Tetramethylpyrazine inhibits tumor growth of lung cancer through disrupting angiogenesis via BMP/Smad/Id-1 signaling

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Abstract. The underlying mechanisms of inhibitory effects induced by tetramethylpyrazine (TMP) on angiogenesis and tumor growth of lung cancer were investigated. In vitro cell proliferation, migration, and tube formation of human microvascular endothelial cells (HMEC-1) were evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), wound healing, Transwell, and Matrigel assays. The expression of BMP/Smad/Id-1 signals was detected by RT-PCR and western blotting. In an A549 xenograft tumor model, TMP (40 and 80 mg/kg/day) was intraperitoneally injected into mice. The expressions of CD31, phosphorylated Smad1/5/8, and Id-1 were measured by immunohistochemistry. We demonstrated that TMP inhibited proliferation, migration, and capillary tube formation of HMEC-1 in a dose- and time-dependent manner. Furthermore, treatment of HMEC-1 cells with TMP (0.4 mg/ml) significantly upregulated BMP2 expression and downregulated BMPRIA, BMPRII, phosphorylated Smad1/5/8, and Id-1 expression. In addition, administrations of TMP remarkably inhibited tumor growth of A549 xenograft in nude mice. The CD31, phosphorylated Smad1/5/8, and Id-1 expression were significantly inhibited in TMP-treated xenograft tumors compared with the vehicle. In conclusion, our results indicated that TMP suppressed angiogenesis and tumor growth of lung cancer via blocking the BMP/Smad/Id-1 signaling.

Introduction

Lung cancer is the second most common cancer threat to human health and life in the world and its incidence is growing in many countries (1). Non-small cell lung cancers (NSCLCs) accounts for almost 80% of all lung cancer cases (2). In recent years, lung cancer is still a great challenge in clinical treatment. Surgery resection is only applicable for the patients with early stage. Due to lack of characteristic clinical manifestations, a majority of lung cancer patients are diagnosed with serious symptoms in the late stages. Moreover, chemo-resistance to many common chemo-agents is emerged in the treatment of lung cancer (3). Therefore, an effective and safe strategy is required for the diagnosis and therapy of lung cancer.

Tetramethylpyrazine (TMP) is one of the active compounds extracted from the Chinese medicinal plant Ligusticum chuanxiong (4). It has been widely used as an active ingredient in the clinical treatment of neurovascular and cardiovascular diseases (4,5). The soluble salts tetramethylpyrazine hydrochloride (TMPH) and TMP phosphate, administrated by injection or oral tablets, have been widely used in clinical treatment (6). The underlying mechanisms involve inhibition of platelet aggregation, suppression of apoptosis, and scavenging peroxyl radicals and superoxide and hydroxyl radicals (1,7). A substantial amount of evidence has revealed that TMP has various biological activities, such as antioxidant activity, antitumor activity including glioma, osteosarcoma, hepatocyte carcinoma, gastric, breast and lung cancer (4,8-13). However, its effectiveness of lung cancer and the molecular mechanisms related to tumor growth are still far from completely known.

The development and metastasis of lung cancer cells are always related to dysregulation of cell proliferation and abnormal tumor microenvironment. The vascular niche acts as a major compartment of tumor microenvironment and has an important role in the initiation, progression and metastasis of tumors. Endothelial cells (ECs) are composed of the majority of vascular cells. The interaction between cancer cells and endothelial cells is associated with the process of angiogenesis within the tumor microenvironment (14). Angiogenesis, the process of new capillary blood vessel formation, is considered to be crucial for growth, maintenance, and metastasis of solid tumor. The strategy of blocking angiogenesis is an effective approach to inhibit tumor growth in lung cancer. It has been investigated that COX-2 is related to the regulation of cancer angiogenesis, which has an effect on the prognosis of patients with non-small cell lung cancer (15). In recent studies, TMP

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has been found to inhibit lung cancer cell proliferation via suppressing cell cycle progression and a significant inhibition of cancer cell invasion and metastasis via COX-2 pathway (1). Moreover, it was reported that the inhibition of neovascularization and fibrosis of microcirculation was mediated by TMP via its regulation of the SDF-1/CXCR4 pathway. These results suggest that TMP may be used as a promising candidate for tumor suppression via angiogenesis. However, the molecular mechanism underlying the TMP functional roles in human lung carcinoma needs to be further investigated. Thus, in this study, we investigated the effect of TMP on angiogenesis and tumor growth of lung cancer.

