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Effect of tetramethylpyrazine combined with cisplatin on VEGF, KLF4 and ADAMTS1 in Lewis lung cancer mice

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ABSTRACT

Objective: To further explore the function of combine use of tetramethylpyrazine (TMP) and cisplatin (DDP) in lung carcinoma.
Methods: We used the combination drug to treat Lewis lung cancer mice, investigated the expression level of vascular endothelial growth factor (VEGF), Kruppel-like factor 4 (KLF4) and A disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1) and to further explore the inhibitory effects and potential mechanism of TMP combined with DDP on tumor angiogenesis.
Results: The tumor growth was suppressed in TMP group, DDP group and TMP combined with DDP group. Furthermore, the weights and volume of tumor, the expression level of VEGF, KLF4 and ADAMTS1 were found significantly changed between experiment group and control group. These findings suggest that TMP with DDP had additional or synergistic effects to inhibit the tumor growth effectively, might be achieved through reducing the expression of angiogenesis promoting factor VEGF and increasing expression of angiogenesis inhibitors KLF4 and ADAMTS1.
Conclusion: KLF4 and ADAMTS1 may be synergically involved in the angiogenesis in mouse Lewis lung cancer through the different signal ways.

1. Introduction

Pulmonary carcinoma is one of the most common causes of cancer-related deaths around the world, with a low survival rate in 5 years [1,2]. It is difficult to remove the tumor by surgery, because most of the patients are diagnosed in an advanced stage. The main treatment of lung cancer is cisplatin (DDP)-based chemotherapy, it has produced a significant survival benefit; however, chemotherapeutic drugs result in significant side effects, owing to their lack of specificity. To overcome this problem, more and more researches focus on combination drug therapy [3]. The development and application of Chinese medicine that can reduce the side effects of chemotherapeutic drugs have garnered increased attention [4,5].

Tetramethylpyrazine (TMP), one of the major bioactive components of traditional Chinese medicine Chuanxiong, has been applied in the treatment of cerebral vascular and cardiovascular diseases. Some previous studies have indicated that TMP has been reported to have strong antitumor activities, such as the inhibition of proliferation and the promotion of the apoptosis of tumor cells in various types of cancer [6,7]. Previous study found that TMP might be a potential chemopreventive and therapeutic agent for osteosarcoma [8]. Moreover, some studies reported that combination treatment with TMP enhances sensitivity to DDP and promotes apoptosis in lung cancer [9]. However, little has been reported on the effects of TMP on vascular endothelial cells from the point of view of tumor angiogenesis. By observing the expression of vascular endothelial growth factor (VEGF), Kruppel-like factor 4 (KLF4) and A disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1) in Lewis lung cancer rats before and after the treatment of TMP combined with DDP, and through dynamic observation of weight and tumor growth in different groups of Lewis lung cancer C57BL/6 mice model, the aim of the present study was to investigate the effect of KLF4

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and ADAMTS1 in lung cancer, further to explain sensitization mechanism of TMP to DDP tumor angiogenesis.

2. Materials and methods

2.1. Materials

A total of 40 male C57BL/6 mice were selected, clean level, 6–8 weeks, 18–22 g (from Academy of Military Medical Sciences). Lewis lung cancer cells from Chinese Academy of Sciences. TMP was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, and DDP from Sigma medical Co. (St, Louis; MO, USA). DMEM medium from Gibco Co.; FBS from Zhejiang Sijiqing; rabbit anti-rat VEGF antibody from Wuhan Boshide Co.; rabbit anti-rat CD105, KLF4 and ADAMTS1 antibody from Beijing Boaosen Co. Anti-β-actin was obtained from Jiamei Biotech Technology (Hunan, china). DAB from Wuhan Boster Co.; super clean bench SWCJ-2FD from Suzhou Antai Co.; inverted phase contrast microscope from Japan Olympus Co.; desk centrifuge from Sigma Co.; carbon dioxide incubator HEPA100 from United States; Image-pro plus 6.0 image processing software; Leica-DM2500B microscope from Germany.

