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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