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FULL LENGTH ARTICLE

Tetrandrine inhibits proliferation of colon cancer cells by BMP9/ PTEN/ PI3K/AKT signaling

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Abstract Despite advances in screening and treatment, colon cancer remains one of the leading causes of cancer-related death. Finding novel and useful drug treatment targets is also an urgent need for clinical applications. Tetrandrine (Tet) is extracted from the Chinese medicinal herbal medicine, which is a well-known calcium blocker with a variety of pharmacological activities, including anti-cancer. In this study, we recruited cell viability assay, flow cytometry analysis, cloning formation to confirm that Tet can inhibit the proliferation of Tetrandrine (Tet) SW620 cells, and induce apoptosis. Mechanically, we confirmed that Tet up-regulates the mRNA and protein level of BMP9 in SW620 cells. Over-expression BMP9 enhances the anticancer effects of Tet in SW620 cells, but these effects can be partly reversed by silencing BMP9. Also, Tet reduces phosphorylation of Aktl/2/3 in SW620 cells, which could be elevated by overexpressed BMP9 and impaired by silencing BMP9. Furthermore, we demonstrated that Tet reduces phosphorylated PTEN, which can be promoted by overexpressed BMP9, analogously also be attenuated through silencing BMP9. Finally, we introduced a xenograft tumor model to investigate the anti-proliferative effect of Tet, further to explore the effects of BMP9 and PTEN in SW620 cells. Our findings suggested that the anti-cancer activity of Tet in SW620 cells may be mediated partly by up-regulating BMP9, followed by inactivation PI3K/ Akt through up-regulating PTEN at least.

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Introduction

Colon cancer is one of the most common malignancies in the world.¹ Because of lifestyle, environmental changes, and aging, the incidence of colon cancer is continually increasing in some low- and middle-income countries.² Meanwhile, the incidence and mortality of colorectal cancer are reduced due to effective cancer screening measures in some developed countries, but the number of young patients diagnosed with colon cancer are increasing with unknown causes. So it's extremely necessary to explore new treatment programs.³

Natural compounds from herbs and their derivates are one of the primary sources of anti-cancer drugs. Nowadays, several products have been applied to the treatment of cancer, such as taxol, catechins and vincristine.⁴⁻⁶ Tetrandrine (bis-benzyliso-guinoline alkaloid, Tet) is extracted from the dried root of Chinese herbal medicine (han fang ji), which possesses multiple pharmacological activities, such as anti-oxidation, anti-inflammation and anti-hypertension.⁷⁻⁹ Besides, it was reported that Tet could inhibit proliferation and induce apoptosis in many cancer cells, such as prostate cancer, gastric cancer, colon cancer and neuroblastoma.^{10–13} In terms of mechanism, some signaling pathways or key molecules may participate in the anticancer activities, such as Wnt, EMT and PI3K/Akt.14-16 Furthermore, our previous studies have shown that Tet can inhibit the proliferation of human colon cancer cells, but the exact mechanism of this effect remains to be further explained.

As a sub-group of the transforming growth factor (TGF- β) super-family, bone morphogenetic proteins (BMPs) are related to multiple physiological functions. For example, bone morphogenetic protein-2 promotes osteosarcoma growth, BMP5 regulates neural crest cell survival, BMP7 possesses anti-cancer activity through non-canonical BMPs/Smads signaling pathway and BMP9 induces osteogenic differentiation.¹⁷⁻²⁰ But there is an uneven distribution of concerns about the various aspects of the role of BMP9 in cancers.^{21,22} Moreover, it was reported that the anti-cancer activity of Res(resveratrol), which is also isolated from the Chinese herbal medicine, may directly up-regulate BMP9 expression to activate p38 MAPK in colon cancer.²³ so whether BMP9 participates in the anti-cancer effect of Tet has aroused our concern.

In this study, we determined the proliferation-inhibiting and apoptosis-inducing effects of Tet in SW620 cells and uncovered possible mechanisms for Tet's anti-cancer effects. Our results indicated that Tet could suppress proliferation and induce apoptosis in SW620 cells, which may be mediated in part by enhancing the BMP9 expression, followed by reducing the activation of PI3K/Akt through upregulating PTEN at least.

