Tetramethylpyrazine inhibits the proliferation, invasiveness and migration of cervical cancer C33A cells by retarding the Hedgehog signaling pathway

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Abstract. Cervical carcinoma (CC) ranks among the top four most common cancers in women worldwide. Over the last 10 years, several studies have confirmed the inhibitory effects of tetramethylpyrazine (TMP) on numerous types of cancer. To investigate the inhibitory effect of TMP on the CC C33A cell line, MTT and colony formation assays were performed to determine how TMP affects C33A cell survival and proliferation. Proliferation-, migration- and hedgehog (Hh) signaling pathway-related protein expression levels were analyzed via western blotting. Wound-healing and Transwell assays were used to detect the migration and invasion abilities of C33A cells, respectively. The results indicated that TMP markedly reduced the C33A cell survival rate compared with the cervical epithelial Ect1 cell line, which was unaffected by TMP treatment. C33A cell proliferation was downregulated by TMP treatment in a dose-dependent manner. TMP treatment also significantly inhibited C33A cell migration and invasiveness in a dose-dependent manner. Furthermore, TMP inhibited the Hh signaling pathway, as demonstrated by a dose-dependent reduction in Hh-related protein expression levels following TMP treatment. Subsequently, treatment with smoothened agonist increased the proliferation, invasiveness and migration abilities of TMP-treated C33A cells. In conclusion, TMP inhibited the proliferation, migration and invasiveness of CC cells via inhibition of the Hh signaling pathway.

Introduction

Cervical carcinoma (CC) ranks among the top four most common cancer types in women worldwide (1), and is characterized by high incidence and fatality rates (2). At present, the primary treatments for CC are limited to radiotherapy,

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chemotherapy and surgery (3-5). Although they are considered relatively effective, these modalities inevitably give rise to serious side effects, and are not sufficient to prevent cancer metastasis (6). With the development of modern molecular biology and genomics, immunotherapy has become a hot spot in the treatment of advanced and recurrent CC; this type of treatment is in a stage of rapid development and is considered to bring new hope to patients with advanced CC (7). In addition, prophylactic vaccines against numerous strains of high-risk human papillomavirus (HPV) have been developed, but are not a completely effective treatment option for CC (8). The HPV vaccine does not protect against all types of HPV infection, can only be administered within a certain age range, and is unavailable in numerous developing countries (9). Therefore, it is necessary to investigate other effective means of preventing and treating CC.

Tetramethylpyrazine (TMP) is a natural compound derived from *Ligusticum wallichii* (*L. Wallichii*), which is also known as Rhizoma Chuanxiong (Chuanxiong) (10). Chuanxiong is believed to possess numerous beneficial properties, which are recorded in ancient Chinese medical works, including Annotation of *Materia Medica* and Compendium of *Materia Medica* (11). Chuanxiong has also been used in Traditional Chinese Medicine for thousands of years (12). Along with ferulic acid, Chuanxiong contains TMP and is reported to be able to prevent and attenuate the progression of numerous diseases, such as cardiovascular diseases, ischemic stroke and diabetes (13-16). Furthermore, over the last 10 years, several studies have confirmed the inhibitory effects and mechanisms of TMP on numerous types of cancer, including prostate (17), lung (18) and bladder cancer (19). For example, a study reported that TMP treatment reduces the viability and increases the apoptosis of prostate cancer (PCa) cells in a dose-dependent manner (20), indicating that TMP may be a promising therapeutic agent for PCa. TMP has also been demonstrated to significantly decrease the viability, migration and invasiveness of breast adenocarcinoma MDA-MB-231 cells, and to increase their apoptosis in a dose-dependent manner (21). Therefore, we hypothesized that TMP may also exert an inhibitory effect on CC cells.

Hedgehog (Hh) signaling molecule is a local protein ligand secreted by signaling cells, and its signaling pathway controls cell fate, proliferation and differentiation (22). The abnormal activation of the Hh signaling pathway has been demonstrated

to be involved in tumor occurrence and development (23). For example, activation of the Hh signaling pathway is associated with tissue invasion and possible metastasis in gastric cancer (22). The regulation of the Hh signaling pathway by TMP has also been investigated in numerous studies. For example, TMP was demonstrated to effectively inhibit the Hh signaling pathway in hepatic fibrosis (24), has also been reported to be activated in cervical carcinoma cell lines (25). However, the role of TMP in CC, and the associated underlying mechanisms, remain unclear. Therefore, the aim of the present study was to investigate the role of TMP in inhibiting the development of CC by blocking the Hh signaling pathway, which may provide the foundations of novel therapeutics for the prevention and treatment of CC.

