RESEARCH ARTICLE

Anti-Proliferation Effects and Molecular Mechanisms of Action of Tetramethypyrazine on Human SGC-7901 Gastric Carcinoma Cells

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Abstract

Aim: To investigate the effects of tetramethypyrazine (TMP) on proliferation and apoptosis of the human gastric carcinoma cell line 7901 and its possible mechanism of action. Methods: The viability of TMP-treated 7901 cells was measured with a 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) and cell apoptosis was analyzed by flow cytometry. The distribution of cells in different phases of cell cycle after exposure of TMPs was analyzed with flow cytometry. To investigate the molecular mechanisms of TMP-mediated apoptosis, the expression of NF-κBp65, cyclinD1 and p16 in SGC-7901 cells was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and western blotting. Results: TMP inhibited the proliferation of human gastric carcinoma cell line 7901 in dose and time dependent manners. Cell growth was suppressed by TMP at different concentrations (0.25, 0.5, 1.0, 2.0 mg/ml), the inhibition rate is 0.46%, 4.36%, 14.8%, 76.1% (48h) and 15.5%, 18.5%, 41.2%, 89.8% (72h) respectively. When the concentration of TMPs was 2.0mg/ml, G1-phase arrest in the SGC-7901 cells was significant based on the data for cell cycle distribution. RT-PCR demonstrated that NF-κBp65 and cyclin D1 mRNA expression was significantly down-regulated in 7901 cells treated with 2.0 mg/ml TMP for 72h ($p<0.05$), while the p16 mRNA level was up-regulated ($p<0.05$). The protein expression of NF-κBp65 and cyclin D1 decreased gradually with the increase in TMP concentration, compared with control cells ($p<0.05$), while expression of protein p16 was up-regulated ($p<0.01$). Conclusion: TMP exhibits significant anti-proliferative and pro-apoptotic effects on the human gastric carcinoma cell line SGC-7901. NF-κBp65, cyclinD1 and p16 may also play important roles in the regulation mechanisms.

Keywords: Tetramethypyrazine - SGC-7901 - proliferation - apoptosis - NF - κBp65 - cyclinD1-p16

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Introduction

Gastric cancer is the second most common cause of cancer-related death worldwide. So far it is the fourth most frequently diagnosed cancer as each year more than one million patients are annually diagnosed with gastric cancer (Ferlay et al., 2010; Hernandez et al., 2010; Basiri et al., 2014; Behnampour et al., 2014; Calik et al., 2014; Deng et al., 2014; Khoshbaten et al., 2014; Karim et al., 2014; Lu et al., 2014; Unal et al., 2014; Yang et al., 2014; Zhou et al., 2014). The incidence of stomach cancer varies geographically, with a much higher prevalence in Eastern countries than in the Western ones (Parkin et al., 2005). In 2005, the incidence of gastric cancer (0.3 million deaths and 0.4 million new cases) ranked third among the most common cancers in China (Yang et al., 2005). Cancer is a kind of diseases that the cell cycle is out of control, and all intracorporal and extracorporeal factors can be attributed to the regulation of cell cycle. The development of gastric cancer is no exception. Tetramethypyrazine (TMP) is a kind of alkaloid that extracts from Chuanxiong, Pharmacological studies showed that it has many biological activities of treating cerebral ischemia, improving microcirculation, anti-thrombosis, protecting the coronary artery, protecting ischemic myocardial cells and the preventing myocardial cell injury. In recent years, some experts have found that TMP has anti-cancer activity and play an active role in the treatment and prevention of lung cancer, stomach cancer, breast cancer and other cancers. They have carried out some explorations on its mechanism, but there is few research and report for the mechanism of TMP on gastric cancer. We select TMP as our object to observe its effect on the proliferation and apoptosis of human gastric cancer cell line SGC-7901 and determine the expression of NF-κ Bp65, cyclinD1 and p16 to study the mechanism of TMP from the angle of cell cycle and provide a theoretical basis for clinical use of TMP in the treatment of gastric cancer.