Materials and methods

Chemicals and reagents. Tetramethylpyrazine (TMP), L-glutamine, penicillin, streptomycin, noggin, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and epidermal growth factor (EGF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MCDB131, DMEM culture medium and fetal bovine serum were purchased from Gibco (New York, NY, USA). Horseradish peroxidase (HRP) conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines and cell culture. Human microvascular endothelial cell line (HMEC-1) was purchased from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in MCDB131 medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 ng/ml EGF and antibiotics (100 µg/ml of penicillin, and 100 µg/ml of streptomycin). A549 human NSCLC cell line was obtained from Cell Bank in the Type Culture Collection Center in Chinese Academy of Sciences (Shanghai, China). A549 cell line was cultured in DMEM medium supplemented with 10% fetal bovine serum and antibiotics. All the cells were maintained in a humidified atmosphere of 5% CO2 at 37°C. In all experiments, exponentially growing cells were used.

MTT assay of cell growth. HMEC-1 cell growth was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) following the manufacturer's instructions. Briefly, cells were incubated in a 96-well plate at a density of 5x10^3 cells /well with 100 µl culture medium. The cells were incubated for 24 h at 37°C with 5% CO2 in the humidified incubator. The next day, cells were treated with 0, 0.2, 0.4 and 0.8 mg/ml of TMP for 24, 48 and 72 h, respectively. At the end of detection time-point, 20 µl of MTT solution (5 mg/ml) was added into each well and incubated for 4 h at 37°C with 5% CO2 in the humidified incubator. Then supernatant was discarded and 200 µl DMSO was added, followed by incubating for 5 min on the oscillator. Finally, the optical density (OD) value was measured at a wavelength of 490 nm using a microplate reader. The OD values of treatment groups were normalized to that of control group.

Wound healing assay. HMEC-1 cells (5x10^5 cells/well) were seeded into a 6-well plate. After incubation for 24 h, a yellow tip was used to create a wound in each well. The cells were incubated with vehicle (control) or new MCDB131 medium containing 0.2, 0.4 and 0.8 mg/ml of TMP after rinsing with PBS three times. After incubation for 12 h, three randomly selected regions were photographed under a microscope. The distance of the wound was then estimated quantitatively using Image-Pro Plus software. Migration rate was calculated as follows: migration rate (%) = (D0-D12)/D0 x 100%.

Migration assay. The migration assay was further carried out on a Transwell assay using cell culture chambers (Corning, Cambridge, MA, USA). HMEC-1 cells (2x10^3 cells/well) were cultured in the inner chamber in the vehicle (control) or MDCB 131 medium containing 0.2, 0.4 and 0.8 mg/ml of TMP. The outer chamber was maintained the same medium with 15% FBS. After incubated at 37°C with in a humidified 5% CO2 atmosphere for 12 h, the cells in the upper chamber and on the Matrigel were removed mechanically with a cotton swab. The migrated cells adherent to the outer surface of the membrane were fixed with 90% ethanol and stained using 0.1% crystal violet, then washed with distilled water till the water was colorless. Images of migrated cells were photographed by microscopy.

Tube formation assay. The effect of TMP on angiogenesis in vitro was measured using the HMEC-1 cells capillary-like tube formation assay. Briefly, a 96-well plate was pre-coated with 50 µl Matrigel per well and solidified at 37°C for 30 min. HMEC-1 cells (5x10^5 cells/well) and samples (TMP of 0, 0.2, 0.4 and 0.8 mg/ml) were added into each well correspondingly, and incubated for another 12 h. The enclosed capillary networks of tube formation were recorded by an Olympus IX51 digital camera (Tokyo Japan).

RT-PCR. After TMP treatment for 24 h, total RNA was extracted using a TRIZol method according to the manufacturer's protocol. Subsequently, first-strand cDNA was synthesized from 1 µg of total RNA using avian myeloblastosis virus reverse transcriptase (Takara Biotechnology, Dalian, China). The PCR primers and the corresponding conditions for BMP2, BMP receptor 1A (BMPR1A), BMP receptor 1B (BMPR1B), BMP receptor II (BMPR1I), Smad 4, Id-1, and 18S rRNA were based on previous reports (16,17).

Western blotting. Total proteins were extracted and further subjected to concentration determination using the bicinchoninic acid (BCA) protein assay reagent kit (Beyo- Biotechnology, China). Then the total cellular protein extraction was separated using SDS-PAGE gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, USA). After blocking with TBST containing 5% non-fat milk for 20 min, the membrane was treated with antibodies against Id-1 (Santa Cruz Biotechnology), phospho-Smad1/5/8 Ser463/465, Smad4 (Cell Signaling Technology, Shanghai, China), and β-actin (Santa Cruz Biotechnology) overnight at 4°C. Protein bands were detected by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h, and visualized with SuperSignal Chemiluminescent HRP substrates (Thermo Fisher Scientific, Shanghai, China).