Materials and methods

Cell culture and treatments. The human cervical epithelial Ect1 cell line and the CC C33A cell line were purchased from the American Type Culture Collection. Cells were cultured in DMEM supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere of 95% air and 5% CO_2. TMP (purity ≥99%; Fig. 1A) was purchased from Beijing Solarbio Science & Technology Co., Ltd., dissolved in DMSO solution and used to treat C33A cells at doses of 25, 50 and 100 μM (26). Hh and smoothened agonist (SAG) was purchased from Sigma-Aldrich (Merck KGaA) and diluted to 100 nM (27).

MTT assay. To determine the effect of TMP on cell viability, Ect1 and C33A cells were separately collected in the logarithmic phase and 100 μl cell suspension was added to each well of a 96-well plate. The density of cells was adjusted to $2x10^3$ cells/well. The cells were then incubated in 5% CO_2 at 37˚C until a monolayer covered the bottom of the well. TMP (25, 50 and 100 μM) or TMP (100 μM) + SAG was subsequently added, and the cells were incubated at 37˚C for 24 h. Then 0.5% MTT solution (Beyotime Institute of Biotechnology) was added and the cells were incubated for a further 4 h. Then, the medium was removed and 150 μl DMSO was added to each well. The plate was oscillated at low speed on a shaker for 10 min to fully dissolve the formazan crystals, and the absorbance value of each well was analyzed at 570 nm.

To determine the effect of TMP on cellular proliferation, C33A cells were treated with TMP (25, 50 and 100 μM) or TMP (100 μM) + SAG, followed by culture for 24, 48 and 72 h. Samples were then treated with MTT as aforementioned.

Colony formation assay. C33A cells in the logarithmic growth phase were digested with 0.25% trypsin. The cell density was adjusted to $1x10^5$ cells/ml in DMEM containing 20% FBS and TMP (25, 50 and 100 μM) or TMP (100 μM) + SAG. Low melting point agar solutions of 1.2 and 0.7% were prepared using distilled water, and were maintained at 40˚C to prevent solidification. A 3-ml mixture of 1.2% agarose and 2X DMEM (containing 2X antibiotics and 20% FBS) at a 1:1 ratio was cooled, solidified and placed in a CO_2 incubator for use as the bottom agar layer. Subsequently, 0.7% agarose and 2X DMEM were mixed in a sterile test tube at a 1:1 ratio, and 0.2 ml cell suspension was added, mixed and injected into the 1.2% agarose layer to form a double agar layer. Once solidified, the plates were placed in an incubator at 37˚C (5% CO_2) for 14 days. The plates were then placed under an inverted microscope (magnification, x100; Olympus Corporation) to observe the colonies. Subsequently, the cells were stained with 0.1% crystal violet (Thermo Fisher Scientific, Inc.) at room temperature for 2 min and imaged. The number of colonies (>50 cells) was quantified using ImageJ v1.8 software (National Institutes of Health).

Western blotting. C33A cells were washed three times with 3 ml PBS and precooled at 4˚C to remove the medium. The PBS was discarded and the flask containing the cells was placed on ice. To each flask, 400 μl PMSF-containing Cell Lysis Buffer (Cell signaling Technology, Inc.) was added at 4˚C for 20 min, followed by centrifugation at 12,000 x g at 4˚C for 5 min. The supernatant was stored at -20˚C. Protein concentration was quantified using a BCA kit (Beyotime Institute of Biotechnology). 10% SDS-PAGE was then performed for 5 h at 40 V to separate total protein (30 μg/lane). The proteins were then transferred to a PVDF membrane and blocked with 5% non-fat milk for 2 h at room temperature. The membranes were incubated with primary antibodies at 4˚C overnight, followed by secondary antibodies for 2 h at room temperature; all antibody details are presented in Table I. The membranes were washed with TBS-0.1% Tween for chemiluminescence detection with an ECL kit (Beyotime Institute of Biotechnology). The molecular weight and net optical density of the target bands were analyzed using ImageJ v1.8 software (National Institutes of Health).

Wound-healing assay. C33A cells ($5x10^5$ cells/well) were seeded into a 6-well plate and cultured in DMEM containing 10% FBS at 37˚C until 80% confluent, and subsequently serum-starved overnight (28). A 200-μl pipette tip was used to create a wound in the cell monolayer, and the cells were then cultured in serum-free DMEM with TMP (25, 50 and 100 μM) or TMP (100 μM) + SAG. Following incubation at 37˚C for 48 h, the cells were washed with PBS, and the wounds were imaged using an inverted light microscope (magnification, x100; Olympus Corporation). The results were analyzed using ImageJ v1.8 software (National Institutes of Health), and migration rate was calculated as follows: Cell migration rate=wound area difference between 0 and 48 h/wound area at 0 h x100%.

