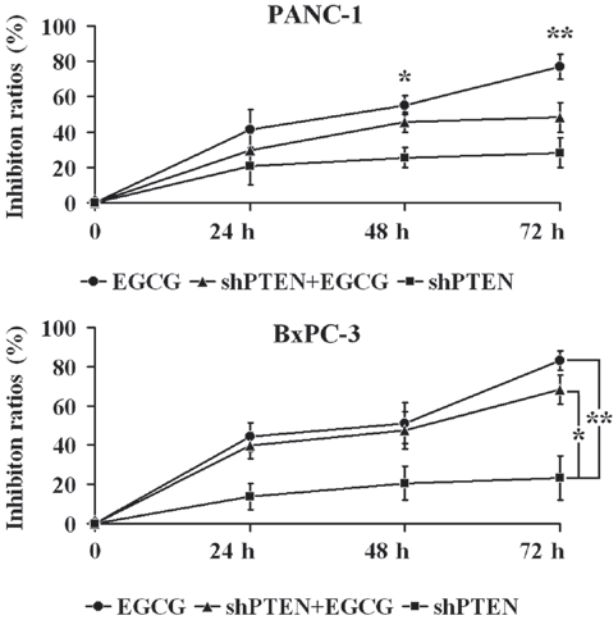

Figure 2. Effect of EGCG on prostate cancer cell proliferation via PTEN. PANC-1 and BxPC-3 cells with or without PTEN knockdown were cultured in medium with or without 40 μg/ml EGCG. Cells were harvested at 24, 48 and 72 h, and the proliferative potential was assessed. Data presented are the mean of three independent experiments. *P<0.05, **P<0.01 vs. shPTEN group. EGCG, (-)-epigallocatechin-3-gallate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; sh, short hairpin.

Effect of EGCG on PC cell apoptosis via PTEN. PANC-1 and BxPC-3 cells with or without PTEN knockdown were cultured in medium with or without 40 μg/ml EGCG for

