

## ORIGINAL ARTICLE

# Anticancer effects of Rosmarinic acid in OVCAR-3 ovarian cancer cells are mediated via induction of apoptosis, suppression of cell migration and modulation of lncRNA MALAT-1 expression

Yan Zhang<sup>1</sup>, Min Hu<sup>1</sup>, Liu Liu<sup>1</sup>, Xiao-Ling Cheng<sup>2</sup>, Jing Cai<sup>3</sup>, Jian Zhou<sup>4</sup>, Tao Wang<sup>5</sup>

<sup>1</sup>Department of Obstetrics and Gynaecology, Renmin Hospital of Wuhan University, Wuhan 430060, China; <sup>2</sup>Institute of Biological Medicine, Wuhan University of Science and Technology, Wuhan, Hubei 430065, China; <sup>3</sup>Department of Obstetrics and Gynecology, Wuhan Medical & Health Care Center for Women and Children (Wuhan Children's Hospital Wuhan Women and Children Care Hospital) 430016, China; <sup>4</sup>Department of Infectious Disease, Tianyou Hospital, Wuhan University of Science and Technology, Wuhan 430081, China; <sup>5</sup>Medical School, Wuhan University of Science and Technology, Wuhan, Hubei 430065, China.

## Summary

**Purpose:** The main objective of the present research work was to study the anticancer properties of rosmarinic acid in OVCAR-3 human ovarian cancer cells and also to evaluate its effects on apoptosis induction, cancer cell migration and modulation of lncRNA MALAT-1 expression.

**Methods:** MTT assay was used to study the effects of the agent on OVCAR-3 cell viability, while inverted phase contrast microscopy and fluorescence microscopy were used to study the effects on cell morphology. Scanning electron microscopy (SEM) was used to study the effects of rosmarinic acid on cell surface morphology in OVCAR-3 cells. In vitro wound healing assay was used to study the effects on cell migration.

**Results:** Rosmarinic acid induced time-dependent and concentration-dependent cytotoxic effects in these malignant cells. The IC<sub>50</sub> values at 48 and 72 hrs time intervals were

found to be 34.6 and 25.1  $\mu$ M respectively. Rosmarinic acid-treated cells revealed significant changes in cell morphology including cellular shrinkage and cell rounding. The cells also lost attachment with the plate surface. Doses of 10, 40 and 160  $\mu$ M rosmarinic acid led to a substantial increase in bright blue fluorescence which is a signpost of chromatin condensation and DNA fragmentation. Rosmarinic acid treatment also led to a significant suppression of cell migration corresponding to 46.5% and 86.2 % cell migration inhibition at 40 and 160  $\mu$ M doses, respectively.

**Conclusion:** In conclusion, the current study showed that rosmarinic acid induced potent anticancer effects in OVCAR-3 cancer cells by inducing apoptosis, inhibiting cell migration and modulating lncRNA Malat-1 expression.

**Key words:** apoptosis, cell migration, chromatin condensation, ovary cancer, rosmarinic acid

## Introduction

Ovarian cancer is considered to be the most fatal gynecological malignancy. The high death rate in ovarian cancer arises from the fact that in the majority of the cases the disease is diagnosed at an advanced stage where treatment regimens are mostly unsuccessful. Despite recent advances

in the biochemical pathways underlying ovarian cancer, the origin and pathogenesis of this malignancy is still not fully understood [1,2]. Ovarian cancer was labelled among the top 7 incidence and mortality-related malignancies in females in 2012. According to a survey in 2011, the estimates of

new ovarian cancer patients were 45,000 and new ovarian cancer-related deaths were around 18,000 in 2011 in China. It has also been observed that the incidence and mortality of this disease were lower in rural areas of China as compared to urban areas [3,4]. The standard treatment of ovarian cancer involves debulking surgical resection followed by platinum-based chemotherapy. Despite a significant patient initial response rate to first-line chemotherapy, the majority of the cases relapse, due to drug resistance which eventually leads to treatment failure. Therefore, the overall 5-year survival rate of advanced-stage ovarian cancer is 10-25% only [5,6]. Keeping this in mind, there is an urgent need of novel and effective chemotherapeutic agents which can target ovarian cancer cells without acquiring resistance. Natural products have always played pivotal role in the anticancer drug discovery. The majority of the anticancer drugs (more than 50%) are either pure natural products or their synthetic/semisynthetic derivatives. It has also been reported that dietary consumption of plant compounds has been linked with reduced risk of malignancy and increased survival of cancer patients [7,8].