Transwell assay. Matrigel (Corning, Inc.) was added into the upper chamber of the Transwell plate at 37˚C for 30 min, and C33A cells ($5x10^4$ cells) were pre-incubated at 37˚C in DMEM with TMP (25, 50 and 100 μM) or TMP (100 μM) + SAG for 48 h. The cells were then resuspended in DMEM, seeded into the upper chamber and incubated at 37˚C for 48 h. The cells remaining in the upper chamber were removed with a cotton swab, and those in the lower chamber were fixed with 10% formaldehyde at room temperature for 30 min. The cells were then stained with crystal violet at room temperature for 20 min, and subsequently observed and counted under an inverted light microscope (magnification, x100; Olympus Corporation). The result was analyzed using ImageJ v1.8 software (National Institutes of Health), and the invasion rate

Table I. Antibodies used for western blotting.

Name	Cat. no.	Dilution	Species	Company
Ki67	orb389335	1:500	Rabbit	Biorbyt
PCNA	orb48485	1:1,000	Rabbit	Biorbyt
MMP2	orb13578	1:1,000	Rabbit	Biorbyt
MMP9	orb13583	1:250	Rabbit	Biorbyt
PTCH1	orb389650	1:500	Rabbit	Biorbyt
SMO	orb46519	1:1,000	Rabbit	Biorbyt
GLI1	ab134906	1:1,000	Rabbit	Abcam
Shh	ab53281	1:1,000	Rabbit	Abcam
GAPDH	orb555879	1:10,000	Rabbit	Biorbyt
IgG H&L (HRP)	ab6721	1:3,000	Goat anti-rabbit	Abcam

PCNA, proliferating cell nuclear antigen; PTCH1, patched 1; SMO, smoothened homolog precursor; GLI1, GLI family zinc finger 1; Shh, sonic hedgehog; H&L, heavy chain & light chain; Biorbyt, Wuhan Biorbyt Biotechnology Co., Ltd.
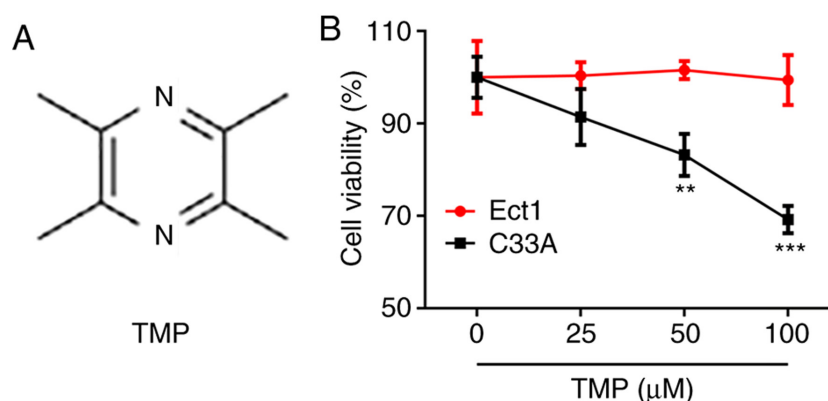

Figure 1. Effect of TMP on CC cell viability. (A) Structure of TMP. (B) Survival of normal cervical epithelial Ect1 cells and CC C33A cells following TMP treatment was detected using an MTT assay. **$P<0.01$ and ***$P<0.001$ vs. 0 μM. N≥3. TMP, tetramethylpyrazine; CC, cervical cancer.

was calculated as follows: Cell invasion rate=the number of invasive cells/number of inoculated cells x100%.

Statistical analysis. All experiments were performed in triplicate; the data were analyzed using SPSS 20.0 (IBM Corp.) and are presented as the mean ± SD. One-way ANOVA followed by a Tukey's post hoc test was used to analyze statistical differences, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

TMP inhibits the survival of C33A cells. To investigate how TMP affects the survival of CC cells, an MTT assay was performed. The results demonstrated that TMP had no significant effect on Ect1 cell viability, whereas TMP decreased C33A cell survival in a dose-dependent manner (Fig. 1B). These results indicated that TMP impeded the survival of CC cells.