Materials and methods

Pharmaceutical and cell culture

Tetrandrine (Tet) was purchased from the company of Hao-xuan Bio-tech (Xi'an, China). Tet dissolved in DMSO in the concentration of 100 mmol/l for in vitro test and stored at -20 °C. Tet's suspension was composed of 0.5% carboxymethylcellulose sodium (CMCNa) to apply in vivo experiments. All the cell lines (HCT116, SW620, SW480, LoVo, FHC and HEK293) used were attained from the American Type Culture Collection (ATCC) in the USA. The total primary antibodies were bought from Santa Cruz Biotechnology (SCB) company in the USA. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 12% fetal bovine serum (FBS), including 100 μ g/ml of penicillin and streptomycin in the environment of 37 °C and 5% CO2.

Cell viability assay

Cell viability was determined with CCK-8 assay. Briefly, SW620 cells were cultured in 96-well plates and coped with diverse concentrations of Tet, recombinant adenovirus or DMSO. At the specified time-point, 10 μ l of CCK-8 and 90 ul DMEM were added into per well and cultured 3 h after treatment. At 450 nm, the absorbance was determined with the microplate reader. Per assay was done three times.

Cloning formation assay

Clonal formation assay was used to detect the ability of cell division and colony-forming in a specific group. Briefly, cells were pre-coped with diverse concentrations of Tet for 1 day and then re-planted in 6-well plates, and then these cells were cultured without Tet 14 days until the colonies emerged. Finally, the plate was lightly washed with PBS, crystal violet formalin solution coped with it about 18 min, and finally washed and dried it. Per assay was done three times.

Flow cytometric analysis

SW620 cells were scattered in 6-well plates, then coped with diverse concentrations of Tet for 48 h. In terms of cell cycle analysis, cells were gathered, washed with cold phosphate-buffered saline (PBS) and immobilized with cold ethanol(4 °C, 70%). Finally, fluorescence-activated cell sorting (FACS) was used to detect cells, which were stained for 30 min by PI. In terms of apoptosis analysis, cells were gathered and washed with cold phosphate-buffered saline (PBS), followed by coping with Annexin V-EGFP and PI reagent from Nanjing KeyGen Biotech Company in China. Finally, the cells were detected with fluorescenceactivated cell sorting (FACS). Per assay was done three times.

Recombinant adenoviral construction for BMP9, PTEN, GFP, and RFP

According to the AdEasy system as described above, the recombinant adenoviral vectors were performed.^{24,25} Briefly, the coding sequence (CDS) of human BMP9 was broadened and sub-cloned into the pAdTrace vector, and the siRNA-knockdown oligonucleotide cassette was cloned into the pSES1 vector. Then, the vectors were transfected into HEK293 cells to form the recombinant adenoviruses, such as AdBMP9, AdsiBMP9, AdPTEN, AdsiPTEN. The total recombinant adenoviruses were labeled with a green or red fluorescent protein (GFP or RFP). AdGFP or AdRFP was used as vehicle control.

Real-time polymerase chain reaction (PCR) analysis

Cells were seeded in T25 flasks and diverse concentrations of Tet coped with it. At the designated time, total RNA was distilled with a reagent of TRIzol (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and then RT reaction was done to generate cDNA. The cDNA product as a template for RT-PCR was used to test the mRNA level of the target gene. The data of each sample was standardized with the relevant expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences for this study were listed in Table 1. Per assay was done three times.

Western blot assay

SW620 cells were scattered in 6-well plates, then diverse concentrations of Tet coped with it for 24 h or 48 h. Then cells were decomposed, gathered and boiled for 15 min. The samples were tested with SDS-PAGE and transferred to a polyvinylidene fluoride membrane (PFM), followed by imprinting of a specific antibody and succeeded in a secondary antibody. Finally, the target bands were detected

Table 1	The primers used for PCR assay.
Gene	Primer sequence (5'-3')
GAPDH	F: CAACGAATTTGGCTACAGCA
	R: AGGGGAGATTCAGTGTGGTG
PCNA	F: GGCTCTAGCCTGACAAATGC
	R: GCCTCCAACACCTTCTTGAG
Bad	F: CGGAGGATGAGTGACGAGTT
	R: CGGAGGATGAGTGACGAGTT
Bcl-2	F: GGATGCCTTTGTGGAACTGT
	R: AGCCTGCAGCTTTGTTTCAT
PTEN	F: CATAACGATGGCTGTGGTTG
	R: CGGGGTAAGGCTGTTTTACA
BMP9	F: CCTGGGCACAACAAGGAC
	R: CCTTCCCTGGCAGTTGAG
	F, forward; R, reverse.

by imager from Thermo Fisher Scientific (TFS). Per assay was done three times.

