RESEARCH ARTICLE

Inhibitory Effects of Phenolic Alkaloids of Menispermum Dauricum on Gastric Cancer *in Vivo*

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Abstract

The present study was conducted to investigate effects and mechanisms of action of phenolic alkaloids of *Menispermum dauricum* (PAMD) on gastric cancer *in vivo*. *In vitro,* cell apoptosis of human gastric cancer cell line SGC-7901 was observed using fluorescence staining. *In vivo*, a mice model was constructed to observe tumor growth with different doses. Cell apoptosis was examined using flow cytometry and K-RAS protein expression using Western blotting. The mRNA expression of P53, BCL-2, BAX, CASPASE-3, K-RAS was examined by real-time PCR. PAMD significantly suppressed tumor growth in the xenograft model of gastric cancer in a dose-dependent manner ($p<0.01$). Functionally, PAMD promoted cell apoptosis of the SGC-7901 cells and significantly increased the rate of cell apoptosis of gastric tumor cells ($p<0.05$). Mechanically, PAMD inhibited the expression of oncogenic K-RAS both at the mRNA and protein levels. In addition, PAMD affected the mRNA expression of the cell apoptosis-related genes (P53, BCL-2, BAX, CASPASE-3). PAMD could suppress gastric tumor growth *in vivo*, possibly through inhibiting oncogenic K-RAS, and induce cell apoptosis possibly by targeting the cell apoptosis-related genes of P53, BCL-2, BAX, CASPASE-3.

Keywords: Gastric cancer - cell apoptosis - *in vivo* - K-RAS - phenolic alkaloids of menispermumdauricum

Asian Pac J Cancer Prev, **15 (24)**, 10825-10830

Introduction

Gastric cancer is regarded as the fourth most common cancer around the world, which is also the second most common cause of cancer-related death (Ferlay et al., 2014). Thus, gastric cancer is still an international public healthcare problem. It has been reported that there are many risk factors for gastric cancer, such as Helicobacter pylori infection (Li et al., 2014), dietary habits (Kweon et al., 2013), tobacco (Guggenheim et al., 2013), obesity (Bickenbach et al., 2013) and so on. The overall incidence of gastric cancer in recent years presents the declining trend, but the mortality rate remains high due to its poor prognosis (Crew et al., 2006).

More recent attractions have been paid to the traditional Chinese medicine due to the advantage of proven safety in the prevention and treatment of cancer. Phenolic alkaloids of menispermum dauricum (PAMD) are a mixture of fat-soluble alkaloid extract isolated from dried roots of menispermum dauricum, and the main active components have been demonstrated to be the dauricine and daurisoline (Shan et al., 2006). Initially, this Chinese herb was widely used in some inflammatory and arrhythmic diseases (Fu 1991; Jia-Qing 2002; Yang et al., 2007). Recently, many studies have demonstrated that dauricine has potential anti-cancer effects in many tumors, through suppression of tumor growth, cell invasion and angiogenesis, induction of cell apoptosis, and prevention of multidrug resistance (He et al., 2002; Yang et al., 2010; Wang et al., 2012). As far as we know, the study of the effect and mechanism of PAMD on gastric cancer remains few.

Cell apoptosis is the process of programmed cell death, whose disturbance has been demonstrated to be involved in the process of tumor growth and development (Evan et al., 2001). It has been reported that dauricine exerts its biological effects by introducing cell apoptosis. Li et al have reported that dauricine may induce apoptosis in cultured human bronchial epithelial cells and in lungs of CD-1 mice by targeting CYP3A (Jin et al., 2012). Yang et al have demonstrated that dauricine could induce apoptosis by targeting NF-ϰB signaling pathway in colon cancer cells (Yang et al., 2010). However, the study of the mechanism of PAMD on cell apoptosis in gastric cancer is still rare.

