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*Full Length Research Paper*

# Inhibitory effect of oxymatrine on the proliferation and vascular endothelial growth factor A (VEGF-A) expression in human lung adenocarcinoma (SPC-A-1) cell

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**The aims of this study were to investigate the effect of oxymatrine (OMA) on human lung adenocarcinoma (SPC-A-1) as well as to explore the underlying mechanism. The inhibitory effects of OMA on the growth of SPC-A-1 cells were tested by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, apoptosis assay and the expressions of vascular endothelial growth factor A (VEGF-A). OMA with different concentrations had significant inhibitory effects on the growth of SPC-A-1 and could also induce apoptosis. In addition, real time-polymerase chain reaction (RT-PCR) analysis indicated that OMA dramatically reduced the secretion of VEGF dose-dependently. The results suggested that OMA might have the therapeutic application in the treatment of human lung adenocarcinoma cell lung cancer.**

**Key words:** Oxymatrine, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), real time-polymerase chain reaction (RT-PCR), vascular endothelial growth factor A (VEGF-A), human lung adenocarcinoma (SPC-A-1).

## INTRODUCTION

Lung cancer is one of the most lethal tumors, causing nearly 1.4 million deaths each year, and lung adeno-carcinoma is one of the most common types which accounts for about 40% of the total cases (Rothschild and Gautschi, 2012). High morbidity is closely related to smoking habit and life style. Currently, the therapies for lung cancer mainly focus on chemotherapy and thoracic surgery, such as radical surgical resection. However, patients will suffer from operative trauma and associated inflammatory reactions (Schneiter and Weder, 2012). To date, there is no effective therapeutic agent but chemotherapy, which could destroy some normal cells as well. And the drugs that could reverse or delay the occurrence and development of the disease were considered to be the best measures to decrease the morbidity or mortality of lung adenocarcinoma. Therefore, better therapies and/or anticancer agents for lung adenocarcinoma. To date, some natural products have been used as effective antitumor agents due to their wide range of biological activities and low toxicity.

Oxymatrine (OMA) is a naturally occurring small-molecule compound from traditional Chinese medicine, *Sophora flavescens* Ait. It was widely used to treat cancer as well as other diseases such as viral hepatitis, cardiac arrhythmia and skin inflammations (Liu et al., 2003; Zhu et al., 2010). Recently, interests in studying the

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anti-cancer mechanism of OMA seem to be mounting (Bao et al., 2012). It was reported that OMA have broad activities in human malignant melanoma (Liu et al., 2008), gastric cancer (Luo et al., 2007), hepatoma (Ma et al., 2008) and lung cancer (Zhang et al., 2009). The inhibition of cellular proliferation, induction of apoptosis and regulation of related protein expression may contribute to the anti-cancer mechanism of OMA. However, studies about the effects of OMA on lung cancer are barely reported, and the mechanism is unknown. It was reported that early metastasis, which is related to abnormal growth, migration, invasion and angiogenesis in the cancer cells, attributed to the high mortality (Travis et al., 2002). Reports revealed that Bcl/Bax protein and vascular endothelial growth factor (VEGF) expressions may be linked to the development of human lung adenocarcinoma (Yang et al., 2009; Huang et al., 2011). VEGF is in close correlation with angiogenesis, viability and growth of various solid tumors (Saintigny and Burger, 2012). Its suppression leads to retrogression of neoplastic vessels and tumor growth restriction (Chekhonin et al., 2012).

In this study, we investigate the inhibitory effect of OMA on human lung adenocarcinoma (SPC-A-1) cell. Meanwhile, the effect of OMA on the secretion of vascular endothelial growth factor A (VEGF-A) in SPC-A-1 cells was also studied.

## MATERIALS AND METHODS

OMA was purchased from the National Institute of Control of Pharmaceutical and Biological Products (Batch NO. 110780-201007). Roswell Park Memorial Institute medium (RPMI) 1640 medium, penicillin and real time-polymerase chain reaction (RT-PCR) kit were purchased from GIBCO, Grand Island, NY, USA; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Tris were purchased from Ameresco; 0.25% pancreatic enzymes were purchased from Gino's biomedical technology Co., LTD; Annexin V-FITC/propidium iodide (PI) Apoptosis Assays Kit was purchased from Nanjing Keygen Biotech. Co., Ltd. Fetal bovine serum (FBS) and trypsin/EDTA were from Sigma. VEGF-A and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) upstream and downstream primer were synthesized by Shanghai ShengGong Biological Engineering Technology Service Company (Shanghai, China).