Immunocytochemical staining

Cells were scattered in 48-well plates, then diverse concentrations of Tet coped with it for 48 h. Cells were immobilized with cold methanol (4 °C) for 18 min and treated with cold PBS and 0.5% Triton X-100. Cells were blocked with 5% BSA, BMP9 or PTEN antibody, and secondary antibody at 37 °C. Finally, cells were stained with DAPI to stain. The inverted microscope was used to record the fluorescence images. Per assay was done three times.

Xenograft model of colorectal cancer and histological assessment

Animal experiments complied with the policies of Chongqing Medical University Institutional Animal Care and Use Committee. SW620 cells were harvested, prepared and injected into the flank subcutaneous of athymic female nude mice (five mice per group). After 7 days, the mice have administrated Tet and CMC-Na mixture (80 mg/kg) intragastrically once a day for 14 days. Then animals were sacrificed and the tumor masses were removed, immobilized and embedded. Next tissue sections were subjected to hematoxylin and eosin (H&E) staining for histological assessment.

Statistical analysis

All experiments were implemented in triplicates. Data was revealed as mean \pm standard deviation (SD), and statistical analysis of results were conducted by using *t*-test. A value of P < 0.05 was considered statistically significant.

Results

Effects of Tet on proliferation in SW620 cells

It has been reported that Tet has robust anti-cancer activity in different cancer cells so that we first validated the impact of Tet on the proliferation in SW620 cells. The CCK-8 assay results revealed that Tet could suppress the proliferation in SW620 cells (Fig. 1A), even at the minimum concentration of 1 $\mu\text{M}.$ The PCR assay and Western blot analysis results showed that the proliferation cell nuclear antigen (PCNA) level is down-regulated substantially by Tet treatment in SW620 cells (Fig. 1B and C). Also, we conducted a flow cytometry analysis to survey and evaluate the impact of Tet on cell cycle to uncover that Tet holds back the cell cycle chiefly at the G1 phase in SW620 cells (Fig. 1D). The colony formation assay revealed that the proliferation activity of SW620 cells is remarkably decreased by Tet (Fig. 1E). These data suggested that Tet can availably suppress the growth in SW620 cells and may be applied to treat colon cancer as a promising chemotherapeutic drug.



Figure 1 Effects of Tet on proliferation in SW620 cells. (A) The anti-proliferation effect of Tet in SW620 cells tested by CCK-8 assay. (B) The mRNA level of PCNA in SW620 cells treated with Tet and tested by RT-qPCR. (C) The protein level of PCNA in SW620 cells treated with Tet and tested by Western blot. (D) The effect of Tet on the cell cycle arrest in SW620 cells tested by flow cytometry analysis. (E) The effect of Tet on the anti-proliferation activity of SW620 cells tested by clonogenic formation. GAPDH was used as control. Error bars are the means \pm SD, n = 3. **P < 0.01 or * P < 0.05 vs. control.

Effects of Tet on apoptosis in SW620 cells

We next implemented more analysis to determine whether Tet could induce apoptosis in SW620 cells. The results of PCR showed that Tet up-regulate Bad mRNA levels, but down-

regulate Bcl-2 mRNA levels (Fig. 2A and B). Moreover, the results of Western blot demonstrated that Tet up-regulate the protein expression level of Bad, but down-regulate the protein expression level of Bcl-2 (Fig. 2C). Therefore, these results suggested that Tet can induce apoptosis in SW620 cells.



Figure 2 Effects of Tet on apoptosis in SW620 cells. The mRNA level of Bad (A) and Bcl-2 (B) in SW620 cells treated with Tet and tested by RT-qPCR. (C) The protein level of Bad and Bcl-2 in SW620 cells treated with Tet and tested by Western blot. GAPDH was used as control. Error bars are the means \pm SD, n = 3. **P < 0.01 or * P < 0.05 vs. control.