The main objective of the present study was to investigate the anticancer and apoptotic effects of rosmarinic acid in OVCAR-3 cancer cells along with studying its effects on cell migration, and modulation of lncRNA Malat-1 expression.

## Methods

### *Chemicals and other reagents*

Rosmarinic acid (purity  $\geq 98\%$ ), Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit, MTT, Hoechst 33342 stain and formalin solution were procured from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, trypsin and phosphate buffered saline (PBS), supplemented with calcium chloride and magnesium chloride were procured from Gibco Life Technologies, Grand Island, NY, USA. All other chemicals and solvents used were of the highest purity grade.

### *Cell line and cell culture conditions*

The OVCAR-3 cancer cell line was procured from the Shanghai Institute of Cell Resource Center of Life Science, China, and maintained in RPMI-1640 supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 100  $\mu\text{g}/\text{ml}$  streptomycin) at  $37^\circ\text{C}$  in a humidified incubator.

### *MTT assay for cell proliferation*

The antiproliferative effects of rosmarinic acid were tested against OVCAR-3 human ovarian cancer

cells by MTT assay. In brief, OVCAR-3 cells at a density of  $2 \times 10^6$  cells per well were seeded in a 96-well plate and incubated for 12 hrs for cell adherence. After incubation, the cells were treated with increasing doses (0, 5, 10, 20, 40, 80 and 160  $\mu\text{M}$ ) of rosmarinic acid for 48 and 72 hrs. Subsequently, MTT solution (20  $\mu\text{l}$ ) was added to the cell culture sample and further incubated for 4 hrs at  $37^\circ\text{C}$ . The formazan crystals so formed were solubilized with DMSO and the absorbance was calculated on a microplate reader at a wavelength of 490 nm.

The effects of rosmarinic acid on cell viability were calculated as an inhibition ratio (I%) using the following formula:

$$I\% = \frac{[\text{OD}_{490}(\text{control}) - \text{OD}_{490}(\text{treated})]}{\text{OD}_{490}(\text{control})} \times 100$$

where OD<sub>490</sub> is the optical density at 490 nm.

### *Inverted phase contrast microscopy*

The morphological changes induced by rosmarinic acid in OVCAR-3 cancer cells was evaluated by inverted phase contrast microscopy. In brief, the cells were seeded at a density of  $2 \times 10^5$  cells/well and incubated for 12 hrs to achieve full adherence. The cells were then treated with different doses (0, 10, 40 and 160  $\mu\text{M}$ ) of rosmarinic acid for 48 hrs, while the control cells were treated with 1.5% dimethyl sulfoxide only. Afterwards, the cells were incubated for 24 hrs and then finally analyzed by inverted phase contrast microscope (Nikon, Tokyo, Japan).

### *Morphological evaluation by fluorescence microscopy*

Fluorescence microscopy using Hoechst 33342 staining is an excellent tool to evaluate apoptosis-related morphological changes in cancer cells. In this study, OVCAR-3 cancer cells were grown on coverslips in 12-well plates and incubated for 24 hrs. The cells were treated with 0, 10, 40 and 160  $\mu\text{M}$  of rosmarinic acid for 48 hrs. Following drug treatment, the cells were washed twice with PBS and then stained with Hoechst 33342 for 40 min at room temperature. Finally, the changes in cellular morphology were detected by fluorescence microscope (Nikon, TS-100-F) equipped with a digital camera using several visual fields.