### Cell culture

SPC-A-1 cells (Shanghai Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI-1640 medium, containing 10% heat-inactivated FBS, 100 U/ml penicillin and 2 mM glutamine in 25 $cm^2$ culture flasks at 37℃ in a humidified incubator with 95% air/5% $CO_2$ atmosphere. The cells in the control group were treated with dimethyl sulfoxide (DMSO) (0.1%, final concentration).

### MTT assay measured *in vitro* cell growth

The *in vitro* cell growth was measured by MTT assay kit. SPC-A-1 cells in exponential growth stage were suspended to $5 \times 10^4$ $ml^{-1}$ and were seeded into 96-well plates (100 µl/well). After cultured for 24 h, phosphate buffered saline (PBS) solution was used to wash the non-adherent monolayer cells twice and was cultured in 100 µl of the fresh medium containing different concentrations of OMA solutions (OMA was dissolved with DMSO for 10 mg/ml and were freshly diluted to gain the final concentrations of 50, 100, 200 and 400 µg/ml, respectively) for 24, 48, and 72 h, respectively. Every concentration was repeated 5 times. And the control group was added with the same volume of DMSO without OMA, and DMSO without cells was added to the blank group. At the end of each time point, the drug-containing medium was replaced by fresh medium. MTT (20 µl of 5 mg/ml) was then added to each well to culture for another 4 h at 37℃. After abandoning the medium, 150 µl of DMSO were added to each well following a gently vibration for 10 min. The relative proliferation rate of SPC-A-1 cells was evaluated after measuring the absorbency at a wavelength of 570 nm of the dissolved solutions with a micro plate reader. Results are expressed as the percentage growth inhibition with respect to the untreated cells: growth inhibition rate (%) = (negative group's OD-test group's OD)/negative group's OD × 100%.

### Annexin V-FITC/PI double staining measured SGC-7901 cells apoptosis rate

The cell apoptosis rate was determined by Annexin V-FITC/PI double staining. After suspended SPC-A-1 cells to $2 \times 10^5$ $ml^{-1}$, they were seeded into 6-well plates (2 ml/well). Cells were washed with PBS twice to obtain adherent monolayer cells after being cultured for 24 h. After incubation with the new medium containing 0 and 100 µg/ml OMA (2 ml) for 48 h, the harvested cells were washed with PBS twice and then centrifuged at 1500 g/min for 5 min to remove PBS. Followed by the addition of 70% iced ethyl alcohol which was added slowly, centrifuged to harvest the cells, and washed with PBS twice. Finally, the cells were treated with 10 µl Annexin V-FITC and 5 µl PI according to the Annexin V-FITC/PI apoptosis detection kit. The early apoptosis rate of the cells was analyzed by flow cytometer. Cells positive for Annexin V, but negative for PI fluorescence were identified as apoptotic.

### RT-PCR for detection of human VEGF-A secretion in SPC-A-1 cells

Quantitative real-time RT-PCR was performed to detect the VEGF-A mRNA in SPC-A-1 cells. Total cellular RNA was extracted with Trizol, and the purity of the total RNA was determined by ultraviolet UV) in A260/280 (>1.8). The primer of VEGF-A was used: sense, 5'-ATGAACTTTCTGCTGTCTTGG-3' and antisense, 5'TCACCGCCT-CGGCTTGTCACA-3'. The primer of probe sequence was used: 5'ACGAAGTGGTGAAGTTCATGGATGTCTATCACC-3'. As internal standard to normalize mRNA levels, amplification of GAPDH was used, and the primer of GAPDH was: forward primer, 5'GAAGGTGAAGGTCGGAGT-3' and reverse primer, 5'-GAAGATGGTGATGGGATTTC-3'. RT-PCR was performed according to the manufacturer's instruction, that is, 50 µl cDNA (One step RT-PCR kit, Takara, Japan) was diluted by 1:500, and 5 µl was used as a template. The final volume of the reaction was 50 µl, and final volume of $MgCl_2$, 5' primer, 3' primer, GAPDH and LC-Red probe were 6 µl (25 mM), 1 µl (20 µm), 1 µl (20 µm) and 1 µl (20 µm), respectively. Then, DEPC (Sigma) was added to 50 µl. The RT-PCR reaction consisted of denaturing for 10 min at 94℃, followed by 45 cycles of denaturing for 30 s at 94℃, annealing for 30 s at 62℃, and extension for 1 min at 72℃.