Effects of Tet on BMP9 expression in SW620 cells

According to the above data, Tet can effectively suppress the growth and induce apoptosis in SW620 cells, so we next explored the underlying molecular mechanisms. Based on the reported that BMP9 is involved in the development of colon cancer,²⁶ we further explore BMP9 endogenous protein level in LoVo, HCT116, SW480, SW620, and FHC cell lines. Results showed that BMP9 mRNA level in cancer cells is much higher than that of FCH cells (Fig. 3A) and BMP9 endogenous protein levels in SW620 cells are much more than FHC cells (Fig. 3B). Thus, we made further investigation of whether Tet can impact the mRNA and protein levels of BMP9. The results of PCR demonstrated that Tet can upregulate BMP9 mRNA level in SW620 cells (Fig. 3C) and the results of Western blot showed that Tet also up-regulate the protein level of BMP9 (Fig. 3D). The further consequences of cell immunofluorescence staining analysis showed that Tet up-regulate the protein level of BMP9 (Fig. 3E). These data potentially suggested that Tet may inhibit the growth of HCT116 cells possibly by regulating the level of BMP9.

Effects of BMP9 on the proliferation-suppressing and apoptosis-inducing affected by Tet in SW620 cells

Because of Tet significantly increasing the level of BMP9 in SW620 cells, next, we explored how BMP9 affects the growth and apoptosis of Tet in SW620 cells. In addition, we obtained and utilized BMP9 over-expressing and/or silencing recombinant adenoviruses. CCK-8 assay showed that the over-expression BMP9 partly increases the antiproliferation of Tet in SW620 cells while silencing BMP9 increases survival rate and slightly inverses the antiproliferation of Tet (Fig. 4A and B). Besides, SW620 cells treated with Tet united over-expression BMP9 or silencing BMP9 were collected and applied to apoptosis analysis to reveal that over-expression BMP9 enlarges the number of apoptotic cells induced by Tet, but silencing BMP9 decreases the number of necrotic cells (Fig. 4C). Thus, our data indicated that the BMP9 might mediate the antiproliferation process of Tet in SW620 cells, although the explicit mechanism underlying this effect remains unknown.



Figure 3 Effects of Tet on BMP9 expression in SW620 cells. The endogenous mRNA and protein levels of BMP9 in the FHC, LoVo, HCT116, SW480 and SW620 cells tested by Real-time PCR (A) and Western blot (B). (C) RT-qPCR and Western blot (D) analyses of BMP9 level in SW620 cell lines treated with Tet. (E) Immunofluorescent stain showing the protein expression level of BMP9 in SW620 cells treated with Tet. GAPDH was used as control. Error bars are the means \pm SD, n = 3. **P < 0.01 or * P < 0.05 vs. control.

Effects of BMP9 on PI3K/Akt signaling affected by Tet in SW620 cells

Although BMPs usually produce their biological functions through BMPs/Smads pathway, pre-test results demonstrated that Tet didn't change the protein level of phosphorylated Smad1/5/8 (didn't show). Therefore, BMPs may affect the anti-cancer activity of Tet through the noncanonical BMPs/Smads pathway, especially the PI3K/Akt signaling. PI3K/Akt signaling plays a significant role in regulating cell survival, differentiation, and apoptosis, but it is unclear whether the effect of Tet on inhibiting SW620 cells growth can also be mediated through this pathway. Hence we detected the PI3K/Akt pathway affected by Tet in SW620 cells, the results showed Tet significantly reduces phosphorylated Akt1/2/3 (p-Akt1/2/3) in a concentration-dependent manner, but has no remarkable effect on the total protein level of Akt1/2/3 (Fig. 5A). Further analysis showed that over-expressed BMP9 enhances the anti-cancer activity of Tet on reducing phosphorylated Akt1/2/3 (Fig. 5B) while silencing BMP9 reverses these effects of Tet in SW620 cells (Fig. 5C). These results showed that the anti-cancer activity of Tet might be derived from inhibiting PI3K/Akt signaling by up-regulating BMP9 in SW620 cells.