TMP inhibits C33A cell proliferation. A series of experiments were performed to determine whether TMP affected the proliferation of CC cells. C33A cell proliferation was quantified at 24, 48 and 72 h, demonstrating a general ascending trend over time. However, in response to an increase in TMP dose, the results demonstrated significantly decreased C33A cell proliferation at 72 h (100 μM; Fig. 2A). Furthermore, the colony formation assay demonstrated that TMP treatment inhibited the clonogenicity of C33A cells in a dose-dependent manner (Fig. 2B and C). The expression of proliferation-related proteins, Ki67 and proliferating cell nuclear antigen (PCNA), was detected via western blotting and both exhibited reduced expression levels following TMP, which occurred in a dose-dependent manner (Fig. 2D). These results demonstrated the possible inhibitory effect of TMP treatment on CC cell proliferation.

TMP inhibits the invasion and migration abilities of C33A cells. The invasiveness and migration of C33A cells were detected using Transwell and wound-healing assays, respectively. Both invasion and migration ability were significantly decreased in response to an increase in TMP concentration (Fig. 3A and B). Furthermore, the protein expression levels of migration-related proteins were further evaluated using
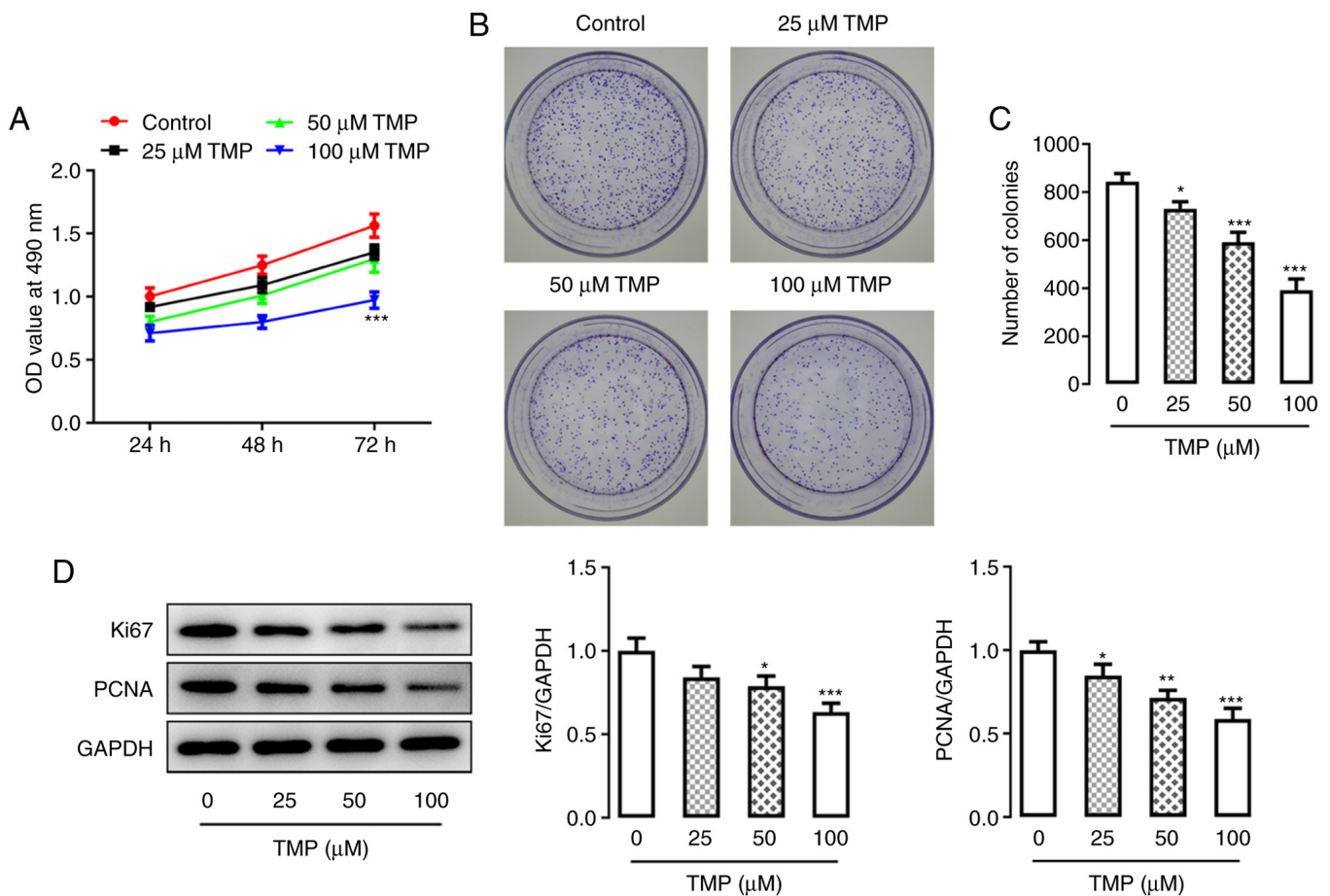
Figure 2. TMP treatment inhibits C33A cell proliferation. (A) C33A cell proliferation following TMP treatment was detected using an MTT assay. ***P<0.001 vs. control 72 h. (B and C) C33A colony formation following TMP treatment was detected using a colony formation assay. (D) Protein expression levels of proliferation-related proteins, Ki67 and PCNA, in C33A cells following TMP treatment were detected via western blotting. *P<0.05, **P<0.01 and ***P<0.001 vs. control (0 μM). N≥3. TMP, tetramethylpyrazine; PCNA, proliferating cell nuclear antigen.