Electrophoresis of the PCR products was analyzed by Syngene image analysis system. The threshold cycle (Ct) values of VEGF-A/GAPDH were used for calculation of the relative expression

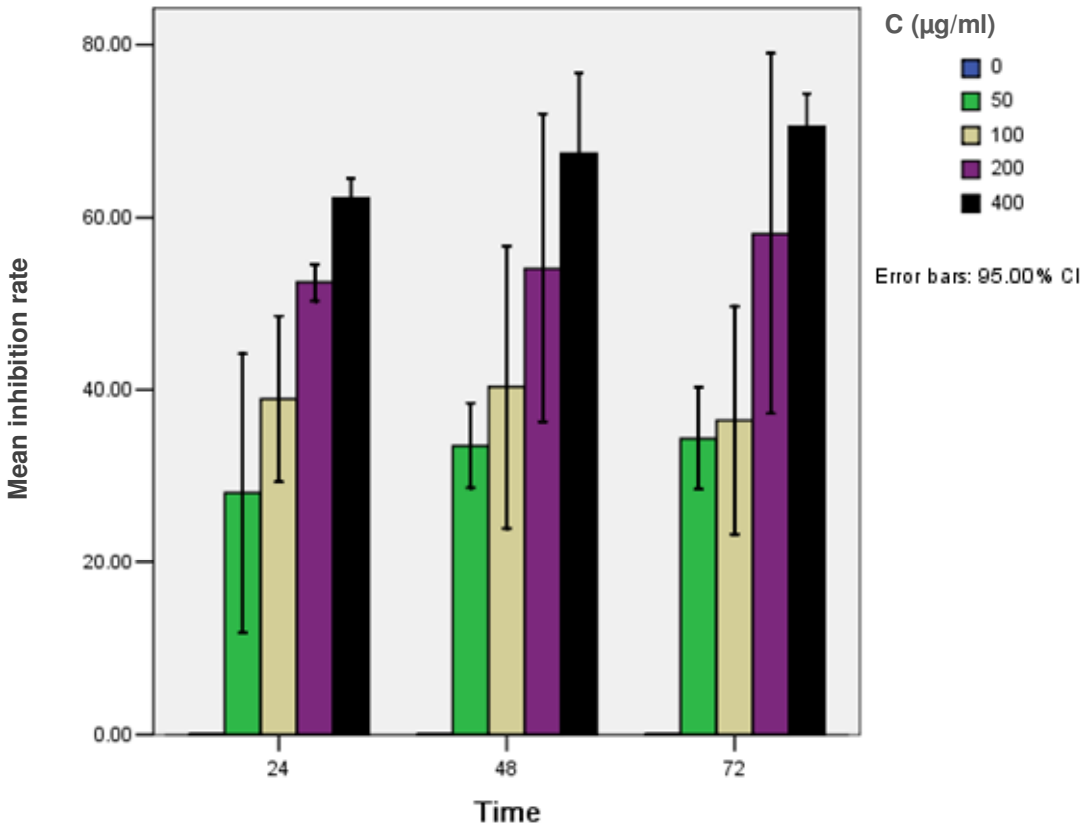


**Figure 1.** Inhibition of SPC-A-1 cells proliferation by OMA. Cells were treated with 0, 50, 100, 200 and 400 μg/ml of OMA. The optical densities of the cells were determined at 24, 48 and 72 h, respectively by MTT assay. Analysis of variance of factorial design showed that the growth of SPC-A-1 cells was inhibited by OMA in a dose- and time- dependent manner ($P < 0.05$).

ratios. All quantitative PCR reactions were performed in triplicate.

#### Statistical analysis

Experimental data were expressed with mean ± standard deviation ($\bar{X} \pm S$). Statistical analysis was performed with analysis of variance of factorial design and one-way or two-way analysis of variance (ANOVA), followed by the appropriate post hoc test (Bonferroni) using the statistical software SPSS 13.0. Significant level $\alpha = 0.05$, $P < 0.05$ was considered statistically significant. All statistical tests were two-sided.

## RESULTS

### Effect of OMA on the SPC-A-1 cells proliferation

Cell growth inhibition was evaluated by MTT assay, which showed that 50 and 100 μg/ml of OMA inhibited cells growth only slightly at 24 h. In addition, the proliferation inhibition rate of SPC-A-1 cells increases greatly as the concentration of OMA increases. In this study, 50% inhibitory concentration ($IC_{50}$) for the growth of SPC-A-1 cells was more than 1.0 mg/ml after the cells were treated with OMA for 72 h. But it was interesting that the rate in 72 h was a little lower than that in 48 h at the treatment group of 100 μg/ml. It was suggested that OMA reduced the viability of SPC-A-1 cells dose- and time-dependently after the cells were treated with OMA at 0 to 400 μg/ml for 24, 48 and 72 h, respectively (Figure 1).