A549 tumor xenografts in nude mice. Male BALB/c nude mice, aged 4-6 weeks, were used in this study and purchased...
from the Shanghai Laboratory Animal Center. All of the experiments were approved by the Experimental Animal Research Committee of our affiliation. A549 cancer cells (1x10^6 cells/0.2 ml serum-free DMEM medium) were inoculated into the right thigh of nude mice. As the tumors reached an average volume of about 100 mm³, the mice were randomized into control and treatment groups with 10 mice each group: a) vehicle; b) TMP (40 mg/kg); c) TMP (80 mg/kg).
TMP was injected intraperitoneally (i.p.) into the mice in the treatment group daily, and mice in control group were subjected to same volume of physiological saline daily. The diet and mental status of the mice were examined every day, and the tumor was measured with a caliper every two days. The tumor volume (in mm³) was calculated as follows: volume = (width)² x length/2.

**Immunohistochemistry (IHC).** At the end of the experiment, the mice were sacrificed. Then, the xenograft tissues were excised, and fixed in 4% paraformaldehyde. Next, the tissues were embedded in paraffin, and sectioned for immunohistochemical analysis. The staining was carried out on a section (5 µm) of xenograft tissues according to standard methods. We incubated the section in an appropriate dilution of primary antibody against CD31 (Proteintech, Wuhan, China), phosphorylated Smad1/5/8, or Id-1 (1:100) overnight at 4˚C, and subsequently stained them with HRP-conjugated secondary antibody (Santa Cruz Biotechnology). The sections were then visualized with 3,3’-diaminobenzidine and counterstaining with hematoxylin.

**Statistical analysis.** The results are presented as mean ± standard deviation (SD). Statistical analyses were performed using Student's t-test for comparison of two groups or one-way analysis of variance (ANOVA) for multiple comparisons by Prism software (GraphPad Software). P<0.05 was considered statistically significant (′p<0.05, ′′p<0.01, ′′′p<0.001).

**Results**

**TMP inhibits HMEC-1 cell proliferation, migration and tube formation.** Angiogenesis involves multiple steps in endothelial cells, including proliferation, migration, and capillary tube formation. First, we used an MTT assay to study the effect of TMP on HMEC-1 cell proliferation. We observed that TMP inhibited the expression of Smad4, Id-1, and 18S rRNA was analyzed by RT-PCR. (B) TMP or noggin inhibited the expression of phosphorylated Smad1/5/8, and Id-1 induced by BMP2 treatment in HMEC-1 cells. The cells were pretreated with vehicle, 0.4 mg/ml of TMP, and 5 µg/ml of noggin for 24 h, and then with 20 ng/ml of BMP2 or vehicle for another 4 h. The above mentioned proteins were analyzed by western blotting. β-actin was used as the control.

**TMP inhibits BMP/Smad/Id-1 signaling in HMEC-1 cells.** As previously reported, the activation of BMP/Smad/Id-1 signaling promoted angiogenesis by upregulation of Id-1 (18,19). In order to investigate whether the inhibition of angiogenesis by TMP was due to the blocking of BMP/Smad/Id-1 signaling in HMEC-1 cells, we evaluated the effect of TMP (0.4 mg/ml) on the signaling pathway. As shown in Fig. 2A, an increased expression of BMP2 was observed in the prior stage of TMP treatment, whereas, a slightly decreased expression of BMP2 was emerged after treatment for 9 h. Additionally, a significant reduction of BMP2 receptor BMPR1B, and BMPRII was observed, while, the receptor BMPR1A did not alter obviously. Furthermore, TMP showed little effect on the expression of Smad4. However, the expression of Id-1 decreased significantly after TMP treatment. The results indicated that TMP modulated the BMP2 expression, and blocked its receptors (BMPR1A and BMPRII), further downregulating Id-1 expression. In order to confirm the role of Smad molecules in the modulation of Id-1, we further investigated the effect of TMP on the phosphorylation of Smad1/5/8 and Id-1 expression. It was observed (Fig. 2B) that TMP showed downregulation of the phosphorylation of Smad1/5/8, but no significant inhibition on Smad4. Moreover, the Id-1 expression was significantly decreased after treated with TMP (0.4 mg/ml). Actually, the effect of TMP blocking BMP/Smad/Id-1 signaling was similar to that of noggin, an endogenous BMP2 antagonist (Fig. 2B). Together, these results suggested that TMP may inhibit angiogenesis via blocking the BMP/Smad/Id-1 signaling in HMEC-1 cells.
Figure 3. TMP suppressed the tumor growth of A549 xenograft in nude mice. (A) Human lung cancer cells A549 were inoculated into the right thigh of nude mice. As the tumors reached an average volume ~100 mm³, the mice were randomized into three groups: a) vehicle; b) TMP (40 mg/kg); c) TMP (80 mg/kg). TMP or same volume of saline was injected intraperitoneally (i.p.) into the mice daily. The diet and mental status of the mice were examined every day, and the tumor was measured with a caliper every two days. The tumor volume (in mm³) was calculated as follows: volume = (width)² x length/2. (B) The tumor weight was significantly inhibited in the xenograft model. Control stands for vehicle group. *p<0.05 vs. group of control, **p<0.01 vs. group of control. (C) The body weights of the three groups showed no significant differences during the experiments [black solid line for vehicle; red dotted line for TMP (40 mg/kg); blue dashed line for TMP (80 mg/kg)].