2.2. Animal experiments

Mice were adapted to live in the room for 1 week. The models were established by subcutaneously injecting Lewis lung cancer cells of logarithmic growth phase (cell confluence 80%) and cell density was adjusted to 1×10^7/L with saline solution from the right axilla of C57BL/6 mice, per 0.2 mL. After the tumor size up to about 8 mm, 40 tumor-bearing mice were randomly divided into the four groups: the model group (A), DDP group (B), TMP group (C) and TMP combined with DDP group (D), with 10 in each group. Mice in the A group were given with TMP 100 mg/kg, 0.2 mL by intraperitoneal injection, once daily. Mice in the B group were given with DDP 2 mg/kg, 0.2 mL by intraperitoneal injection, once daily. Those in the C group were given with TMP 100 mg/kg by intraperitoneal injection, once daily. Those in the D group were given with the same dose as above by intraperitoneal injection, per 0.2 mL, once daily. Before dosing every time, the dosage was adjusted according to weight. All medication was started from the 7th day of inoculation, lasting 14 successive days. All the mice were sacrificed at the 19th day of the experiment. The tumor blocks were taken out, weighed, and then divided into two parts, one section fixed in 4% paraformaldehyde and another section frozen using liquid nitrogen. All experimental protocols and animal care were performed according to authorization granted by the Chinese Ministry of Agriculture.

2.3. The toxic side reaction of chemotherapy and the life quality of the Lewis lung cancer mice

After the treatment according to the plan, we observed the indicators of mouse's fur color, activity, appetite, response to stimuli and weight changes to evaluate chemotherapy side effects and life quality. The results showed that the C group had no obvious toxicity reaction and life quality was the best, followed by the combined treatment group, again as the control group, while the B group had the heaviest toxic side reaction and the worst life quality. The D group was significantly lower than the toxicity of cisplatin, and life quality was better than the B group, suggesting that combination therapy can reduce the toxic side reaction of cisplatin.

2.4. Calculating weight and volume of tumor

Weight was measured daily after treatment, and the maximum diameter a (mm) and the minimum diameter b (mm) of the tumor blocks were calculated with Vernier caliper. The tumor volume was calculated based on $V = ab^2/2$. Time was taken as the horizontal axis and volume as the vertical axis, which depicted tumor growth curves.

2.5. Calculating tumor necrosis rate

ACUSON1228ST type color ultrasound imaging device was used. The probe frequency was 9 MHz. Tumor size, shape and internal echo were observed, and the situation of the liquefaction necrosis was assessed in the tumor tissue. The rate of tumor necrosis (tumor necrosis rate of a tumor necrosis area/total tumor area × 100) was calculated.

2.6. Immunohistochemistry

After all tumor tissue was removed, fixed, dehydrated, paraffin was embedded and each paraffin-embedded tissue specimen was cut into four 3-μm-thick serial sections. Negative control specimens were prepared as well. For immunohistochemistry (IHC), rabbit anti-rat VEGF antibody polyclonal antibody, rabbit anti-rat CD105, rabbit anti-rat KLF4, and rabbit anti-rat ADAMTS1 polyclonal antibody were used as primary antibodies, and normal goat serum as a negative control. After deparaffinization, hydration and incubation with 3% hydrogen peroxide for 30 min, the slices were immersed into citric acid solution (0.01 mol/L) for antigen retrieval for 20 min at 92–94 °C. Then, the sections were incubated with following primary antibodies (rabbit anti-rat VEGF antibody, rabbit anti-rat CD105 antibody, 1:150 dilution; rabbit anti-ratKLF4, rabbit anti-rat ADAMTS1 antibody 1:100 dilution) overnight at 4 °C. The samples were then incubated with biotinylated rabbit-anti-rat IgG for 20 min at room temperature. After incubation with horseradish peroxidase for 30 min, they were exposed to DAB solution according to the manufacturer's protocol and counterstained with hematoxylin.

2.7. Image processing and analyses

With the microscope Leica camera, Image-pro plus 6.0 software was used for image processing and analysis. The amount of the target area of the target protein stain color shades and the size distribution were determined. The number of positive cells in the target area selected, the greater the positive area; the darker the stain, the more strong positive signal; its integrated optical density (integrated option density, IOD) value must be higher. Finally, the IOD statistics was used.