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Materials and Methods

Materials

TMP was purchased from Pharmaceutical and Biological Products Inspection of China (Production batch number: 110817-202006, Specification: 40 mg/2ml). Fetal bovine serum was purchased from Hangzhou Sijiqing. DMEM, MTT, propidium iodide (PI), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Annexin V-FITC Apoptosis Detection Kit was purchased from KeyGEN Biotech (Nanjing, China). Rabbit polyclonal anti-NF-κBp65, anti-cyclinD1, anti-p16 and β-actin antibody were purchased from Abcam, Santa cruz and KeyGEN Biotech, respectively.

Cell culture and inhibition rate

Human gastric cancer SGC-7901 cells were cultured and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 μm/l penicillin, and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere with 0.5% carbon dioxide. Cells were cultured in a 10-cm culture dish and were allowed to grow to 60-80% confluence before experimentation.

The effect of TMP on the viability of cells was examined by MTT assay. SGC-7901 cells were seeded at 5×10^3 cells/well in 96 well plates in RPMI 1640 medium supplemented with 10% FBS. After 24 h, cells were treated with various concentrations of TMP (0.25, 0.5, 1.0, 2.0 mg/mL) in triplicate. RPMI 1640 medium alone served as a control. A MTT assay was performed after 48h and 72h. Twenty microliters of MTT solution (1 mg/mL) was added to each well, followed by incubation for a further 4h. Medium was removed and 150ul dimethyl sulfoxide was added to each well. Absorbance was measured at 570 nm. Results were expressed as the mean of three wells for each group. The inhibition ratio (IR) was calculated by the following equation: Inhibitory ratio (%)= (Acontrol -Atreated)/Atreated ×100%. Where Acontrol-Atreated represents the differential absorbance for both the test group and control at 570 nm and Atreated is the absorbance for the test group at 570 nm. Results were expressed as the mean of three individual experiments.

Cell apoptosis analysis and cell cycle analysis

Determination of apoptosis by flow cytometry. Apoptosis was determined through Annexin V-FITC Apoptosis Detection Kit. SGC-7901 cells were seeded in 6-well plates and were incubated overnight and then treated with various concentrations of TMP (0, 0.5, 1.0, 2.0 mg/mL) respectively for 72h. Cells were harvested by trypsinization, washed with pre-chilled PBS (4˚C) and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in 195 μl of binding buffer and incubated with 5 μl Annexin V-FITC in the dark at room temperature for 10 min. Cells were centrifuged, washed with PBS, and re-suspended in 195 μl of binding buffer containing 10 μl PI solution in the dark and were then analyzed by flow cytometry (Beckman FC400 MPL, USA).

Cell cycle analysis. The distribution of cells in different phases of cell cycle after exposure of TMPs was analyzed with flow cytometry. Briefly, SGC-7901 cells were harvested and washed with PBS after exposure of various concentrations of TMP (0, 0.5, 1.0, 2.0 mg/mL) for 72h. The cells were fixed with 70% cold ethanol at -20˚C overnight and then stained with PI solution consisting of 1 mg/mL PI and RNase A. The fluorescence-activated cells were sorted in the flow cytometry, and the data were analyzed using CellQuest analysis software.

Table 1. The RT-PCR Sense Primer of NF-κBp65, cyclinD1, p16 and GAPDH

Gene	Primer Sequence 5'→3'	Product size (bp)
NF-κBp65	Sense: 5'-TGTCCTTTCTCATCCCATCTTTG-3'	162
	Antisense: 5'-ATGTCCTCTTTCTGCACCTTGTC-3'	
cyclin D1	Sense: 5'-GCCCTCGGTGTCCTACTTCAAAT-3'	112
	Antisense: 5'-CTCCTCCTCGCACTTCTGTTCCT-3'	
p16	Sense: 5'-TGAGAAACCTCGGGAAACTTAGAT-3'	141
	Antisense: 5'-CGGTAGTGGGGGAAGGCATATA-3'	
GAPDH	Sense: 5'-GCACCGTCAAGGCTGAGAAC-3'	181
	Antisense: 5'-TGGTGAAGACGCCAGTGGA-3'	

The mRNA extract and RT-PCR

The mRNA of NF-κBp65, cyclinD1 and p16 was extracted from SGC-7901 cells treated with various concentrations of TMP (0, 0.5, 1.0, 2.0 mg/mL) respectively for 72h by using Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcription of total RNA (2 μg) was performed in 20 μl volume according to the manufacturer's instructions. The primer sets of NF-κBp65, cyclinD1 and p16 are shown in Table 1. GAPDH was amplified as an internal control. Each PCR product was visualized by staining with ethidium bromide after electrophoresis on 2% agarose gels under ultraviolet light.