K-RAS has been known as an oncogene, whose mutations are found in many cancers, such as colon cancer, pancreatic cancer and lung cancer (Pellegata et al., 1994; Johnson et al., 2001; Karapetis et al., 2008). It has also been detected in patients with gastric cancer, which may be involved in the carcinogenesis of gastric cancer (Hiyama et al., 2002; Okumura et al., 2010). In addition, KRAS mutation in gastric cancer has been reported to be related to DNA mismatch repair deficiency (Van Grieken et al., 2013). Neverthless, the effects of PAMD on expression

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of K-RAS in gastric cancer has been poorly understood until now.

In order to investigate the effect and mechanism of PAMD on gastric cancer, a mice gastric model was constructed to test whether PAMD could inhibit tumor growth *in vivo* in gastric cancer. Meanwhile, we also examined the effect of PAMD on cell apoptosis *in vitro* and *in vivo* using fluorescence staining and cell apoptosis kit, respectively. Furthermore, cell apoptosis-related genes (P53, BCL-2, BAX, CASPASE-3) were examined in our study using the method of real-time PCR. In addition, we also tested the expression of K-RAS in the mRNA and protein levels after PAMD treatment.

Materials and Methods

Main reagents

PAMD was provided by Prof Dong Wang from Heilongjiang University of Traditional Chinese Medicine (TCM). PAMD was dissolved in PBS with a certain amount of dimethyl sulphoxide (DMSO, Sigma Chemical Company, St. Louis, Missouri, USA) to make a solution of 400μg/ml for later use.

Cell culture

Human gastric cancer cell line SGC-7901 was purchased from Keygen Biology Co. Ltd (Nanjing, Jiangsu, China). Cells of SGC-7901 were cultured in RPMI 1640 medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Sijiqing Bio-engineering Material Institute, Hangzhou, Zhejiang, China). All cells were cultured in a humidified incubator in an atmosphere of 5% CO_2 at 37°C.

All animal experiments performed throughout the present study were approved by the Animal Research Ethics Committees of Affiliated Tumor Hospital of Harbin Medical University.

Fluorescence staining in SGC-7901 cell using Hoechest and PI

Cells of SGC-7901 were seeded at 1×10^5/ml with a volume of 2.5 ml per well in the 6-well plates. After 24h, they were treated with different concentrations of PAMD (20 mg/L as high dose, 10 mg/L as medial dose, 5 mg/L as low dose). The cells were also treated with 5-fluorouracil (5-FU, Pharmacia Inc., Kalamazoo, Michigan, USA) at the concentration of 50 μg/L, which was defined as positive control group, while the blank group was treated with the same volume of RPMI 1640 medium. After 24 h, the cells in the above groups were stained with Hoechest 33342 (Sigma-Aldrich, St Louis, Missouri, USA) at the concentration of 5 ug/mL for 10 min. Then they were stained with PI at the concentration of 40 ug/mL for 10 min. After that, all the cells were washed with PBS and photographed using fluorescence microscopy.

Xenograft model of gastric neoplasm

Nude mice were sourced from Good Laboratory Practice (GLP) experiment center of Heilongjiang University of Traditional Chinese Medicine (TCM), bred in Heilongjiang University of TCM drug safety evaluation center followed the standard specific pathogen-free breeding protocol. There were a total of 60 nude mice with a mean weight of 20±2 g, and the ratio of male to female was 1:1. All the mice were randomly divided into six groups: the PAMD experimental groups (high, medial and low dose group), model control group, positive control group, and the blank control group. A number of 1×10^6 SGC-7901 cells was i.*p*. injected into the mice from the PAMD experimental groups (high, medial and low dose group), model control group, and positive control group, while the other 10 nude mice from the blank control group did not receive tumor cell injection. After 24 h, high, medial and low dose group were given the PAMD 20 mg/kg, 10 mg/kg, 5 mg/kg, respectively. The positive control group was given 5-FU for 20 mg/kg once a week. Model control group and blank control group were given the same volume normal saline. All the above administrations in different groups except the positive control group were given for 21 d. On the next day after the last final administration, the tumor weight of these mice was recorded. The tumor inhibition rate was calculated according to the following formula.

Tumor inhibition rate (%)= (The average tumor weight in model control group -the average tumor weight in experimental group) / the average tumor weight in model control group.