### Effect of OMA on SPC-A-1 cells apoptosis rate

To understand the mechanisms of action of OMA on the growth in SPC-A-1 cells, we investigated the effects of OMA on apoptosis by staining it with Annexin V-FITC and PI and subsequently analyzed by flow cytometer. The dual parameter fluorescent dot plots showed the viable cell population in the lower left quadrant (Annexin V-PI-). The cells at the early apoptosis are in the lower right quadrant (Annexin V+PI-). As show in Figure 2, apoptosis appeared after the cells were treated for 48 h with OMA at 100 μg/ml, whereas the negative treatment cells did not show the evident apoptotic changes. The cell apoptosis rate of 100 μg/ml OMA treatment cells was 23.12 ± 0.81%, while the negative group was 1.97 ±

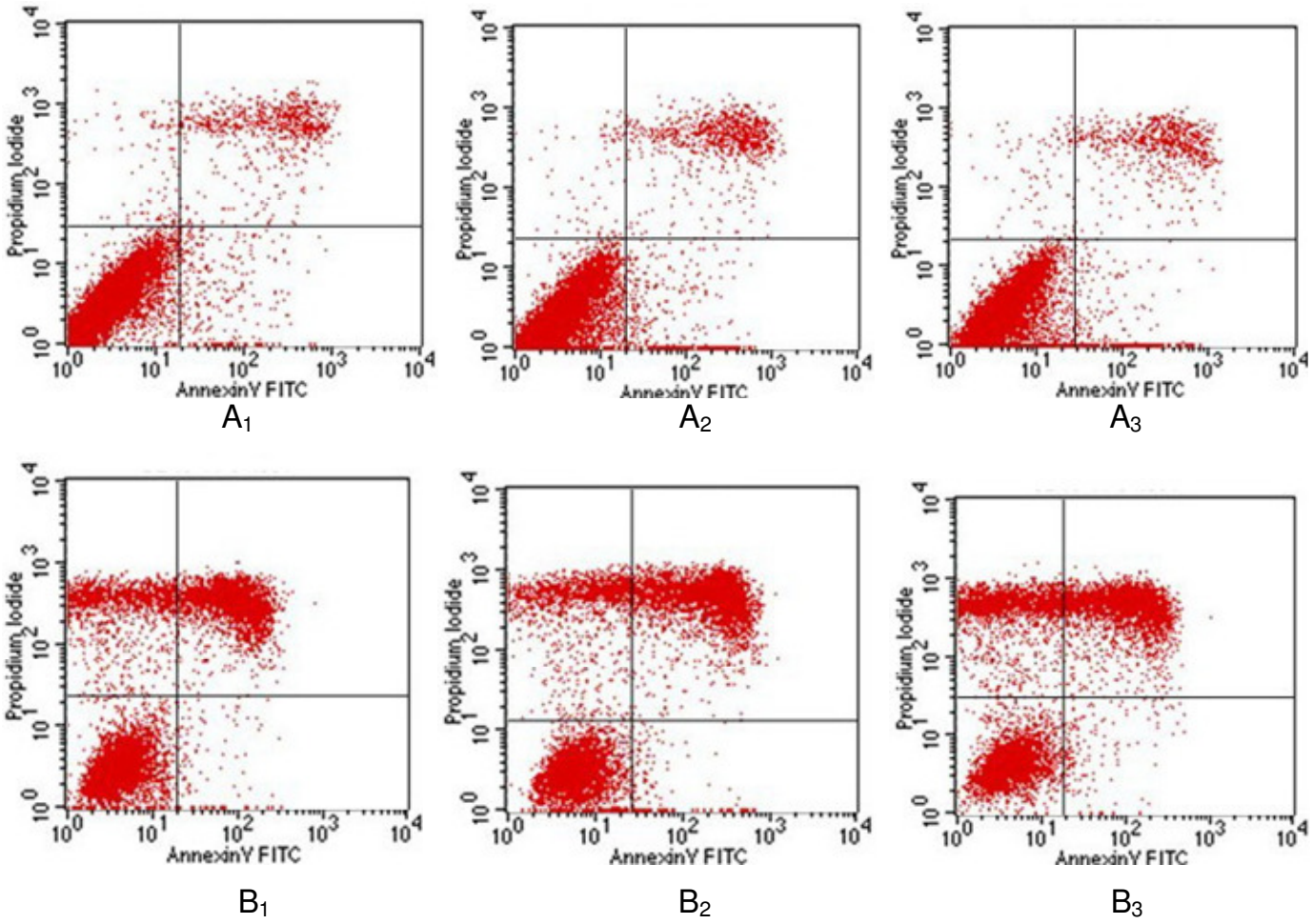


**Figure 2.** The apoptosis of SPC-A-1 cells after treated with OMA for 48 h. (A) negative and (B) cells treated with 100 µg/ml of OMA.

**Table 1.** Apoptosis rate of SPC-A-1 cells treated with OMA for 48 h.

| Group | Apoptosis rate (%) |
|---|---|
| Negative | 1.97 ± 0.39 |
| OMA | 23.12 ± 0.81[a] |
| F | 1318.921 |
| P | 0.000 |

[a]P < 0.01 versus negative group. Apoptosis rate of negative group and 100 µg/ml of OMA-treated cells were determined by Annexin V-FITC and PI staining and flow cytometric analysis. The values expressed were mean ± standard deviation of triplicate measurements.

0.39%, and there were significant difference between the two groups (P = 0.000, Table 1). These results indicated that 100 µg/ml of OMA had positive effects on apoptosis of SPC-A-1 cells.

### OMA down-regulated the VEGF-A expression in the SPC-A-1 cells

For detection of the effects of OMA on the secretion of VEGF-A in SPC-A-1 cells, the cells were treated for 24 h with OMA at the concentrations of 50 to 400 µg/ml. Then, each supernatant of the cell culture was respectively collected and analyzed by RT-PCR, respectively. Changes in VEGF-A mRNA were expressed as the ratio compared to GAPDH (internal control). RT-PCR showed a VEGF-A specific 262 bp band at mRNA level. The group without OMA was set as control group and showed a high expression of VEGF-A in SPC-A-1 cells. Compared to the control group, in the treatment groups with different concentration of OMA, we found that the expression of VEGF-A was significantly down-regulated in the SPC-A-1 cells (P < 0.05; Figure 3).