2.8. Western blot analysis

Whole-tissue lysates were prepared using radioimmunoprecipitation analyses buffer supplemented with protease

and/or phosphatase inhibitors. Tissue proteins were extracted using tissue extraction kit (Applygen Technologies, inc.; Beijing, China) according to the manufacturer's instructions. Protein concentration was determined by BCA Protein kit assay (Hyclone-Pierce, South Logan, U.S.A.). Proteins (100 g/well) were separated by SDS–polyacrylamide gel, transferred to a PVDF membrane (Millipore, Burlington, MA, USA), blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20, transferred to a nitrocellulose, and incubated with monoclonal rabbit anti-rats antibodies against VEGF, ADAMTS1, KLF and β-actin. And subsequently horseradish peroxidase-conjugated secondary antibodies were used. Equivalent loading was confirmed using an antibody against β-actin. Protein bands were visualized by ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) and quantified by densitometry using an electrophoresis image analysis system (FR980, Shanghai Furi Science & Technology, Shanghai, China).

2.9. Statistical analysis

Statistical analysis was performed using SPSS17.0 program (SPSS, USA). The results were analyzed by chi-square test, correction for chi-square test, Fisher probabilities and two-group *t*-test. The measurement data are expressed as mean ± standard deviation (SD). Correlation analysis was analyzed by Pearson correlation, when P value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Tumor weight and tumor inhibition rate of mice from each group

All the mice were sacrificed at the 19th day of the experiment, and tumor blocks were taken out and weighted. The growth of transplanted tumors in the groups B, C, and D were significantly inhibited. The weight of tumor body in the three groups was markedly lower than that of group A. The tumor inhibitory rates were 34.52%, 39.40% and 58.39% in groups B, C and D, respectively. The tumor inhibitory rate of D group was significantly higher than B and C group ($P < 0.01$). The inhibitory effect of TMP combined with DDP on tumor growth was enhanced compared with TMP or DDP alone treatment (Table 1).

3.2. Tumor volume change after treatment

When the Lewis lung cancer cells were inoculated, tumor blocks at inoculation could be touched on the 7th–9th days. And the blocks in each group were increased gradually. After treatment, tumor growth was the lowest in the group D than the other three groups (Figure 1).

Table 1
Tumor weight and tumor inhibition rate of mice in each group ($n = 10$).

Group	n	Tumor weight	Inhibitory rate (%)
A	10	5.26 ± 0.05	0.00
B	10	3.39 ± 0.56[ab]	34.52
C	10	3.31 ± 0.28[ab]	39.40
D	10	1.97 ± 0.07	58.39

[a]$P < 0.01$ *vs* A group; [b]$P < 0.01$ *vs* D group.

3.3. Tumor necrosis rate in each group

Before all mice were sacrificed, the tumor necrosis was observed with color Doppler ultrasound, and the necrosis rate was calculated. The results showed that the D group and B group

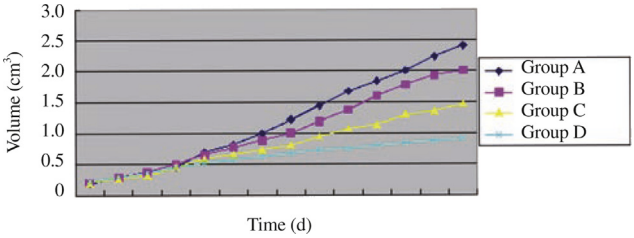

Figure 1. Tumor volume change after treatment.