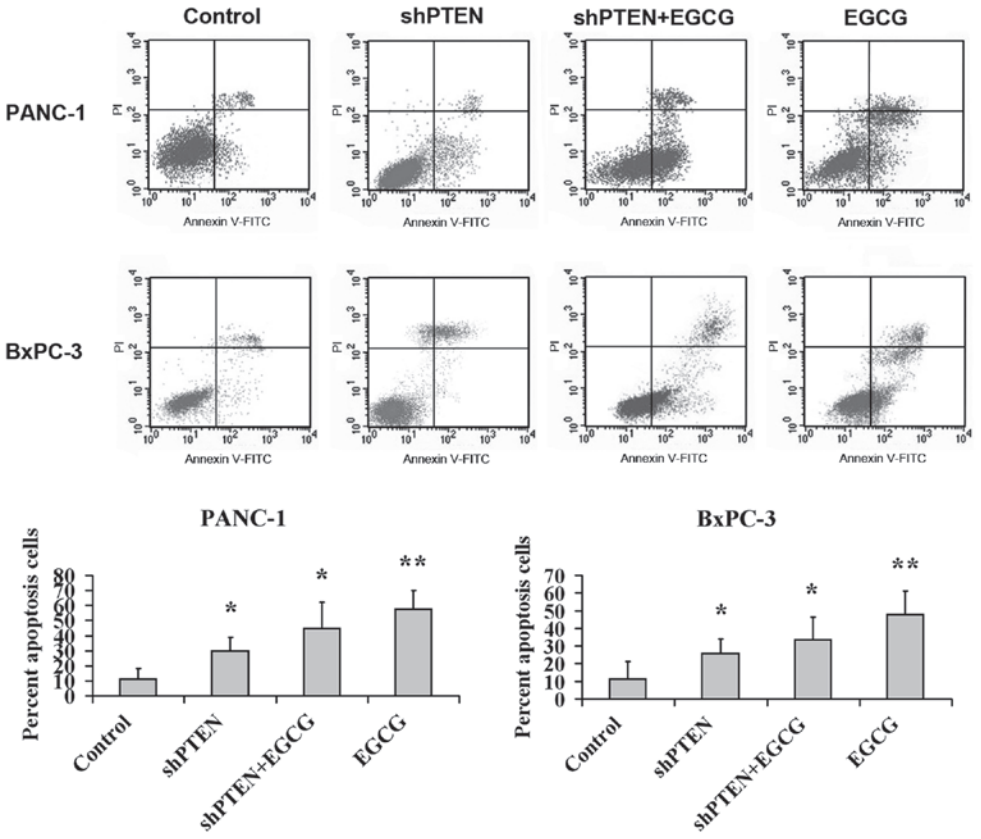

Figure 3. Effect of EGCG on apoptosis of prostate cancer cells via PTEN. PANC-1 and BxPC-3 cells with or without PTEN knockdown were treated with 40 μg/ml EGCG for 48 h. The apoptotic rate of cells was analyzed by flow cytometry. Cells in the lower right quadrant were considered to be in early apoptosis, and cells in the upper right quadrant in late apoptosis. *P<0.05, **P<0.01 vs. control group. EGCG, (-)-epigallocatechin-3-gallate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; sh, short hairpin.

48 h, and the apoptotic rate was analyzed by flow cytometry (Fig. 3). This indicated that the apoptotic ratios in the EGCG group were substantially higher compared with the control (untreated) group (P<0.01). Additionally, the apoptotic rates in the shPTEN and shPTEN+EGCG groups were substantially higher than the control group (P<0.05).

EGCG regulates the expression of genes and proteins in the PI3K/Akt/mTOR pathway in PC cells via PTEN. PANC-1 and BxPC-3 cells with or without PTEN knockdown were cultured in medium with or without 40 μg/ml EGCG for 48 h, and the mRNA expression of PI3K, PTEN, Akt and mTOR is presented in Fig. 4. The mRNA expression levels of PTEN in the shPTEN group were significantly lower compared with the control (untreated) group (P<0.05). Furthermore, the mRNA expression of PTEN in the EGCG group was significantly higher compared with the control group (P<0.01). However, the mRNA expression levels of PTEN in the shPTEN+EGCG group did not differ compared with the control group (P>0.05).

Subsequently, the effect of EGCG on the protein expression of PI3K, PTEN, Akt, p-Akt, mTOR and p-mTOR in PANC-1 and BxPC-3 cells was investigated with or without PTEN knockdown (Fig. 4). The β-actin expression levels in the treated groups were unaltered compared with the control groups (P>0.05). The protein expression levels of PTEN in the shPTEN group were significantly lower compared with the control (untreated) group (P<0.01). Furthermore, the expression of PTEN in the EGCG group was markedly higher compared with the control group (P<0.01). However, the expression of PTEN in the shPTEN+EGCG group did not differ compared with the control group (P>0.05). The expression levels of p-Akt and p-mTOR in the shPTEN group were significantly greater compared with the control (untreated) group (P<0.05). Additionally, the p-Akt and p-mTOR expression levels in the EGCG groups were significantly lower compared with the control group (P<0.05). However, the p-Akt and p-mTOR expression levels in the shPTEN+EGCG group were unaltered compared with the control group (P>0.05).

Discussion

In the present study, using PANC-1 cells the inhibition ratio in normal cells following EGCG treatment at 48 and 72 h was observed to be significantly higher compared with shPTEN cells (P<0.05 and P<0.01, respectively), with the inhibition ratio in shPTEN cells following EGCG treatment unaltered compared with shPTEN cells (P>0.05). In BxPC-3 cells, the inhibition ratios in normal cells following EGCG treatment were significantly higher compared with shPTEN cells (P<0.01), with the inhibition ratios in shPTEN cells following EGCG treatment substantially higher than in the shPTEN cells (P<0.05). These data indicate that PTEN was involved in EGCG inhibiting PC cell proliferation, with the knockdown