Figure 4. CD31, phosphorylated Smad1/5/8, and Id-1 expression reduced significantly in the TMP-treated tumor tissues. Immunohistochemistry method was carried out on the tumor tissues after sacrifice of the TMP-treated (40 and 80 mg/kg) and vehicle-treated mice. Compared with the vehicle (A), CD31 expression reduced significantly in the tumor tissues treated with 40 mg/kg TMP (B), and 80 mg/kg TMP (C). The expression of phosphorylated Smad1/5/8 was also lower in the treatment of 40 mg/kg TMP (E), and 80 mg/kg TMP (F), compared with the vehicle (D). The expression of Id-1 was decreased in the treatment of 40 mg/kg TMP (H), and 80 mg/kg TMP (I), compared with the vehicle (G). All images are at x200 magnification.
TMP inhibits the growth of A549 xenograft nude mice. To further evaluate the anti-angiogenic activity of TMP in vivo, we used an A549 lung cancer cell line xenograft tumor model. As shown in Fig. 3A, a low and high dose of TMP (40, and 80 mg/kg/day) was intraperitoneally administered into the mice. After 22-day treatment, TMP significantly inhibited the growth of A549 xenograft in nude mice, compared to the vehicle group (Fig. 3A). In addition, the tumor weight of TMP-treated groups was remarkably decreased compared to the vehicle-treated group (Fig. 3B). However, the body weight of the mice did not show any significant change in any of the A549 xenograft nude mice during the experiments (Fig. 3C).

TMP inhibits BMP/Smad/Id-1 signaling in A549 xenograft nude mice. In order to confirm the in vivo anti-angiogenic activity of TMP via BMP/Smad/Id-1 signaling, we used an immunohistochemical assay to detect the expression of CD31, phosphorylation of Smad1/5/8, and Id-1 expression in the A549 tumor tissues. CD31 is a positive biomarker related to microvessel density in endothelial cells. As shown in Fig. 4A-C, the CD31 expression of TMP treatment (40 and 80 mg/kg/day) was significantly reduced in the tumor tissues compared to the vehicle-treated tumor tissues. The result suggested that TMP also had an anti-angiogenic activity in vivo. Interestingly, we also observed downregulation of phosphorylated Smad1/5/8 expression in the TMP-treated tumor tissues (Fig. 4D-F). Moreover, the expression of Id-1 in the tumor tissues was also decreased after TMP treatment (Fig. 4G-I). These results indicated that suppression of A549 xenograft in nude mice may be attributed to the impairment of angiogenesis via blocking the BMP/Smad/Id-1 signaling.

Discussion

In this study, we demonstrated that tetramethylpyrazine (TMP) could potently inhibit HMEC-1 cell proliferation and migration in vitro, and capillary structures of HMEC-1 cells using Matrigel model. Most importantly, the inhibition of angiogenesis was found to be associated with the blockade of BMP/Smad/Id-1 signaling, suggesting that the BMP/Smad/Id-1 signaling was a potential target of TMP.

The blood vessel circulatory system fuels the neighboring tissues with nutrients and oxygen. A proper formation of blood vessels is necessary for cell and tissue growth. Thus, the disruption of tube formation has been used as an effective strategy to inhibit tumor growth (20). Angiogenesis involves multiple steps, including vasodilation, basement membrane degradation, endothelial cell migration, endothelial cell proliferation, and capillary tube formation (21). As described in the previous study, TMP significantly decreases migration and tube formation in human umbilical endothelial cell line ECV304 (6). Our results demonstrated that TMP remarkably inhibited human microvascular endothelial cell (HMEC-1) proliferation, migration, and capillary structure formation compared with the control (Fig. 1). In addition, the inhibition of angiogenesis by TMP was dose- and time-dependent.