western blotting. Similar to the results of the wound-healing and Transwell assays, it was demonstrated that the protein expression levels of MMP2 and MMP9 were negatively associated with an increase in TMP dose (Fig. 3C). These results indicated that TMP had an inhibitory effect on the invasion and migration capacities of C33A cells.

TMP treatment inhibits the Hh signaling pathway in C33A cells. To understand the mechanism of TMP, its potential regulatory effects on the Hh signaling pathway were investigated using CC cells. The protein expression levels of Hh signaling-related proteins patched 1, smoothened homolog precursor, GLI family zinc finger 1 and sonic hedgehog, were assessed by western blotting. The results demonstrated that these proteins were all markedly reduced in C33A cells following TMP treatment (Fig. 4), and in a dose-dependent manner. This result revealed that TMP may block the Hh signaling pathway in CC cells.

TMP inhibits the proliferation, invasiveness and migration of C33A cells by retarding the Hh signaling pathway. To further elucidate the mechanism underlying TMP treatment, and to verify its effect on the Hh signaling pathway, C33A cell proliferation, invasion and migration abilities were investigated in the presence of a Hh signaling pathway

agonist (SAG) and 100 μM TMP. Western blotting verified the increased activation of the Hh signaling pathway, which was evidenced by the upregulated protein expression levels of Hh signaling pathway-related proteins in TMP-treated C33A cells following SAG inducement (Fig. 5A). Moreover, the MTT assay