Western blot analysis

SGC-7901 cells (2.5×10^5/ml) were seeded for 72h in 6-well plates prior to experimentation, and incubated with various doses (0, 0.5, 1.0, 2.0 mg/mL) of TMP, as described above. After incubation, the cells were washed three times with ice-cold PBS and harvested in lysis buffer. The lysate was centrifuged at 12,000 rpm for 15 min at 4˚C and the supernatant was collected. Protein concentration was determined by Bradford protein assay. Proteins (70 μg/well) denatured with sample buffer were separated by electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes (0.45 μm). The membranes were blocked with 10% (v/v) dried fat-free milk in PBS containing 0.1% Tween-20 for 1 h and were incubated with anti-NF-κBp65 (1:500 dilution, Abcam), anti-cyclinD1 (1:500 dilution, Santa cruz) and anti-p16 (1:500 dilution, Santa cruz) and β-actin antibody (1:500, KeyGenBiotechCo. Ltd) overnight at 4˚C. After washing with 1×phosphate-buffered saline Tween-20 (PBST), membranes were continuously probed with HRP-conjugated anti-mouse IgG (1:1000 dilution) in PBS for 1h at room temperature, then washed with PBST. The immunobinding signals were detected by a chemiluminescence method (ECL, KeyGenBiotechCo.Ltd). Relative protein expression was quantified densitometrically using the Image-Pro Plus Version 6.0 software and calculated according to the β–actin reference bands.

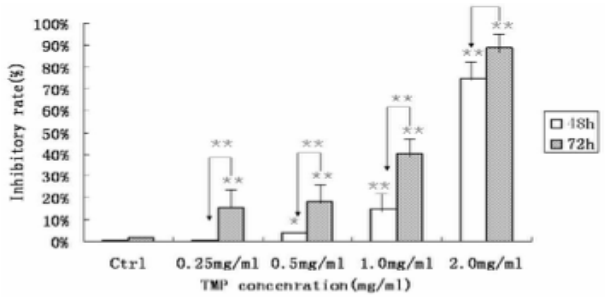

Figure 1. Inhibition Rate of Different TMP Concertrations on SGC-7901(χ±s). SGC-7901 cells were plated in a 96-well plate and treated with various concentrations of TMP (0, 0.25, 0.5, 1.0 and 2.0 mg/ml) for various exposure times (48h, 72h). Cell proliferation was measured by MTT assay. Each group was analyzed in triplicate. *p<0.05, ** p<0.01

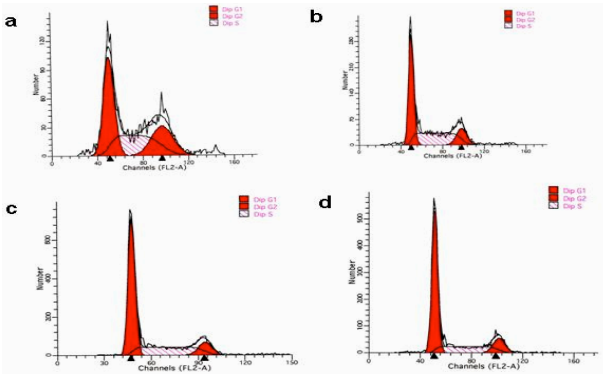

Figure 2. Cell Cycle Progression of Different TMP Concertrations on SGC-7901. Cell cycle phase distribution of the treated cells was measured by flow cytometry. SGC-7901 cells were treated with 0 (a), 0.5 (b), 1.0 (c) and 2.0 (d) mg/ml for 72h