Cell apoptosis test

The tumor tissues in different groups were made into single cell suspension liquid using the net rubbing method. Then a total of 2×10^6 cells each time were stained with PI. At last, the results were analyzed using a flow cytometer (Becton Dickinson, San Jose, California, USA).

RNA extraction

Total RNA from the tumor tissues was extracted using the TRizol reagent (Invitrogen, Carlsbad, CA) according to the provided protocol. RNA quantity and quality were determined by spectrophotometry at 260 nm and agarose gel electrophoresis.

Reverse transcription and Quantitative Real-Time PCR (RT-qPCR)

Reverse transcription for mRNAs was performed using a PrimeScript Reverse Transcription System (Takara, Shiga, Japan). In order to detect mRNA, 500 ng RNA was reverse-transcribed according to the manufacturer's protocol and amplified by RT-qPCR the specific primers, which were shown in Table 1. β-actin was used as an internal control to normalize for differences among input RNAs. The PCR procedures were as follows: 1 cycle at 95°C for 10 sec, 40 cycles at 95°C for 5 sec, and 60°C for 34 sec. Amplication was analyzed using $\triangle\triangle$Ct method (Livak et al., 2001). All the experiments were repeated three times over multiple days.

Western blotting

Total protein lysates were extracted from ground-up tumor tissues. Protein concentrations of individual samples were assessed using a standard BCA assay (Harbin Saituo Biotechnology, Heilongjiang, China). For each sample,

Table 1. PAMD Suppresses Gastric tumor Growth *in vivo*

Group	Dosage (mg/kg)	Weight before the experiment (g)	Weight after the experiment (g)	Tumor weight (g)	Tumor inhibition rate (%)
High dose	20	20.55±0.73	23.35±0.74	0.51±0.12**	37.88
Medial dose	10	20.32±0.66	23.00±0.81	0.52±0.10**	36.86
Low dose	5	20.07±0.66	23.63±1.06	0.52±0.07**	36.46
Positive control	20	20.56±0.73	17.33±1.38**	0.38±0.07**	53.16
Model control	-	20.68±0.97	23.12±1.09▲▲	0.82±0.12	-
Blank control	-	20.75±0.69	25.26±0.94	-	-

*Each dosage group compared with model control group **$p<0.01$; Model control group compared with blank control group ▲▲$p<0.01$

Table 2. The Primes of the Genes Used for Real-Time PCR

Genes	Forward	Reverse
P53	5`-TGTCCCCGGACGATATTGA-3`	5`-TGGCATTCTGGGAGCTTCAT-3`
BCL-2	5`-GCCCCAAAAGGAGAAGAACAT-3`	5`-ACGACCCGATGGCCATAGA-3`
BAX	5`-TGGAGCTGCAGAGGATGATTG-3`	5`-TTGCCGTCAGAAAACATGTCA-3`
CASPASE3	5`-CAGTGGAGGCCGACTTCTTG-3`	5`-ATGAACCAGGAGCCATCCTTT-3`
K-RAS	5`-ACTGAATATAAACCTTGTGGTAG-3`	5`- TCAAAGAATGGTCCTGGACC-3`
β-actin	5`-CGTGGACATCCGCAAAGAC-3`	5`-TGGCATTCTGGGAGCTTCAT-3`

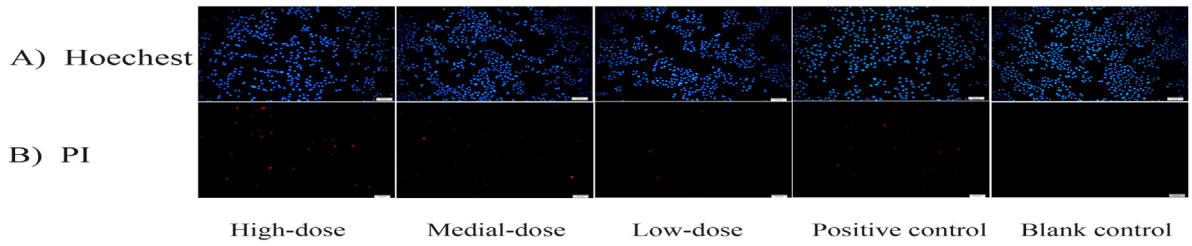

Figure 1. PAMD Induced Cell Apoptosis of SGC-7901 Cells using Fluorescence Staining after Treated with Different Doses. (A) The fluorescence results stained with Hoechest; **(B)** The fluorescence results stained with PI