demonstrated that the inhibited proliferation of C33A cells following TMP treatment was reversed by SAG inducement, compared with the control group (Fig. 5B). Furthermore, the colony formation assay demonstrated a reduction in the number of C33A colonies formed when treated with TMP (compared with the control), which was markedly reversed by SAG treatment (Fig. 5C and D). Western blotting also detected increased protein expression levels of the proliferation-related proteins Ki67 and PCNA in TMP-treated C33A cells induced by SAG, which were originally inhibited by TMP (Fig. 5E). Moreover, wound-healing and Transwell assays demonstrated that both the migration and invasion abilities of TMP-treated C33A cells were increased following SAG inducement (Fig. 5F and G). The protein expression levels of migration-related proteins MMP2 and MMP9 also exhibited a similar trend (Fig. 5H). These results collectively indicated that TMP may block the Hh signaling pathway, and thereby inhibit the proliferation, invasiveness and migration of CC cells.
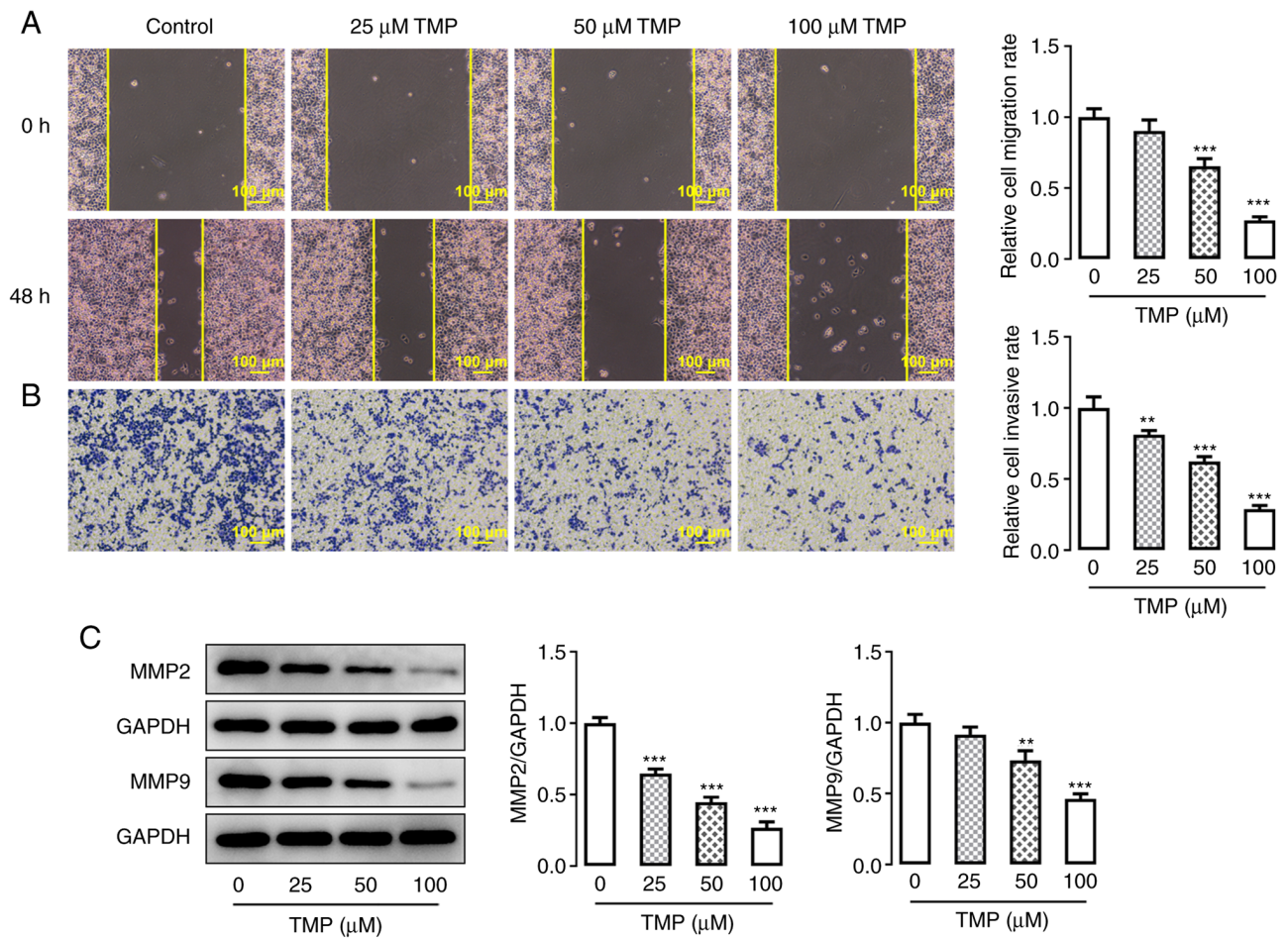
Figure 3. TMP treatment inhibits the invasion and migration abilities of C33A cells. C33A cell (A) migration and (B) invasiveness following TMP treatment was detected using wound-healing and Transwell assays, respectively. Scale bar, 100 μm. (C) Protein expression levels of migration-related proteins MMP2 and MMP9 in C33A cells following TMP treatment were detected via western blotting. **P<0.01 and ***P<0.001 vs. 0 μM. N≥3. TMP, tetramethylpyrazine.
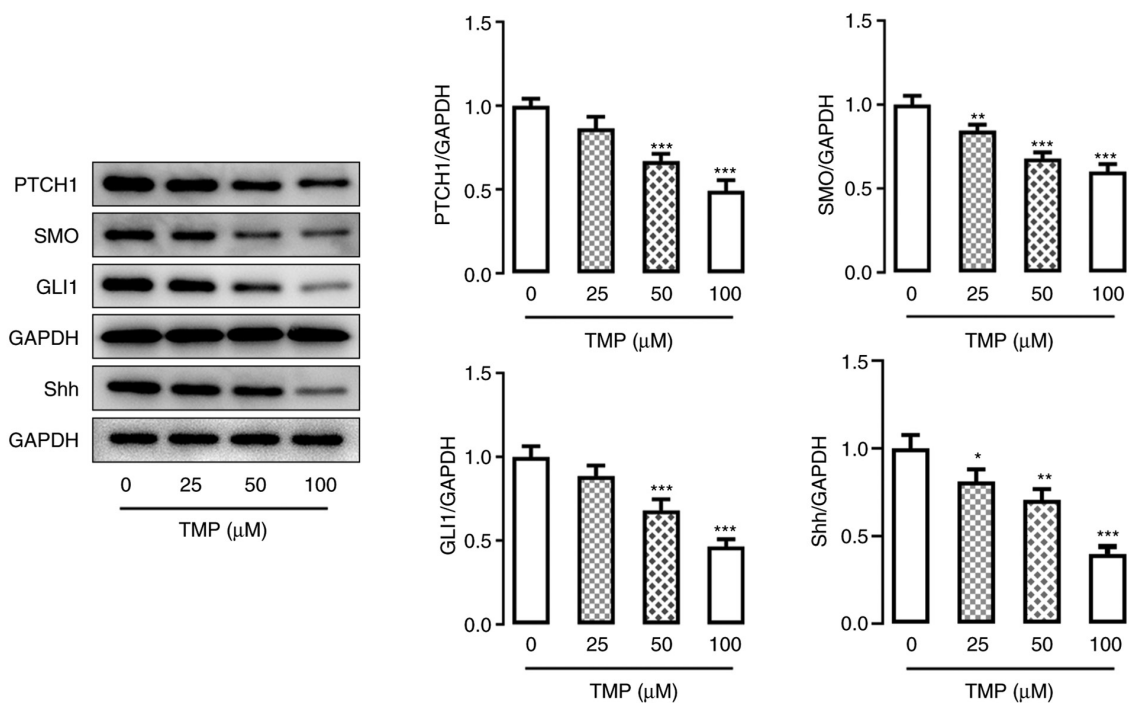