BMPs are secreted extracellular signaling ligands of the transforming growth factor β (TGF-β) family, including four groups: i) the BMP2 and -4 subgroup; ii) the growth and differentiation factor (GDF) 5, -6, and -7 group; iii) the BMP5, -6, -7, and -8 group; and iv) the BMP9 and -10 group (20). Originally, the BMPs were discovered due to their ability to induce bone and cartilage formation (22). Subsequently, BMPs were also found to have an important role in the development of blood vessels. Accumulating evidence has demonstrated that BMPs are essential for angiogenic processes by binding to their receptors on the cells, such as endothelial and smooth muscle cells (22). It is reported that BMP2 can enhance angiogenesis in melanoma (23). The angiogenic effect is exerted via the signaling triggered by the BMP type I receptor (BMP1A, and BMP1B) and BMPRII. Most importantly, it has been demonstrated that BMPRII is required for maintenance of vascular integrity (24). The result obtained in this study demonstrated upregulated BMP2 expression, and downregulated BMPRIB and BMPRII expression after TMP treatment. Interestingly, the expression of BMP2 was upregulated after 3 h of treatment with TMP. However, BMP2 was subsequently decreased slightly after 9 h of the treatment. We hypothesize that BMP compete with BMP2 to bind with BMPs receptors. Thus, BMPRIB and BMPRII expression are downregulated in the early stage of TMP treatment, corresponding to the upregulation of BMP2 expression. Whereas, with the downregulation of BMP receptors (BMPRIB and BMPRII), the expression of BMP2 was further decreased slightly. Actually, this hypothesis still needs to be confirmed by future study.

BMP signal binding to type I and II transmembrane serine/threonine kinase type receptors further triggers phosphorylation of the receptor-regulated Smads, such as Smad1, -5, and -8, and initiation of the canonical or Smad-mediated pathway (20). The other Smads involved in the BMP signaling include the common-mediator Smad4 and the inhibitory Smad6, and -7 (25,26). Once activated, the phosphorylated Smad1/5/8 forms a hetero complex with Smad4, and translocates into the nucleus, where they interact with different co-activators, repressors, then positively or negatively regulate transcription of target genes, such as the genes for Id-1 (inhibitor of differentiation-1 or inhibitor of DNA binding-1), inhibitory Smads (Smad6 and -7), and Serpinel (plasminogen activator inhibitor-1) (27). Id-1 activated by BMPs is crucial for the stimulation of endothelial cells (28). In addition, loss of Id-1 has been demonstrated to result in the downregulation of proangiogenic genes in tumor endothelial cells (29). It suggests that Id-1 has an important role in tumor metastasis via promoting angiogenesis (30). The expression of Smad4 did not show any significant changes in our data (Fig. 2A). On the contrary, the phosphorylation of Smad1/5/8 was downregulated significantly after treated with TMP at 0.4 mg/ml (Fig. 2B). Most importantly, the expression of Id-1 decreased significantly induced by the treatment of TMP (Fig. 2), whereas, the effect of TMP on the BMP/Smad/Id-1 signaling was similar to that of BMP2 antagonist noggin (Fig. 2B).

In addition, Id-1 has a pivotal role in cell proliferation, differentiation, invasion, and metastasis (31,32). Further, TMP-exerted anti-A549 activity in vivo was confirmed by the A549 xenografts in nude mice. The inhibitory effect of TMP on the growth of A549 lung cancer cells in nude mice was attributed to the inhibition of tumor growth (Fig. 3A and B). Moreover, inhibition of angiogenesis in tumor tissues was also demonstrated. CD31, a well-established biomarker in endothel-
in vivo. Suggested that the angiogenesis was also inhibited by TMP. Compared with the control, the CD31 expression was significantly reduced in the TMP-treated mice (Fig. 4A−C), which suggested that the angiogenesis was also inhibited by TMP in the A549 tumor xenograft model. The in vivo result was in accordance with that of in vitro result (Fig. 1). Furthermore, the expression of phosphorylated Smad5/5/8, and Id-1 in the TMP-treated tumor tissues was lower than that of vehicle-treated tissues. Thus, TMP was confirmed to inhibit BMP/Smad5/Id-1 signaling in vivo.

In conclusion, in this study, we illustrated that TMP was a potent angiogenesis inhibitor. This anti-angiogenic activity was correlated with the blockade of BMP/Smad5/Id-1 signaling. In addition, TMP presented a significant inhibition of the nude mouse tumor xenograft model of A549 human lung cancer cells. Hence, TMP may be a potential drug candidate for human lung cancer therapy.

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References