50μg protein was separated on a 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and blocked in 5% milk in 0.1% Tris-buffered saline-Tween 20 (TBST) at room temperature for 1.5 hours. After washing with TBST for three times, the membranes were incubated at 4°C at the appropriate dilution with the following primary antibodies: rabbit anti mouse K-ras monoclonal antibody (Santa Cruz Biotechnology Inc.,California, USA) at dilution of 1:200, and rabbit anti mouse β-actin monoclonal antibody (Xiangsheng Biotechnology Inc., Shanghai,China) at dilution of 1:200. Then the membranes were washed with 1×TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Harbin Saituo Biotechnology Inc., Heilongjiang, China). The immunoreactive bands were visualized by chemiluminescence and detected by an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK). All the experiments were repeated three times over multiple days.

Analysis of tumor morphology using transmission electron microscopy

The marginal tumor tissues were selected and fixed in 2.5% glutaraldehyde at 4°C for 2 h. Then the tumor mass was washed with 0.1mol/L phosphate buffer. After that, the samples were fixed again in 1% osmic acid for 1.5 h and then dehydrated, penetrated and embedded. Then the samples were sliced using ultra-thin slicing machine and dually stained with uranium acetate and lead citrate. Finally, the treated tissues were observed using transmission electron microscopy.

Statistical analysis

Data were presented as the mean±standard deviation (SD) from at least three separate experiments, and significant difference was analyzed using one-way Analysis of Variance (ANOVA). A p-value<0.05 was considered statistically significant. All the analyses were carried out with the SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

PAMD inhibits gastric tumor growth in a mouse model

To confirm whether PAMD could suppress gastric cancer growth, we examined the effects of PAMD on the growth of xenograft gastric tumor in nude mice. As shown in Table 2, it was found that PAMD could suppress gastric tumor growth significantly compared with the model control group. The tumor inhibition rates in the groups of PAMD high-dose, middle-dose, low-dose were 34.76%, 32.38%, 31.00%, respectively. In addition, our results also indicated that PAMD inhibited gastric tumor growth in a dose-dependent manner *in vivo*.

PAMD induces cell apoptosis in vitro and in vivo

To investigate whether PAMD affected cell apoptosis, which attributes greatly to the suppression of gastric tumor growth, the SGC-7901 cells were stained with Hoechest and PI after treated with different doses of PAMD. In addition, the cell apoptosis from the tumor tissues in different groups were also examined using flow cytometer. As shown in Figure 1, the fluorescence staining outcomes

showed that more apoptotic cells occurred in the cells treated with different doses of PAMD when compared with that in the model group, suggesting that PAMD could induce cell apoptosis in the gastric cells. In addition, it was also found that there were the most apoptotic cells in the high dose PAMD group, while there were the fewest apoptotic cells in the low-dose PAMD group, indicating that the PAMD may induce the cell apoptosis of cells from the tumor tissues in a dose-dependent manner. As shown in Figure 2, it was also found that the cell apoptosis rates in the high dose and medial dose were significantly higher than that in the model control group. All the above results suggested that PAMD could promote cell apoptosis *in vitro* and *in vivo*.

PAMD promotes the expression of apoptosis-related genes in the mRNA level in vivo

To confirm the effect of PAMD on cell apoptosis, we examined whether PAMD affect the expressions of apoptosis-related genes (P53, BCL-2, BAX, CASPASE-3) using the method of RT-qPCR. As shown in Figure 3, our results showed that the mRNA expression level of P53 was up-regulated in the PAMD experimental groups with different doses when compared with that in the model control group, but with no significant difference.