Figure 4. TMP treatment blocks the Hh signaling pathway in C33A cells. Protein expression levels of Hh signaling pathway-related proteins in C33A cells following TMP treatment were detected via western blotting. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 μM. N≥3. TMP, tetramethylpyrazine; PTCH1, patched 1; SMO, smoothened homolog precursor; GLI1, GLI family zinc finger 1; Shh, sonic hedgehog.
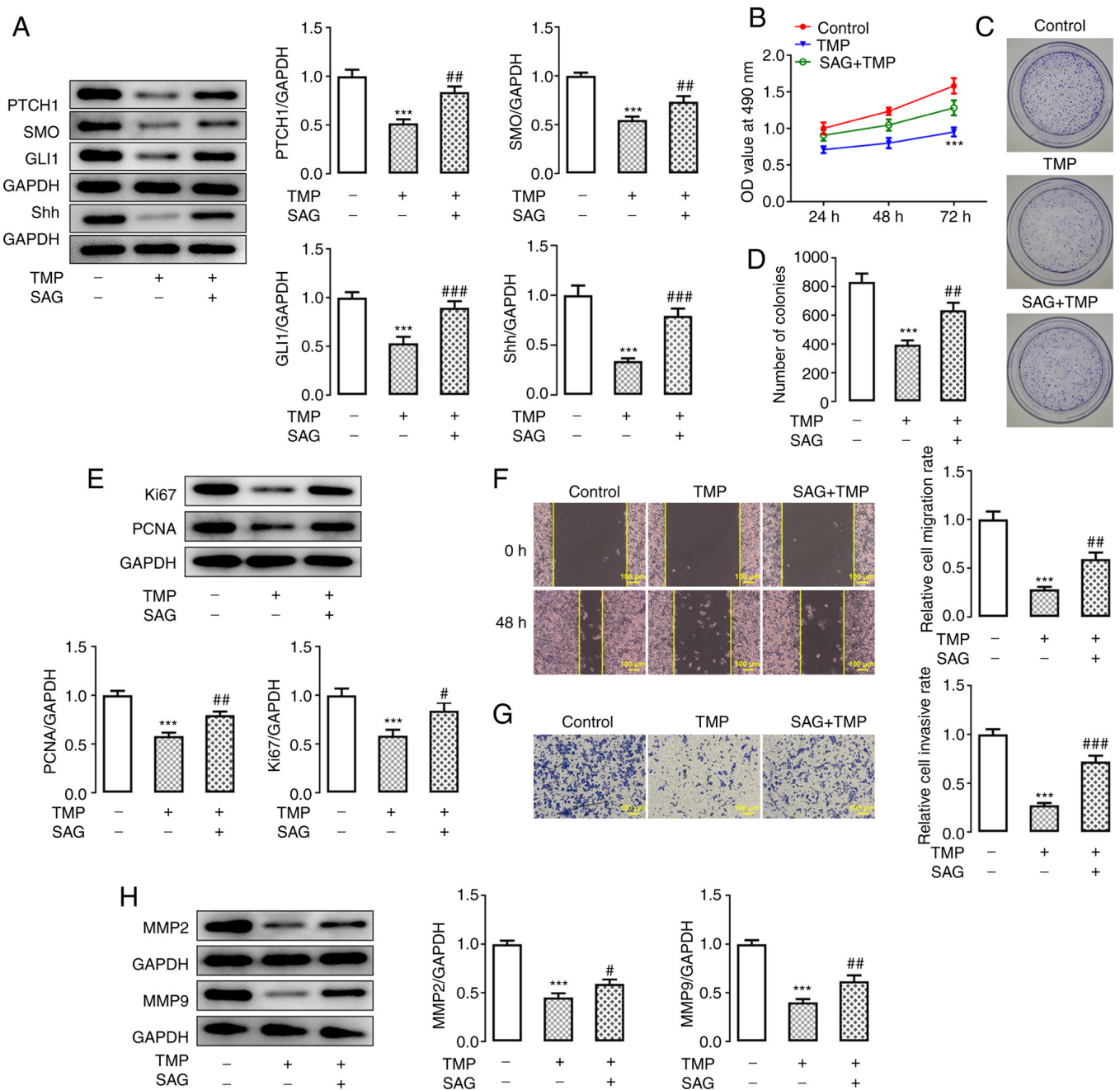
Figure 5. TMP inhibits the proliferation, invasion and migration abilities of C33A cells by retarding the Hh signaling pathway. (A) Protein expression levels of Hh signaling pathway-related proteins in TMP-treated C33A cells, before and after SAG inducement, were detected via western blotting. (B) Proliferation of TMP-treated C33A cells before and after SAG inducement was detected using an MTT assay. (C and D) Colony formation in TMP-treated C33A cells, before and after SAG inducement, was detected using a colony formation assay. (E) Protein expression levels of proliferation-related proteins in TMP-treated C33A cells, before and after SAG inducement, were detected via western blotting. (F and G) Migration and invasiveness of TMP-treated C33A cells, before and after SAG inducement, were detected using wound-healing and Transwell assays, respectively. (H) Protein expression levels of migration-related proteins in TMP-treated C33A cells, before and after SAG inducement, were detected via western blotting. $^{***}P<0.001$ vs. control; $^{#}P<0.05$, $^{##}P<0.01$ and $^{###}P<0.001$ vs. TMP. N≥3. TMP, tetramethylpyrazine; PTCH1, patched 1; SMO, smoothened homolog precursor; GLI1, GLI family zinc finger 1; Shh, sonic hedgehog.