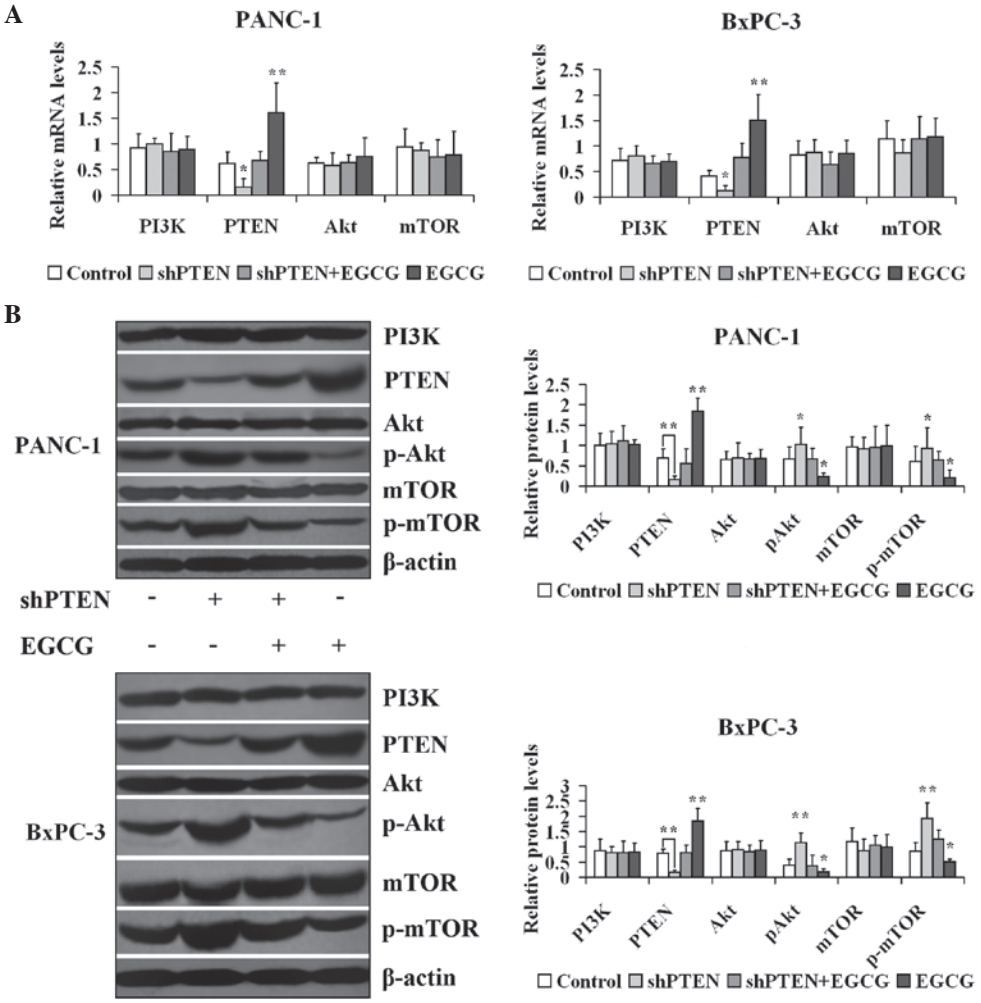

Figure 4. Effect of EGCG on expression of genes and proteins involved in the PI3K/Akt/mTOR pathway in prostate cancer cells via PTEN. (A) PANC-1 and BxPC-3 cells with or without PTEN knockdown were treated with 40 μg/ml EGCG for 48 h. PI3K, PTEN, Akt and mTOR mRNA expression levels were measured by reverse transcription-polymerase chain reaction. (B) PANC-1 and BxPC-3 cells with or without PTEN knockdown were treated with 40 μg/ml EGCG for 48 h. PI3K, PTEN, Akt, pAkt, mTOR and p-mTOR expression levels were measured by western blotting. *P<0.05, **P<0.01 vs. control group. EGCG, (-)-epigallocatechin-3-gallate; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; mTOR, mechanistic target of rapamycin; PTEN, phosphatase and tensin homolog deleted on chromosome 10; sh, short hairpin; p-, phosphorylated.

of PTEN reducing the inhibitory effect of EGCG on PC cell proliferation. Furthermore, the previous findings support the present study. In a previous study, the proliferation of PANC-1 cells was inhibited following treatment with 40 μg/ml EGCG for 24, 48 and 72 h (22). In addition, Zhang *et al* (25) reported that loss of PTEN promoted proliferation and invasion in PC cells, and Ma *et al* (26) demonstrated that knockdown of PTEN was able to upregulate cell invasiveness and proliferation in PC cells. Furthermore, Lyn-Cook *et al* (27) demonstrated that EGCG suppressed pancreatic cell growth by approximately 90%. Differences in the methods or cell lines used in these studies may explain the discrepancies between these studies and the present study.