## DISCUSSION

Cancer is characterized by the imbalance between cell

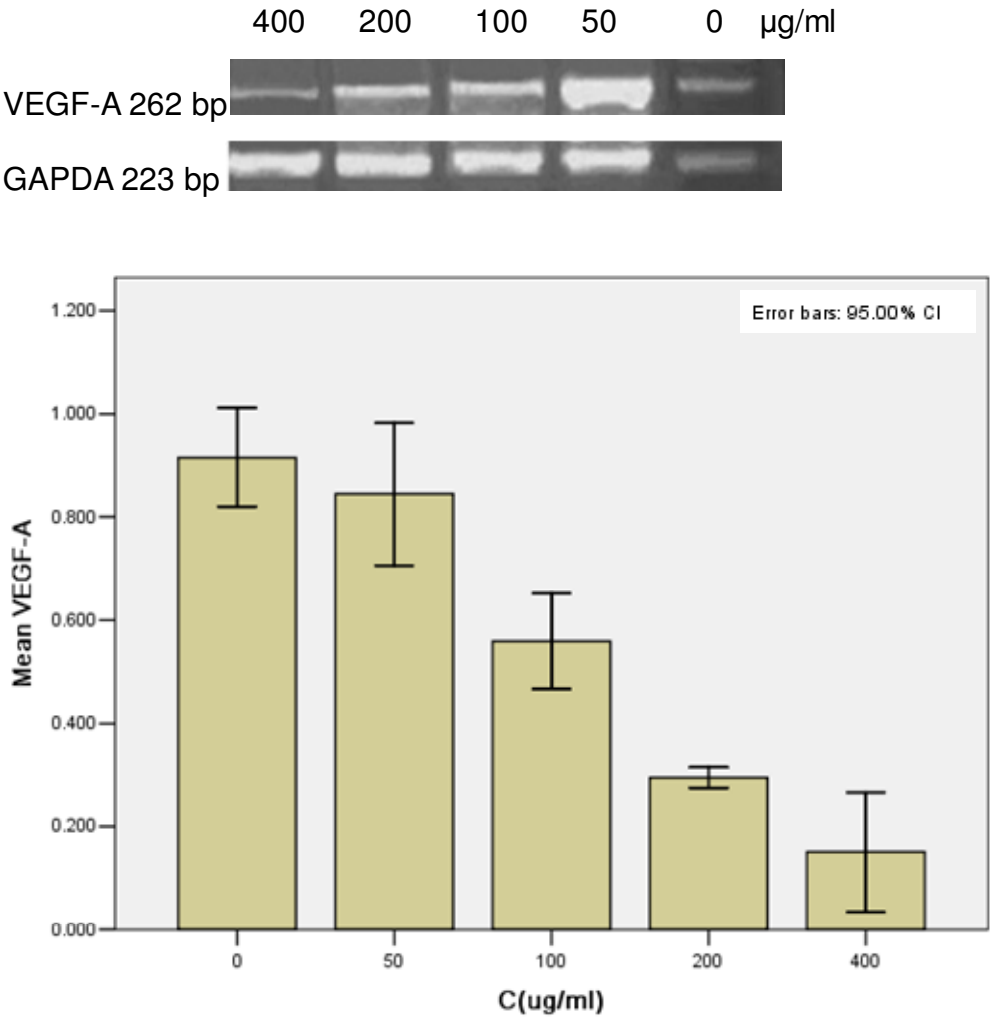


**Figure 3.** The down-regulated effects of OMA on the secretion of VEGF-A in SPC-A-1 cells. The cells were treated for 24 h with OMA at the concentrations of 50 to 400 μg/ml and the untreated group was set as control group. Then, RT-PCR was used to analyze VEGF-A mRNA expression in SPC-A-1 cells in response to different concentrations of OMA. Changes in VEGF-A mRNA were expressed as the ratio compared to GAPDH (internal control). Bars represented mean ± SD in triplicate.

proliferation and apoptosis (Claudia et al., 2009). It was reported that matrine and oxmatrine, the active constituents of *Radix sophorae flavescentis*, a kind of traditional Chinese medicine, has antitumor activity. The underlying mechanism may be that it inhibits cell proliferation and increase apoptosis in tumors via several ways, such as EGF/VEGF-VEGFR1-AKT-NF-kappaB signaling pathway (Yu et al., 2009), reducing the ratios of Bcl-2/Bax protein and mRNA levels in the cancer cells (Fan et al., 2012; Wang et al., 2000). In this study, the MTT assay results showed that OMA could inhibit the proliferation of SPC-A-1 cells in a dose- and time-dependent manner. Moreover, Annexin V-FITC and PI staining showed that after 100 μg/ml of OMA treating SPC-A-1 cells for 48 h, the cell apoptosis rate (23.12 ± 0.81%) had statistical significance when compared with the negative group (1.97 ± 0.39%). These findings suggested that it may be a potential anti-tumor agent.