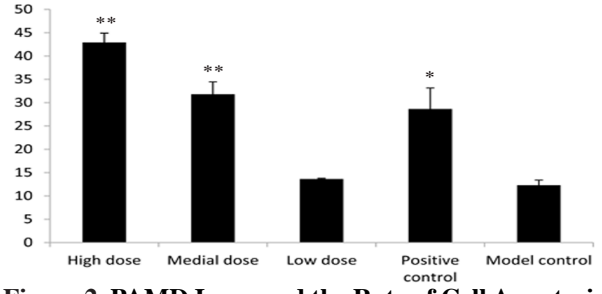

Figure 2. PAMD Increased the Rate of Cell Apoptosis *in vivo*. ** indicates $p<0.01$

The expression of BCL-2 was lower in the PAMD experimental groups with different doses than that in the model control group, without significant difference. The expressions of BAX in the PAMD medial and low dose groups were significantly higher than that in the model control group. The expression of CASPASE3 was significantly higher in the PAMD experimental groups with different doses than that in the model control group. The above results suggested that PAMD may promote cell apoptosis by targeting the apoptosis-related genes in gastric tumor.

PAMD down-regulates the expression of K-RAS in vivo

To further understand the molecular mechanism how PAMD inhibit the tumor growth *in vivo*, we tested whether PAMD affect the expression of K-RAS using the methods of RT-qPCR and western blotting. Our results showed that the expression of K-RAS was down-regulated both in mRNA and protein level in the PAMD experimental groups compared to that in the model control group (Figure 4). The results indicated that PAMD may suppress gastric tumor growth by targeting K-RAS.

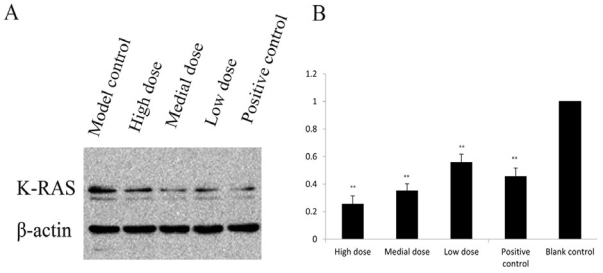

Figure 4. PAMD Suppressed the Expression of Oncogenic K-RAS. (A) The protein expression of K-RAS after treated with different doses of PAMD using western blotting; **(B)** The mRNA expression of K-RAS after treated with different doses of PAMD compared with the blank control group using real-time PCR. ** indicates $p<0.01$