Statistical analysis

For the statistical analysis of data, comparison between results from different groups were analysed with SPSS for Window Version 15.0. The Student's t-test was employed to determine the statistical significance of the difference between different experimental groups and control group at P<0.05 value being regarded as statistically significant. All experiments were repeated at least three times. Data are presented as means ± standard deviation (SD). We have enough experience in conducting medical researches, and have published some results elsewhere (Chen et al., 2013; Dai et al., 2013; Deng et al., 2013; Gu et al., 2013; Fei et al., 2013; Huang et al., 2013; Huang et al., 2013; Liu et al., 2013; Liu et al., 2013; Liu et al., 2013; Lu et al., 2013; Lu et al., 2013; Shen et al., 2013; Sun et al., 2013; Wei et al., 2013; Wu et al., 2013; Wu et al., 2013; Yan et al., 2013; Yang et al., 2013; Yang et al., 2013; Yang et al., 2013; Yin et al., 2013; Yin et al., 2013; Yang et al., 2013; Gong et al., 2014; Xu et al., 2014; Xu et al., 2014; Zhang et al., 2014).

Results

Growth inhibition of TMP on the SGC-7901 cells

The SGC-7901 cells were treated with various concentrations of TMP for 48h and 72h, cell viability was determined by Alamar Blue assay. Cell growth was

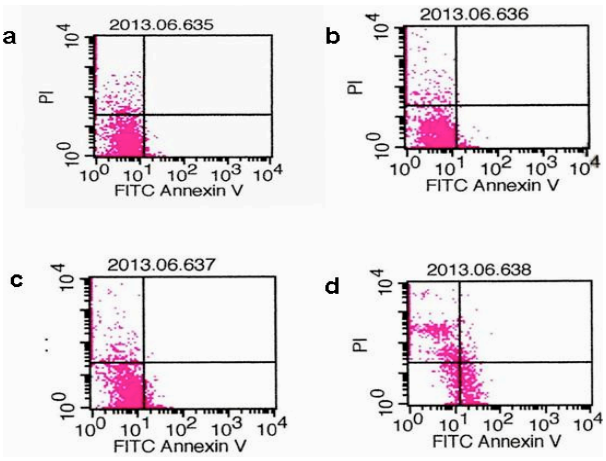

Figure 3. Cell Apoptosis of the SGC-7901 Cell Line Treated with TMPs. SGC-7901 cells were treated with 0 (a), 0.5 (b), 1.0 (c) and 2.0 (d) mg/ml for 72h. Cells were collected and stained with annexin V-FITC/PI

suppressed by TMP of different concerntrions (0.25, 0.5, 1.0, 2.0 mg/mL), the inhibition rate is 0.46%, 4.36%, 14.76%, 76.13% (48h) and 15.45%, 18.46%, 41.23%, 89.78% (72h) respectively. The inhibition rate of 2.0 mg/mL TMP for tumour cell growth was 89.78%. TMP inhibited the growth of gastric cancer cells in a dose-dependent manner (Figure 1).

Effects of TMP on cell apoptosis and cell cycle progression

The inhibition of cell growth may be a result of the induction of apoptosis that is mediated by cell cycle arrest. To determine whether the inhibitory effects of TMPs on the proliferation of the SGC-7901 cell line involved cell cycle changes, we examined the cell cycle phase distribution of the treated cells by flow cytometry. The cells were treated with various concentrations (0.5, 1.0, 2.0 mg/mL) of TMPs for 72 h. The results showed that TMPs were capable of inducing an increase in the percentages of G1-phase cells and the number of apoptotic cells (p<0.05; Figure 2). When the concentration of TMPs was 2.0 mg/mL, G1-phase arrest in the SGC-7901 cells was significant based on the data of cell cycle distribution.

To further quantify TMP-induced apoptosis of SGC-7901 cells, cells were stained with annexin V-FITC and PI, followed by flow cytometry. A representative result of flow cytometry is presented in Figure 3. The lower right quadrant depicts the percentage of early apoptotic cells (annexin V-FITC-stained cells) and the upper right quadrant represents the percentage of late apoptotic cells (annexin V-FITC- and PI-stained cells). The fully apoptotic cells are those in the lower right and upper right quadrants. As shown in the quantitative result, only a small number of apoptotic cells were detected in the control group. However, 72h after treatment with 0.5, 1.0, 2.0 mg/mL TMP, cell apoptosis was 5.5%, 7.94%, and 16.56%, respectively. This result suggests that rhein induced significant apoptosis of SGC-7901 cells in a dose-dependent manner.