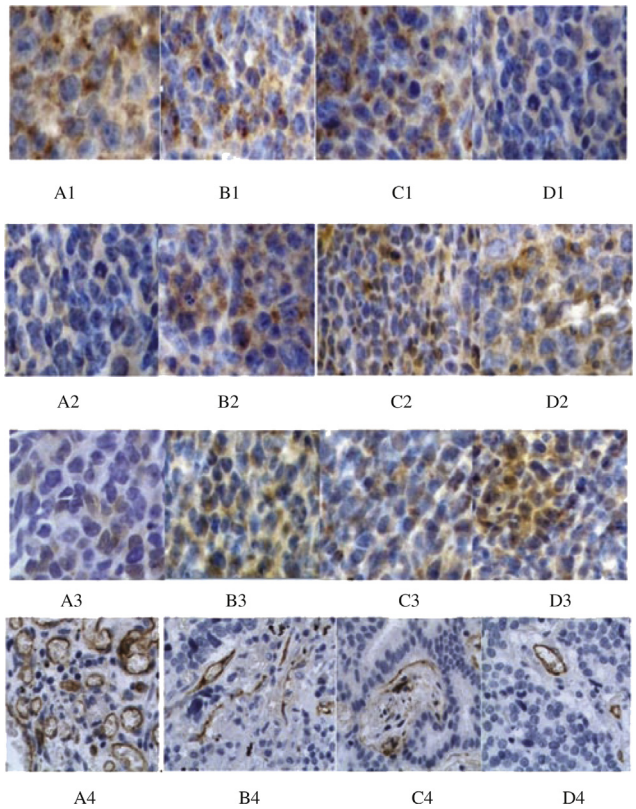

Figure 2. Immunohistochemical staining of VEGF, KLF4, ADAMTS1 and MVD in NSCLC tissues (from the same source, Sp, ×400).
The expression of VEGF in NSCLC tissues (A1) was decreased as compared with the other three groups (B1, C1 and D1). KLF4 and ADAMTS1 in NSCLC tissues (D2 and D3) was highly expressed as compared with that in A, B and C group tissues. A4–D4 indicated the MVD expression in NSCLC tissues.

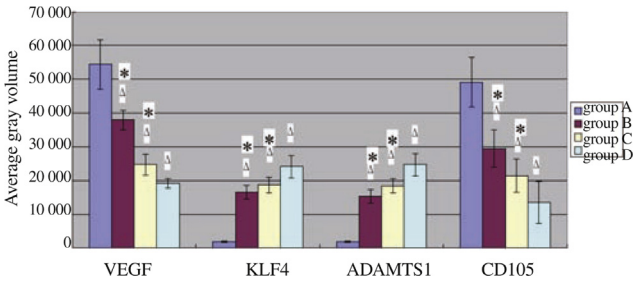

Figure 3. The expression levels of VEGF, KLF4, ADAMTS1, and MVD in Lewis lung cancer tissues.
$^{\Delta}P < 0.05$ *vs* A group; $^{*}P < 0.05$ *vs* D group.

Table 2
Correlation of VEGF, KLF4, ADAMTS1, and MVD expression in NSCLC tissues.

Values	VEGF/MVD	VEGF/KLF4	VEGF/ADAMTS1	MVD/KLF4	MVD/ADAMTS1	KLF4/ADAMTS1
r	0.519	−0.685	−0.573	−0.568	−0.548	0.253
P	0.018*	0.023*	0.009*	0.004*	0.006*	0.120

*$P < 0.05$.

were seen clearly multifocal tumors larger area of necrosis, and the C group and A group only a small area of the tumor necrosis. The tumor necrosis rates in the groups A, B, C, and D were 10.37 ± 1.36, 16.58 ± 3.20, 23.64 ± 11.96, and 30.76 ± 15.20, respectively. The tumor necrosis rate in the D group was significantly higher than that in the C group ($P < 0.05$), but compared with the B group, the difference was not significant ($P > 0.05$).

3.4. Expression of VEGF, KLF4 and ADAMTS1, and microvessel density (MVD) in Lewis lung carcinoma tissues

The cell membrane and cytoplasm were stained brown in cells positive for VEGF and ADAMTS1, and expression of KLF4 was mainly in the cell nucleus, few in the cell cytoplasm, stained brown in cells positive. The expression levels of VEGF in Lewis lung carcinoma tissues of A group were remarkably higher than the other three groups ($P < 0.05$). The results showed that TMP and DDP could decrease VEGF expression, and their combination might further enhance the effect of inhibition. The expression levels of KLF4 and ADAMTS1 in the D group were remarkably higher than the other three groups ($P < 0.05$), and the results demonstrated that TMP and DDP could increase KLF4 and ADAMTS1 expression, and their combination might further enhance their expressions (Figures 2 and 3).