Vascular endothelial growth factor is a positive regulator of angiogenesis, and its expression is up-regulated in many carcinomas. There has been a report showing that suppression of VEGF expression in human lung cancer cell lines including SPC-A-1 cells leads the inhibition of growth in the lung cancer *in vitro* and *in vivo*. The VEGF-A, -B, -C, and -D, and placental growth factor have been reported to be associated with tumor progression and poor prognosis, because of its angiogenic and lymphangiogenic properties in most solid tumors. VEGF-A, a key regulator of blood vessel growth, is commonly secreted by a variety of solid tumors. It is

conceivable that VEGF increases tumor progression not only by stimulating tumor angiogenesis, but also by direct stimulation of VEGFR signaling on various types of tumor cells (Ellis and Hicklin, 2008; Ferrer et al., 1999; Lacal et al., 2000). In this study, the mRNA expression of VEGF-A decreased with the concentration of OMA increasing, indicating that OMA can inhibit VEGF-A expression. Therefore, we assumed that OMA may inhibit tumor angiogenesis by down-regulating VEGF-A mRNA expression.

Given the positive results, we demonstrated that OMA could inhibit the growth of the SPC-A-1 cells and induce apoptosis. Moreover, OMA could down-regulate the expression of VEGF-A mRNA in the SPC-A-1 cells. The results indicated that OMA had a positive anticancer activity *in vitro* and may be a potential effective and safe material for anticancer agent. Nevertheless, the real mechanism of OMA on cells apoptosis should be clear by more works, such as evaluating the levels of Bcl-2 and Bax in the SPC-A-1 cells treated with OMA, which play important roles in the mitochondrial pathway resulting apoptosis.

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