Effects of the TMP on the DNA and mRNA expression of NF-κBp65, cyclinD1 and p16

Real-time quantitative PCR was used to detect the

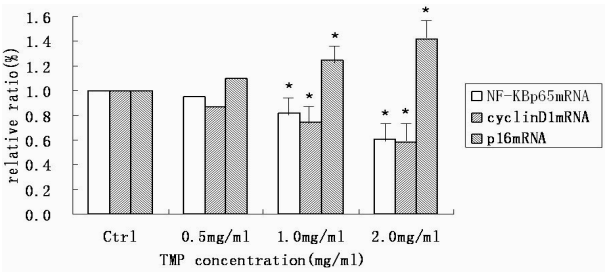

Figure 4. Effects of the TMP on the mRNA Expression of NF-κBp65, cyclinD1 and p16 in SGC-7901 Cell. TMP decreased gene expression of NF-κBp65, cyclinD1 and increased p16 in SGC-7901 cells in a dose-dependent manner. SGC-7901 cells were treated with TMPs (0.5, 1.0, and 2.0 mg/ml) for 72h. The expression of mRNAs was analyzed by RT-PCR and normalized by GAPDH expression. *$P < 0.05$ versus control group

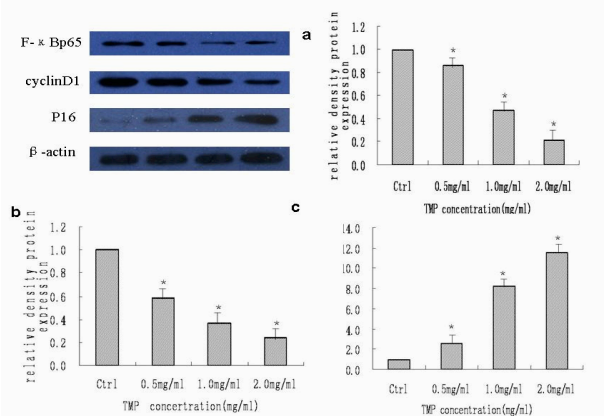

Figure 5. Western Blot Analysis for the Expression of NF-κBp65, cyclinD1 and p16. TMP decreased the expression of NF-κBp65, cyclinD1 but increased the expression of p16 in SGC-7901 cells (a: NF-κBp65, b: cyclinD1, c: p16). SGC-7901 cells were treated with TMPs (0.5, 1.0, 2.0 mg/ml) for 72h and the expression of proteins in treated cells was determined by Western blot analysis. Data are reported as the means ± SEM of at least three experiments. *$P < 0.05$ versus control group

mRNA expression of NF-κBp65, cyclinD1 and p16 at 72h after TMP treatment at concentrations of 0.5, 1.0, or 2.0 mg/mL, The change in mRNA expression was normalized by GAPDH expression. The results revealed a significant down-regulation of mRNA expression of NF-κBp65, cyclinD1, and up-regulation of the mRNA expression levels of p16 on TMP concentrations of 1.0, or 2.0 mg/mL as compared with the controls (Figure 4).

Effects of the TMP on the protein expression of NF-κBp65, cyclinD1 and p16

To clarify the mechanisms of anti-proliferation and apoptosis by TMPs in the SGC-7901 cell line treated with various concentrations of TMPs (0.5, 1.0, 2.0 mg/mL) for 72h, we assayed the protein expression of NF-κBp65, cyclinD1 and p16. To ensure equal loading of proteins in all the samples, β-actin control was used. After the SGC-7901 cells were treated with TMPs, western immunoblotting was used to analyze the expression of NF-κBp65, cyclinD1 and p16. Figure 5 shows the effect of TMPs on the protein expression of NF-κBp65, cyclinD1 and p16 in SGC-7901 cells. The protein expression of NF-κBp65, cyclinD1 decreased gradually with the increase in TMPs concentration compared with the control cells ($p<0.05$). The expression of p16 was upregulated by TMPs compared with the control cells ($p<0.01$). These results demonstrate that TMPs have anti-proliferative and apoptotic effects in the SGC-7901 cell line (Figure 5).