Effects of PTEN on BMP9 affected by Tet in SW620 cells

PI3K/Akt signaling is subtly impacted by many factors, including PTEN. PTEN removed phosphorylatedrylated from phosphatidylinositol-3, 4, 5-triphosphate (PIP3) to PI-4, 5-bisphosphate (PIP2), which brought about PI3K/Akt signal inactivation. The above exhibition data implied that Tet reduces phosphorylated Akt1/2/3 protein level in SW620 cells, which may be controlled by up-regulating BMP9. Since PTEN is a center-negative moderator for PI3K/ Akt signal pathway, it implied that the effect of BMP9 on PI3K/Akt signaling might be impacted through upregulating PTEN in SW620 cells. So we detected the effect of BMP9 on PTEN and phosphorylated PTEN (p-PTEN). PCR results demonstrated that Tet up-regulates the mRNA level of PTEN in SW620 cells (Fig. 6A), and Western blot showed that Tet exerts no substantial effect on the total protein level of PTEN, but reduces the protein level of phosphorylated PTEN (Fig. 6B). Further analysis results



Figure 4 Effects of BMP9 on the proliferation-suppressing and apoptosis-inducing affected by Tet in SW620 cells. (A) The antiproliferation effect of Tet and AdBMP9 in SW620 cells tested by CCK-8 assay. (B) The anti-proliferation effect of Tet and AdsiBMP9 in SW620 cells tested by CCK-8 assay. (C) The effect of Tet with AdBMP9 or AdsiBMP9 on apoptosis in SW620 cells tested by flow cytometry analysis. Error bars are the means \pm SD, n = 3. **P < 0.01 or * P < 0.05 vs. control.

showed that over-expressed BMP9 could enhance the anticancer activity of Tet through decreasing the protein level of p-PTEN, but silencing BMP9 partly reverse the effect of Tet-induced (Fig. 6C and D). Above mentioned data showed that the up-regulation of BMP9 might enhance the anti-cancer activity of Tet in SW620 cells by partly improving the level of PTEN. Also, it showed that the effect of silencing BMP9 on the anti-cancer activity of Tet can be slightly decreased through somewhat increasing the level of p-PTEN in SW620 cells. Further Western blot assay results revealed that silencing PTEN can partly increase the level of phosphorylated Akt1/2/3 when combined with Tet and over-expressed BMP9 (Fig. 6E). These data showed that BMP9 might mediate the anti-cancer activity of Tet in SW620 cells through slightly downregulating the level of p-PTEN, followed by decreasing the level of phosphorylated Akt1/2/3.

In vivo Tet inhibits tumor growth

We next investigate the role of BMP9 in the anti-cancer activity of Tet with xenograft model. The results showed that the tumors volume in the mice who received a combination of Tet and over-expressed BMP9 are significantly smaller, compared with those who were treated by Tet alone; silencing PTEN reverses the anti-cancer effect of Tet combined with over-expressed BMP9 (Fig. 7A). H&E staining revealed that BMP9 enhances Tet-induced karyopyknosis, but silencing PTEN reverses the effect of Tet combined with over-expressed BMP9 in the tumors mass (Fig. 7B). This information showed that the over-expressed BMP9 might mediate Tet's anti-cancer activity with a PTENdependent manner partly.

Discussion

Colon cancer is one of the most common malignancies in the world. Although the treatment of colon cancer has been improved dramatically in the past, the prognosis remains unsatisfied yet. In this study, we demonstrated that Tet has distinct anti-proliferative and apoptosis induction activities in SW620 cells, which may be mediated by up-regulating BMP9 to partly decrease the level of p-PTEN, followed by inactivating the PI3K/Akt signal.

Tet, a bis-benzylisoquinoline alkaloid was extracted from the dried root of a Chinese herb (*han fang ji*). Also, Tet is one of the well-known calcium blockers and possesses multi pharmacological activities, such as anti-oxidation,



Figure 5 Effects of BMP9 on PI3K/Akt signaling affected by Tet in SW620 cells. (A) The protein level of Akt1/2/3 and p-Akt1/2/3 in SW620 cells treated with Tet tested by Western blot. (B) The protein level of Akt1/2/3 and p-Akt1/2/3 in SW620 cells treated with Tet and/or AdBMP9 tested by Western blot. (C) The protein level of Akt1/2/3 and p-Akt1/2/3 in SW620 cells treated with Tet and/or AdsiBMP9 tested by Western blot. GAPDH was used as control. Error bars are the means \pm SD, n = 3. **P < 0.01 or * P < 0.05 vs. control.