Discussion

CC is a primary malignant tumor of the cervix, which is mainly caused by persistent high-risk HPV infection (29). Regular CC screening, management of cervical precancerous lesions and HPV vaccinations are currently the primary preventative measures for CC (29). Screening and management of precancerous lesions contribute to the prognosis of CC, whereas HPV vaccinations prevent infection from certain strains of HPV;

however these preventative measures have limitations (30). For example, a considerable number of CCs have been confirmed to be HPV negative (31). As aforementioned, TMP was found to serve an inhibitory role in numerous types of cancer (17-19). The present study demonstrated that TMP reduced the survival rate of CC cells in a dose-dependent manner. TMP also effectively inhibited CC cell proliferation and colony formation, and reduced proliferation-related protein expression. Furthermore, the dose-dependent attenuation of CC cell

migration and invasiveness by TMP was also demonstrated. These results indicated that TMP may exert a protective effect against CC.

In order to further investigate the mechanism underlying the effects of TMP in CC, its effects on the Hh signaling pathway were determined. A previous study demonstrated that TMP disrupted the Hh signaling pathway to reduce sinusoidal tubule angiogenesis, and to inhibit the angiogenic properties of liver sinusoidal endothelial cells (32). Another study reported that a compound extracted from Artemisia plants reduced the proliferative index of HeLa and Caski CC cells through the Hh signaling pathway, and inhibited tumor growth in tumor-bearing mice (33). Furthermore, sonic Hh gene silencing has been demonstrated to inhibit the epithelial-mesenchymal transition, as well as the proliferation, invasiveness and migration of CC cells by inhibiting the Hh signaling pathway (34). In the present study, the levels of Hh signaling pathway-related protein expression in CC cells were negatively associated with TMP in a dose-dependent manner. To further verify the roles of the Hh signaling pathway and TMP, CC cells were treated with the Hh agonist SAG (27). SAG induction of TMP-treated CC cells reversed the anti-proliferative, anti-invasive and anti-effects of TMP on CC cells.

In conclusion, the present study indicated that TMP protected against CC, and inhibited the proliferation, invasiveness and migration of CC cells by blocking the Hh signaling pathway. These results highlighted the potential for a novel therapy for the prevention or treatment of CC. In order to fully utilize TMP and apply it in clinical practice for CC, more in-depth research is required to determine its mechanism of action. future research will include establishing animal cervical cancer models to verify the effects of TMP *in vivo*.

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

JR helped to draft the manuscript and performed the experiments. JC performed the experiments and data analysis. CW revised the manuscript and helped to design the experiments. All authors read and approved the final manuscript. JR and CW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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