3.5. Correlation of VEGF, KLF4 and ADAMTS1, and MVD expression in Lewis lung cancer tissues

Statistical analysis showed that in nonsmall-cell lung cancer (NSCLC) tissues, VEGF expression was related to KLF4 expression ($r = -0.685$) and ADAMTS1 expression ($r = -0.573$), respectively. No significant relationship was noted between KLF4 expression and ADAMTS1 expression ($r = 0.253$, $P > 0.05$).

Additionally, as statistical analysis demonstrated, the expression levels of VEGF, KLF4 and ADAMTS1 were significantly correlated with the MVD in Lewis lung cancer tissues ($P < 0.05$, Table 2).

Table 3
The expression levels of VEGF, KLF4, ADAMTS1, and MVD in Lewis lung cancer tissues of each group ($n = 10$).

Group	VEGF/β-actin	KLF4/β-actin	ADAMTS1/β-actin
Group A	0.93 ± 0.04	0.38 ± 0.16	0.24 ± 0.03
Group B	0.86 ± 0.12[ab]	0.48 ± 0.04[ab]	0.42 ± 0.16[ab]
Group C	0.55 ± 0.34[ab]	0.69 ± 0.04[ab]	0.53 ± 0.05[ab]
Group D	0.42 ± 0.09[a]	0.84 ± 0.06[a]	0.80 ± 0.12[a]

Data were obtained from three independent experiments and are expressed as the mean ± SD. [a]$P < 0.05$ versus the group A, and [b]$P < 0.05$ versus the group D.

3.6. Expression of VEGF, KLF4, ADAMTS1, and MVD-CD105 in Lewis lung cancer tissues

To investigate the mechanism of TMP combined with DDP on tumor angiogenesis, the protein expression of VEGF, KLF4 and ADAMTS1 was determined by Western blot analysis. The results demonstrated that VEGF, KLF4 and ADAMTS1 were expressed in each group. TMP combined with DDP upregulated the protein expression of KLF4 and ADAMTS1, while downregulated the protein expression of VEGF (Table 3), which is consistent with the previous results of immunohistochemistry.

4. Discussion

Angiogenesis, the formation of new blood vessels from preexisting vascular network, plays the key role in the occurrence, progression and metastasis of tumors by supplying sufficient oxygen and nutrients required for tumor growth and metastasis. Specifically, angiogenesis is an important mediator of NSCLC progression, so anti-angiogenic therapy has been proven to be beneficial to patients with NSCLC, and become a research hot spot. TMP is one of the most important active ingredients of the Chinese medicine Ligusticum Chuanxiong, which is used for the treatment of many kinds of cancer. So we chose Chinese medicine angiogenesis inhibitory-TMP in combination with DDP to effect on Lewis lung cancer bearing-tumor mice.

In this study, the experiment showed that the tumor volume of TMP/DDP (group D) was obviously smaller than DDP (group C), but the weight slowly declined in ED/DDP (group D). And we evaluated chemotherapy side effects and life quality of the bearing-tumor mice after the treatment. The results showed that the toxic side reaction and life quality of TMP/DDP (group D) was significantly better than those of DDP (group C), which suggests that TMP could enhance treatment effect, decrease side effects and improve life quality of mouse. In addition, we also found that inhibition rate of mice in TMP/DDP group was significantly higher than that of TMP group and DDP group, suggesting that angiogenesis inhibitor drugs in combination with chemotherapy treatment is better than angiogenesis inhibitor and DDP treatment alone. Using the color Doppler ultrasound observed, the experiment revealed that TMP/DDP group had multifocal large necrotic areas, and DDP group only had a small area of tumor necrosis. Tumor necrosis rate in combined treatment group was significantly higher than the cisplatin group. It might precisely because angiogenesis inhibition and further to inhibit tumor growth and increased tumor-induced ischemia and hypoxia. Based on the above results, it suggested that

combination therapy compared with DDP therapy alone have a stronger antitumor effect.