anti-inflammation, and anti-hypertension.^{7–9} Increasing evidence supports that Tet may be a prospective anticancer reagent to inhibit proliferation and induce apoptosis in many cancer cells, such as neuroblastoma, prostate cancer, colon cancer, and gastric cancer.^{10,11,13,19} However, the mechanism of anti-cancer activity for Tet has not been completely elucidated yet. Our results showed that Tet could suppress proliferation and induce apoptosis in SW620 cells concentration-dependently, which suggested that Tet may be as one of the potential natural chemotherapy drugs. In terms of mechanism, it was reported that some critical molecules or signaling pathways are associated with the pathogenesis of colon cancer, such as Wnt, EMT and TGF- β .^{14,15,26} However, the explicit mechanism keeps unclear yet.

Bone morphogenetic proteins (BMPs) are part of TGF- β super-family firstly described by Ali and Brazil, which include various secreted proteins, such as BMP2, BMP7 and BMP9.²⁷ The biological function of BMPs varies with the microenvironment in which it is located. Reports showed that the anti-cancer activity of HNK (Honokiol) in colon cancer might be mediated by up-regulating BMP7.²⁸ In terms of biological function, BMPs usually exert their function through canonical BMPs/Smads signal pathway. Briefly, BMPs ligands bind to BMPRI or BMPRII receptors to compose a complex, and then the complex activates

Smad1/5/8 by recruiting and phosphorylatedrylating the receptor, which further binds to the Smad4 to form another complex which can translocate to the nucleus and bind with other transcriptional factors to regulate downstream target genes.²⁹ Besides, BMPs can also impact the non-canonical BMPs/Smads signal pathways, including PI3K/Akt.³⁰ Meanwhile, BMPs play a crucial role in regulating differentiation and proliferation in cancer cells.³¹ At present BMP9 is one of the least studied BMP members, and it's the role in tumorigenesis, development, and progression still keep unclearly, whether that is a tumor forwarder or inhibitor or irrelevant.³² Our previous research showed BMP9 partly increase the anti-proliferation activity of Tet in HCT116 cells, but this effect in SW620 cells stills uncover. In this study, we found that Tet can increase the level of BMP9 in SW620 cells. Over-expression of BMP9 can increase the antiproliferative effect of Tet, but silencing BMP9 partially reverse this activity, suggesting that TET achieved its anticancer activity by increasing BMP9 expression. Our data suggested that BMP9 might serve as a potential target in the anti-proliferative process of Tet in cancer cells, but further analysis showed that Tet could not increase the phosphorylated Smad1/5/8 (didn't show), which meant that the canonical BMPs/Smads pathway might not be affected by Tet. And BMPs may regulate the anti-cancer function by means of the non-canonical BMPs/Smads pathway,²³ such as



Figure 6 Effects of PTEN on BMP9 affected by Tet in SW620 cells. (A) The mRNA level of PTEN in SW620 cells treated with Tet tested by PT-qPCR. (B) The protein level of PTEN and p-PTEN in SW620 cells treated with Tet tested by Western blot. (C) The protein level of PTEN and p-PTEN in SW620 cells treated with Tet and/or AdBMP9 tested by Western blot. (D) The protein level of PTEN and p-PTEN in SW620 cells treated with Tet and/or AdsiBMP9 tested by Western blot. (E) The protein level of PTEN and p-PTEN in SW620 cells treated with Tet and/or AdsiBMP9 tested by Western blot. (E) The protein level of PTEN and p-PTEN in SW620 cells treated with Tet and/or AdsiBMP9 tested by Western blot. (E) The protein level of PTEN and p-PTEN in SW620 cells treated with Tet and/or AdsiBMP9 tested by Western blot. (E) The protein level of PTEN and p-PTEN in SW620 cells treated with Tet and/or AdsiBMP9 and/or AdsiPTEN tested by Western blot. GAPDH was used as control. Error bars are the means \pm SD, n = 3. **P < 0.01 or * P < 0.05 vs. control.