Discussion

ACell cycle has four periods (G1, S, G2, M). G1/S and G2/M phase are the two important control points in the cell cycle (Pestell , 2003). Regulational function of Cell cycle mainly depend on the cyclins, cyclin dependent kinase (CDKs) and cyclin dependent kinase inhibitor (CKIs) (Murray et al., 2001). Cyclins have positive regulation for CDKs and CKIs have negative regulation for CDKs. If the balance of positive and negative regulation is broken, cells may be proliferate abnormally and lead to cancer, so abnormal regulation of cell cycle is closely related with cancer (Liu et al., 2004).

CyclinD1 is one of the subtypes of cyclinD, a currently recognized oncogene, which can effect G1/S phase control point in cell cycle. Since cyclin D1 regulates the G1/S transition during cell division, Its overexpression can activate CDK4 or CDK6 and shorten G1 phase, to some extent, which can reduce dependence of mitogen for cell proliferation and resulting in disorder regulation of cell cycle and cell abnormal proliferation. Overexpression of cyclinD1 is commonly reported in malignancies. The basis of such a correlation with cancer development and cyclinD1 amplification relies on the critical role of cyclinD1 gene in cell cycle control (Umit et al., 2013). Nuclear factor κB (NF-κB) is a protein molecule that widely exists in the cell and has a multi-directional function of regulation. NF-kB regulates the expression of both antiapoptotic and proapoptotic genes. It is a family of closely related protein dimers that bind to a common sequence motif in DNA the kB site (Shanmugam et al., 2013). p50p65 dimer is the the most common active form of NF-κB. It can combine certain specific nucleotide sequences of gene promoter region and start gene transcription. Hinz et al. (1999) found that cyclinD1 is the target gene of NF-κB, which can start the transcription of cyclinD1 and promote the transition of G1/G0 phase to S phase, eventually leading to cell proliferation and malignant transformation. P16 is a kind of CKIs that play an important role of negative regulation at the G1 / S phase. P16 combining with CDK4 inhibits the activity of cyclinD1, thereby counteract the phosphorylation of RB protein. It prevent cell entry into S phase and lead to the cell cycle arrest. Inactivation of P16 leads to excessive proliferation of cells. Kishimoto et al. (2008) found that there are at least more than 2 abnormal expression of oncogene in the development of gastric cancer. Nearly 40%- 50% cases were occurred in the absence of expression of P16/Rb and overexpress of Cyclin D1/CDK4.

TMP is an effective monomer that separated from Chuanxiong and can be synthesized and used in clinic widely. In recent years, many experts have used TMP for the treatment of cancer and studied deeply, and found that TMP may play the roles of the inhibition of

cell proliferation, invasion and metastasis, inhibition of vascular endothelial cell growth and proliferation, apoptosis. It has the actions of reversing tumor drug resistance, improving radiotherapy sensitization and chemotherapy effect, reducing chemotherapy toxicity. In this study, we analyze the effect of different concentrations of TMP in human gastric cancer SGC-7901 cells, the results show that TMP has significant inhibition to the proliferation. Meanwhile, TMP affect apoptosis of human gastric cancer SGC-7901 cells in time and dose-dependent manner. TMP can cause the number of G0/G1 phase cells increasing, which makes part of the cell arrested in the period and inhibit the proliferation of human gastric cancer cell SGC-7901. The results of RT-PCR method and Weston blot assay showed that NF-κBp65 and CyclinD1 presented low expression ($p<0.05$, $p<0.01$) and have positive relation ($p<0.05$), because cyclinD1 promoter has the binding sites of NF-κBp65. If the NF-κBp65 is activated, it can directly activate the transcription of cyclinD1 and promote cell proliferation. Therefore, the downregulation of NF-κBp65 expression will directly affect the expression of CyclinD1, resulting in inhibiting the activity of CDK4 or CDK6 and preventing the transition from G1/G0 phase to S phase. Finally, it suppresses the cell proliferation inhibition and promotes the cell apoptosis. As the cyclin-dependent kinase inhibitor, pl6 showed high expression, which can activate the negative feedback of cell cycle. The specific binding of p16 and CDK4 can make CDK4 inactivation and regulate cell cycle activity negatively. Thereby, it prevents the cells from G1/G0 phase to S phase. Anti-tumor effects were occurred when cell proliferation is uncontrolled. The results suggest that TMP may have an effect on the pathway of gastric cancer genesis. Because the mechanism of cancer occurrence and development is complex and the view above is likely to be a tip of the iceberg, further researches were needed in the future.

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