The current study indicated that the apoptotic rates in normal cells following EGCG treatment were significantly higher compared with the control group (P<0.01), and the apoptotic rates in the shPTEN cells with or without EGCG treatment were significantly higher compared with the control group (P<0.05). These results suggested that PTEN was involved in EGCG promoting PC cell apoptosis, and that the absence of PTEN may attenuate the apoptosis-promoting ability of EGCG in PC cells. These results are supported by previous studies. In a previous study, the apoptosis ratio in PANC-1 cells following 40 μg/ml EGCG treatment over 24 h was 28.56±1.56% (22). Qanungo *et al* (28) reported that EGCG induced the apoptosis of human PC Mia Paca-2 cells and that the apoptotic rate was ~2.5-25% following treatment with 0.025-0.2 mM EGCG for 24 h. The differences in the cell types and EGCG concentrations used in these previous studies may account for the variation in these rates.

PTEN is an important negative modulator of the PI3K/Akt/mTOR pathway, as it can weaken upstream signals. Deactivation of PTEN leads to activated PI3K/Akt/mTOR signaling. In the present study, the mRNA and protein expression of PTEN in normal cells following treatment with EGCG were significantly higher compared with the controls (P<0.01). The mRNA and protein expression levels of PTEN in shPTEN cells following EGCG treatment were comparable with the control cells (P>0.05). The expression levels of p-Akt and p-mTOR in shPTEN cells were significantly higher compared with the controls (P<0.05), whilst the p-Akt and p-mTOR expression levels in normal cells treated with EGCG alone were

significantly lower compared with the control cells ($P<0.05$). The expression levels of p-Akt and p-mTOR in shPTEN cells treated with EGCG were comparable with the control cells ($P>0.05$). Previous studies have demonstrated that EGCG is able suppress the PI3K/Akt/mTOR pathway by downregulating p-Akt and p-mTOR expression based on the presence of PTEN, instead of regulating Akt and mTOR (22,29). These data indicate that EGCG-induced upregulation of PTEN expression is a prohibitive mechanism on the PI3K/Akt/mTOR pathway and that the loss of PTEN may attenuate the inhibitory effect of EGCG on the PI3K/Akt/mTOR pathway in human PC cells. However, previous studies support the results of the present study. In a previous study, EGCG upregulated the expression levels of PTEN and downregulated the expressions of p-Akt and p-mTOR in PANC-1 cells (22). Additionally, Zhang *et al* (25) reported that loss of PTEN resulted in increased expression of p-Akt and p-mTOR in PC cells and Shankar *et al* (30) reported that EGCG inhibited the phosphorylation of PI3K and p-Akt in PC tissues and promoted PTEN expression, however with no influence on Akt. Nevertheless, certain studies have indicated that EGCG is able to modulate the expression levels of PI3K, mTOR or Akt in certain types of cancer. Shen *et al* (31) reported that EGCG treatment resulted in a reduction in the mRNA and protein expression levels of PI3K and Akt in hepatoma, and Li *et al* (32) found that loss of PTEN resulted in increased expression levels of Akt, p-Akt, and p-mTOR in endometrial cancer cells. Additionally, Van Aller *et al* (33) indicated that EGCG was able to inhibit the expression of PI3K, mTOR and p-Akt in MDA-MB-231 and A549 cells, and Shimizu *et al* (34) demonstrated that EGCG can inhibit the expression levels of Akt and p-Akt in colorectal cancer xenograft tumors. Furthermore, Shirakami *et al* (35) reported that EGCG can repress Akt expression in human hepatoma HuH7 cell xenografts, and Ichimatsu *et al* (36) reported that EGCG can repress the activation of PI3K in JB6Cl41 cells. However, alterations in the expression levels of PI3K, Akt and mTOR were not observed in the current study, therefore further study is required.

In conclusion, EGCG was able to inhibit proliferation and induce apoptosis in PC cells via PTEN, with the loss of PTEN reducing the ability of EGCG to inhibit proliferation and promote apoptosis in PC cells. In addition, EGCG is able to downregulate the expression levels of p-Akt and p-mTOR to regulate then PI3K/Akt/mTOR pathway via PTEN. Furthermore, this regulatory effect may contribute to the apoptosis-inducing and anti-proliferative properties of EGCG. However, further study is required to fully elucidate the regulatory effect of EGCG on components downstream of the PI3K/Akt/mTOR signal pathway.

Acknowledgements

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