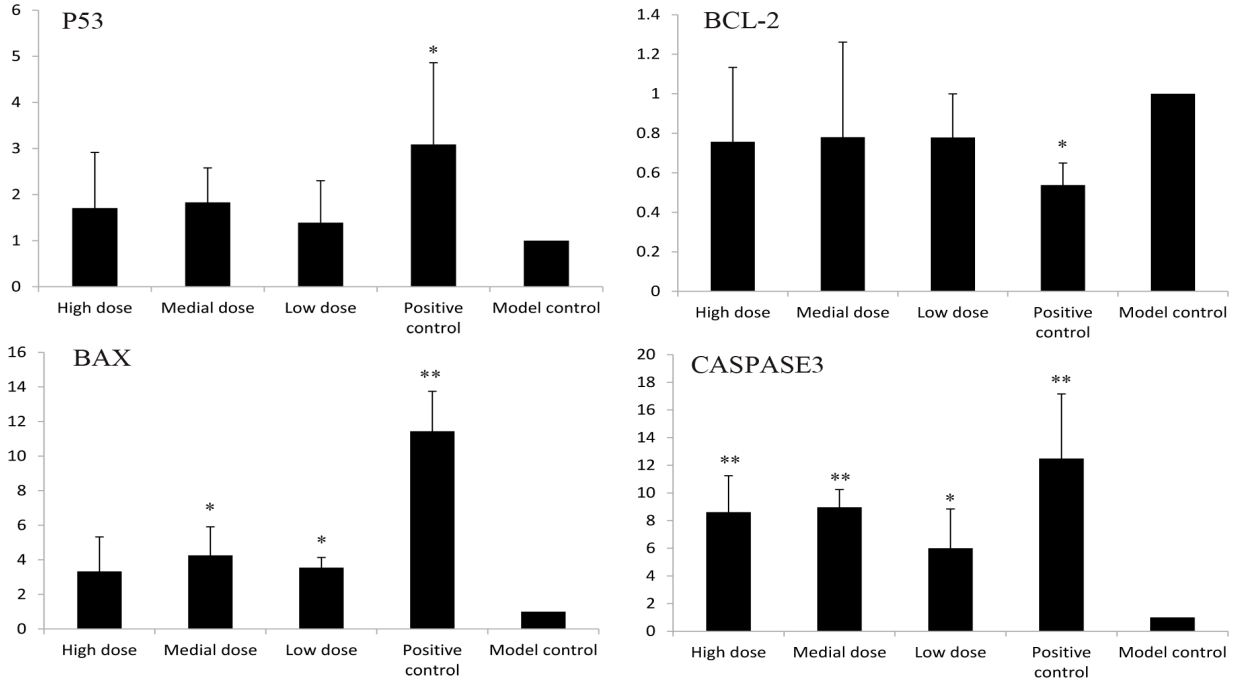

Figure 3. The Effect of PAMD with Different Doses on the mRNA Levels of P53, BCL-2, BAX, CASPASE-3. *indicates $p<0.05$; ** indicates $p<0.01$

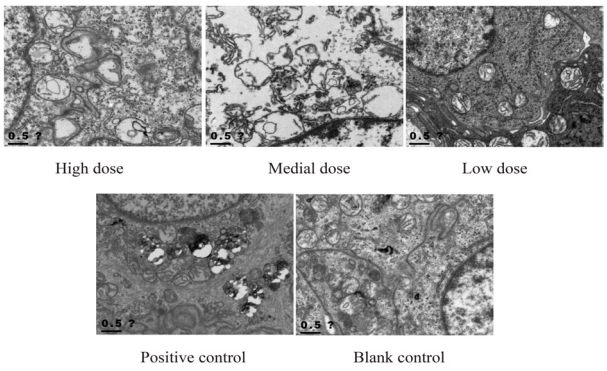

Figure 5. The Tumor Morphology Change of Cells Treated with Different Doses of PAMD Using Transmission Electron Microsco

The effect of PAMD on the tumor morphology using transmission electron microscopy

As shown in Figure 5, results obtained by transmission electron microscopy showed that in the PAMD high dose group, the cell mitochondria vacuoles degenerated, the heterochromatin agglutinated slightly; in the medial dose group, the intracellular organelles decreased significantly, nuclear swell, and the heterochromatin agglutinated in the blocks; in the low dose group, the tumor cells occurred with the liquefied lesions; in the positive control group, the tumor cells narrowed significantly, nucleolus was rare, the volume decreased and the chromatin structure showed turbidity; in the model control group, the mitochondrial of some tumor cells became larger, some of which swell, and the membrane was integral.

Discussion

The outcomes for tumor treatment is to improve life quality, prolong patient survival and reduce adverse reactions, thus exploring antitumor drugs with low toxicity. It has been demonstrated that PAMD exerts its beneficial pharmacological effects not only in the benign diseases, but also in cancers. In our study, it was found that PAMD could inhibit gastric tumor growth *in vivo*. It was also reported that PAMD could promote cell apoptosis *in vitro* and *in vivo*. Furthermore, our real-time PCR results showed that PAMD up-regulated the mRNA expression of some cell apoptosis-related genes (P53, BCL-2, BAX, CASPASE3). In addition, our results also showed that PAMD down-regulated the expression of K-RAS both in the mRNA and protein levels using real-time RCR and western blotting, respectively.