It is well known that VEGF, an important factor in angiogenesis, plays a crucial role in regulating angiogenesis and has become an essential step in anti-angiogenic therapy for the growth and metastasis of cancer [10,11]. MVD, as an indicator of evaluating angiogenesis, is closely related to the tumor recurrence, metastasis and prognosis. In our study, the tumor growth was significantly suppressed, and the expression levels of VEGF and MVD were significantly lower in TMP/DDP group than in DPP group and TMP group, maybe because combined application of TMP and DDP has stronger inhibition on tumor growth than single use. But the exact mechanism is not clear, and studies have shown that it may be caused by the "normalization" of tumor vessels after anti-angiogenesis therapy. The experiment also found that the VEGF and MVD expression was significant weaker in group B, group C and group D than group A, and their expression showed positive correlation ($r = 0.519$, $P < 0.05$). It revealed that TMP could inhibit the growth and microvascular generation in Lewis lung cancer *in vivo*. Wang *et al.* showed that the expression level of VEGF and MVD count levels were positively correlated. The experiment also proves this point [12].

In the present study we investigated the protein expression of ADAMTS1 and KLF4 in different group tissues, as well as their correlation with angiogenesis. To date, this current study is the first report to examine ADAMTS1and KLF4 expression levels in Lewis lung cancer mice. ADAMTS1 is a recently discovered metalloproteinase with antiangiogenic activity. It has been reported that ADAMTS1 is a potent inhibitor of angiogenesis and can inhibit VEGF-induced angiogenesis [13]. Then upregulation of ADAMTS1 may lead to lower levels of bioavailable VEGF by this mechanism, thereby decreasing angiogenesis. ADAMTS-1 through direct binding angiogenic growth factor (VEGF165) to form a complex, then affect the binding of VEGF to the receptor and inhibit VEGF165 to stimulate vascular endothelial growth factor receptor 2 (VEGFR-2) phosphorylation and activation signals, inhibition of endothelial cell proliferation to inhibit angiogenesis. The transcription factor KLF4 is a critical transcription factor that is associated with both tumor suppression and oncogenesis [14], and it has been shown to function as a tumor suppressor gene based on its inhibition of cell proliferation [15]. The experiment found that the ADAMTS1 and KLF4 expression were the strongest in group D. Their expression had negative correlation with the VEGF and MVD expression. In addition, we also found that the expression level of KLF4 was higher than that of ADAMTS1, but their expression was no significant correlation, so we speculate that KLF4 and ADAMTS1 may be synergically involved in the angiogenesis in mouse Lewis lung cancer through the different signal ways. The target of DDP was tumor cells, and it could directly kill tumor cells, reduce tumor cells to secrete VEGF. The target of TMP was vascular endothelial cell [16], and it could interrupt the activity of NF-κB p65, BCL-2, cyclinD1 [17] and activity of endothelial cell selection element, indirectly inhibit VEGF secretion. Then enhanced ADAMTS1 and KLF4 bind VEGF or VEGFR2 on the surface of endothelial cells. As a result, inhibition of new vessels growth leads to reduction of pathological blood vessels and tumor regression. They could inhibit tumor growth at the same time. This could also explain sensitization mechanism of TMP to DDP.

In conclusion, TMP combined with DDP can improve the anti-tumor effect, reduce the toxicity of chemotherapy, and enhance the sensitization of TMP to DDP, but the sensitization mechanism was not clear. The anti-tumor mechanism of TMP combined with DDP might be through downregulating the expression of VEGF, while the expression of ADAMTS1 and KLF4 is regulated to reduce the angiogenesis. The results showed that TMP reaches sensitization by influencing the angiogenesis correlation factor, but the specific mechanism and the key targets still need to be further discussed. It provides experimental basis for widely clinical application.

Conflicts of interest statement

The authors declare that they have no conflicts of interest.