PI3K/Akt signaling pathway which may play a significant role in cell survival and apoptosis.^{33,34} Moreover, it is also reported that Tet can suppress PI3K/Akt signaling pathway activity to directly impact apoptosis in HT29 cells.³⁵ Our data also revealed that Tet could reduce the phosphorylated Akt1/2/3 (p-Akt1/2/3) in SW620 cells, even though it has not any distinct effect on the total protein level of Akt1/2/3. And over-expression BMP9 can enhance the Tetinduced increasing of phosphorylated Akt1/2/3, but silencing BMP9 slightly inverted the Tet-induced decreasing of phosphorylated Akt1/2/3 in SW620 cells. Based on the above results, we inferred that Tet may impact PI3K/Akt signaling pathway, which may be mediate by BMP9 in SW620 cells, and previous studies have shown that the overactivation of the PI3K/Akt signal is due to the functional inactivation of PTEN, which causes the proliferation of tumor cells to no longer under the control.³⁴ Furthermore, it was reported that Tet reduced the level of p-Akt1/2/3, which may be impacted by PTEN in HCT116 cells.²⁶

Phosphatase and tensin homolog with loss of chromosome 10th (PTEN) is frequently disrupted in multiple sporadic tumor types, ^{36,37} which also controls a lot of cellular evolutions, including survival, apoptosis, proliferation, differentiation, and energy metabolism.^{38,39} Commonly known PTEN is a tumor suppressor, which suppresses the phosphorylatedinositide-3-kinase (PI3K)–AKT–mTOR signaling pathway through catalyzing phosphatidylinositol 3, 4, 5-triphosphate (PIP3) de-phosphorylatedrylated to PI-4, 5-bisphosphate (PIP2).^{40,41} Given the reports and our findings, which are that over-expression BMP9 enhance the anti-cancer activity of Tet due to a decrease in phosphorylated Akt1/2/3 and a decrease in the level of p-PTEN, but silencing BMP9 partially reverse this effect, we speculated that BMP9 may regulate the PI3K/Akt signal through impacting PTEN in SW620 cells. Our further studies showed that silencing PTEN can decrease the reduction of phosphorylated Akt1/2/3 by Tet, BMP9 or combined Tet with BMP9 in SW620 cells. In vivo xenograft models of colon cancer also demonstrated that silencing PTEN partially reverse the growth inhibitory effect of Tet combined with BMP9 on the tumor mass. Taken together, these data demonstrated that BMP9 might mediate the anti-cancer activity of Tet in SW620 cells through slightly downregulating the level of p-PTEN to inactivate PI3K/Akt signaling.

In conclusion, our data demonstrated that Tet could inhibit proliferation and induce apoptosis in SW620 cells, which strongly suggested that Tet may be a potential candidate for colon cancer therapy. BMP9 may partly mediate the anti-cancer activity of Tet in SW620 cells through reducing the activation of PI3K/Akt by activating PTEN at least.



Figure 7 In vivo Tet inhibits tumor growth. (A) Representative tumors sample revealed the effect of Tet and/or AdBMP9 and/or AdsiPMP9 and/or AdsiPTEN on the growth of colon cancer. (B) H&E staining analysis revealed the anti-cancer effects of Tet and/or AdBMP9 and/or AdsiPMP9 and/or AdsiPTEN in colon cancer.

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Ethical approval

All experiments involving animals, procedures were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University. A statement of non-retrospective ethical approval obtained for the animal experiments conducted in the study. See further examples of ethical guidelines (Albert Einstein College of Medicine Institute for Animal Studies). In addition, I confirm that the tumor burden did not exceed the recommended dimensions (see University of Pennsylvania guidelines) and that animals were anesthetized and sacrificed using acceptable methods/techniques.

Availability of data and materials

Data sets used or analyzed during the current study can be obtained reasonably request of the corresponding authors.

Data statement

All data generated or analyzed during this study are included in this article which is authentic. Besides, the datasets during the current study are available from the corresponding author on reasonable request.

Authors contribution

BCH and WJS planned the experiments; YZ and LM performed the experiments, prepared the figures and analyzed the data; XLL, QL,YH, FSL, LXD and HCC helped to perform experiments; KW composed the manuscript. All authors read and approved the final manuscript.

Conflict of Interests

The authors declare no conflict of interests.

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