The anti-tumor effects of PAMD have been reported before. Laboratory experiments showed that PAMD could inhibit the proliferations of human tumor cells such as PC-3 and BT5637 (Su et al., 2007). In addition, PAMD also showed an inhibitory effects on mice-transplanted pancreatic cancer BXPC-3 cells through suppressing the expression of K-RAS mRNA (Bian et al., 2010). The main active components of PAMD have been reported to be the dauricine and daurisoline. Yang et al have demonstrated that dauricine could inhibit colon cancer growth both *in vitro* and *in vivo* (YangLi et al. 2010). Another study by Wang et al has also showed that dauricine could inhibit proliferation of urinary tract tumor cells (Wang et al. 2012). In accordance with the above results, our results showed that PAMD, whose main effective component is daucirine, could also significantly suppress the gastric tumor growth when compared with that the model group (Table 1). Besides, it was also found that PAMD may inhibit gastric tumor growth in a dose-dependent manner *in vivo*. Furthermore, the study by Yang et al found that daucirine may have a suppressive effect on NF-κB signaling pathway, resulting in the inhibition of colon cancer growth (Yang et al. 2010). In contrast, our results indicated that PAMD could down-regulate the expression of oncogenic K-RAS both in the mRNA and protein levels, suggesting that PAMD may inhibit gastric tumor growth by targeting K-RAS (Figure 4).

Cell apoptosis contributes greatly to the cell proliferation. Jin et al has reported that dauricine could induce CYP3A-mediated apoptosis in cultured human bronchial epithelial cells and in lungs of CD-1 mice (Jin et al. 2012). Yang et al also have demonstrated that dauricine could induce cell apoptosis by inhibiting NF-κB signaling pathway in colon cancer (Yang et al. 2010). Similar to the above results, our results also showed that PAMD could promote cell apoptosis both *in vivo* and *in vitro* (Figure 1 and 2). P53 gene plays an important role in regulating cell apoptosis when cooperated with a variety of growth factors and oncogene (Wu et al., 1994; Flores et al., 2002). In addition, it has been reported that tumor suppressor p53 is a regulator of BCL-2 and BAX gene expression *in vitro* and *in vivo* (Miyashita et al., 1994). In our study, it was found that PAMD affected the expressions of some cell apoptosis-related genes in the mRNA levels (Figure 3), explaining the possible reasons for the effect of PAMD on cell apoptosis in gastric cancer.

Cancer is a major public health problem in the whole world. Numerous researchers have devoted themselves to exploring the pathogenesis of cancer and attempting to seek effective therapies against various cancers including gastric carcinoma. Multi-factors including abnormal expression, aberrant methylation and single nucleotide polymorphisms (SNPs) of genes that associated with cell proliferation, cell differentiation and other key cell processes have been identified to be related to tumorigenesis, in addition, small regulators such as microRNA also have been shown to play an important role in cancers (Guo et al., 2012; Jing et al., 2012; Chen et al., 2013; Gao et al., 2013; Yao et al., 2013; Zhu et al., 2013). Based on these advances of mechanisms, treatments against cancer are no longer confined to traditional chemotherapy. Nowadays, molecular targeted therapies developed rapidly. Chinese traditional medicine with abundant theoretical foundations and widely proven safety has been applied in more and more cancer researches. Several Chinese traditional medicines or their bioactive constituents including Scutellaria barbata D. Don (S. barbata), vitexicarpin and β-asarone have been shown to inhibit experimental tumorigenesis (Meng et al., 2012; Zou et al., 2012). In our study, we also demonstrated that PAMD, a Chinese traditional medicine, had an anti-tumor effect on mice with gastric cancer. However there are several difficulties on identifying the definite

bioactive constituents and the molecular targets of Chinese traditional medicine, therefore the ability for clearing local lesions is relatively poor. We do suppose that the key improvement for the development of Chinese traditional medicine against cancer may be the confirmation of the bioactive constituents. Thus further advances would be achieved on therapies of Chinese traditional medicine against cancers by identifying the molecular targets as well as improving the formulations.

In conclusion, it was found that PAMD could suppress gastric tumor growth *in vivo*, possibly by suppressing the expression of oncogenic K-RAS. In addition, PAMD could induce cell apoptosis in gastric cancer, possibly by targeting the cell apoptosis-related genes.

Acknowledgements

This study was supported by the Key Research of Science and Technology Plan Projects of Heilongjiang Province (No.GC09C405-3) and Heilongjiang Postdoctoral Grant (No.LBH-Z09007). We wish to express our warm thanks to Fenghe (Shanghai) Information Technology Co., Ltd. Their ideas and help gave a valuable added dimension to our research.

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