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References

[1] Jemal A, Center MM, DeSantis C, Ward EM. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 1893-1907.
[2] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90.
[3] Ren Z, Wang Y, Jiang W, Dai W, Jiang Y. Anti-tumor effect of a novel soluble recombinant human endostatin: administered as a single agent or in combination with chemotherapy agents in mouse tumor models. *PLoS One* 2014; **9**(9): e107823.
[4] Li L, Huang T, Jia A, Hou HL, Pang S, Wang YZ. Effects of ligustrazine combined with cis-dichlorodiamine platinum on anti-proliferation, cell cycle and apoptosis of human gastric carcinoma SGC-7901 cell lines. *Agric Sci Technol* 2014; **15**(9): 1482.
[5] Qi F, Zhao L, Zhou A, Zhang B, Li A, Wang Z, et al. The advantages of using traditional Chinese medicine as an adjunctive therapy in the whole course of cancer treatment instead of only terminal stage of cancer. *Biosci Trends* 2015; **9**(1): 16-34.
[6] Zheng CY, Xiao W, Zhu MX, Liu Y, Pan XJ, Yang ZH, et al. Inhibition of cyclooxygenase-2 by tetramethylpyrazine and its effect on A549 cell invasion and metastasis. *Int J Oncol* 2012; **40**: 2029-2037.
[7] Zhang F, Kong DS, Zhang ZL, Lei N, Zhang XJ, Zhang XP, et al. Tetramethylpyrazine induces G0/G1 cell cycle arrest and stimulates mitochondrial-mediated and caspase-dependent apoptosis through modulating ERK/p53 signaling in hepatic stellate cells *in vitro*. *Apoptosis* 2013; **18**: 135-149.
[8] Wang Y, Fu Q, Zhao W. Tetramethylpyrazine inhibits osteosarcoma cell proliferation via downregulation of NF-κB *in vitro* and *in vivo*. *Mol Med Rep* 2013; **7**: 984-988.
[9] Zhang J, Cao PG, Fu HQ. Experimental research on TMP combination with DDP in treating Lewis lung carcinoma. *J Clin Res* 2006; **23**(8): 1249-1251.
[10] Feng P, Zhang ZL, Zhang ZH, Zhang XL, Xiang F, Tang JH, et al. Effect of endostar combined with cisplatin on expression of VEGF and Sema3A of Lewis lung cancer rats. *Asian Pac J Trop Med* 2013; **6**(1): 57-60.
[11] Lu QL, Liu J, Zhu XL, Xu WJ. Expression of nerve growth factor and hypoxia inducible factor-1α and its correlation with angiogenesis in non-small cell lung cancer. *J Huazhong Univ Sci Technol Med Sci* 2014; **34**(3): 359-362.
[12] Wang J, Bao YX, Geng Y. Effect of dexamethasone on the growth and angiogenesis of transplanted Lewis lung carcinoma in mice. *China Oncol* 2012; **22**(1): 5-9.

[13] Luque A, Carpizo DR, Iruela-Arispe ML. ADAMTS1/METH1 inhibits endothelial cell proliferation by direct binding and sequestration of VEGF165. *J Biol Chem* 2003; **278**: 23656-23665.

[14] Evans PM, Liu C. Roles of Krupel-like factor 4 in normal homeostasis, cancer and stem cells. *Acta Biochim Biophys Sin* 2008; **7**: 554-564.

[15] Hsu LS, Chan CP, Chen CJ, Lin SH, Lai MT, Hsu JD, et al. Decreased Kruppel-like factor 4 (KLF4) expression may correlate with poor survival in gastric adenocarcinoma. *Med Oncol* 2013; **30**(4). 632–626.

[16] Chen L, Lu Y, Wu JM, Xu B, Zhang LJ, Gao M, et al. Ligustrazine inhibits B16F10 melanoma metastasis and suppresses angiogenesis induced by Vascular Endothelial Growth Factor. *Biochem Biophys Res Commum* 2009; **386**(2): 374-379.

[17] Wang XY, Ma ZC, Wang YG, Tan HL, Xiao CR, Liang QD, et al. Tetramethylpyrazine protects lymphocytes from radiation-induced apoptosis through nuclear factor-κB. *Chin J Nat Med* 2014; **12**(10): 730-737.