### *In vitro wound healing assay*

The effect of rosmarinic acid on cancer cell migration in OVCAR-3 cells was evaluated by *in vitro* wound healing assay. OVCAR-3 cells at a cell density of  $2 \times 10^5$  cells per ml were seeded in a 6-well plate and incubated for 12 hrs to acquire 100% confluency. The cells were starved for 18 hrs, after which a straight cell-free wound was made using a pipette. Each well containing cells was washed with PBS twice and then treated with 0, 10, 40 and 160  $\mu\text{M}$  of rosmarinic acid for 48 hrs. The drug-treated cells were then fixed with 3.5% ethanol comprising 1.5% crystal violet for 40 min. Then the cells were analyzed by a light microscope (Nikon Corp., Tokyo, Japan) and the percentage of cells that migrated into the scratched area was measured.

### Scanning electron microscopy (SEM) assay for cell surface morphology

For this assay, OVCAR-3 cancer cells were seeded at a density of  $2 \times 10^6$  cells/well and were covered by RPMI-1640 medium (5 ml). The cells were seeded in two 12-well plates and these plates were incubated for 12 hrs and varying doses (0, 10, 40 and 160  $\mu$ M) of rosmarinic acid were added into each well for 50 min. The supernatant was removed and 5 ml volume of formalin solution was put into each well. The cells were then fixed and all coverslips were soaked in 3.5% tannic acid for 24 hrs. The cells were then counter-fixed using 2.0% osmium tetroxide solution for 2 hrs following which cells were dehydrated in 70% ethanol and then dried using a point dryer. As a final point, the cells on coverslips were coated with gold in an ionic sputter coater (BAL-TEC Co., USA) and analyzed using a scanning electron microscope (Hitachi, Tokyo, Japan).

### Quantitative real-time PCR (qRT-PCR)

Total RNA from tissues was extracted using Trizol reagent (Invitrogen, California, USA). qRT-PCR assays were performed to detect MALAT-1 expression using the Prime Script RT reagent Kit and SYBR Premix ExTaq (Takara, Biomedical Technology, Beijing Co. Ltd) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control, and lncRNA MALAT-1 values were normalized to GAPDH. qRT-PCR reactions were performed by the ABI7900 system (Applied Biosystems, California, USA).

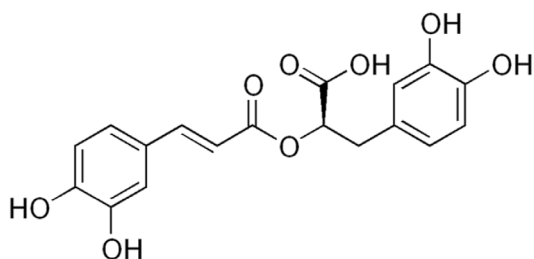
### Statistics

All the data were expressed as mean  $\pm$  standard error of mean (S.E.M.). The significance of difference between groups was estimated by one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* test. A *p* value less than 0.05 indicated statistical significance.

## Results

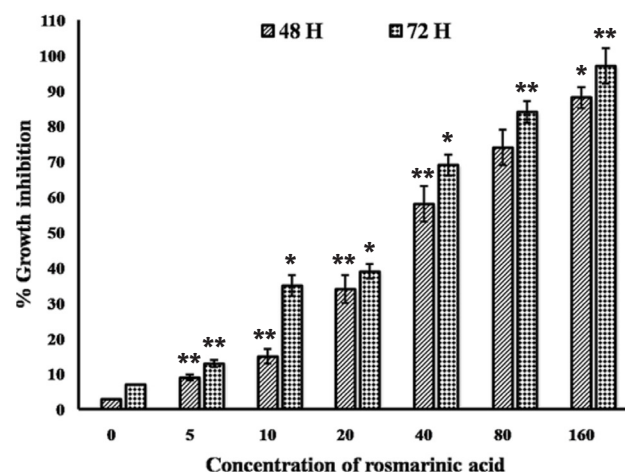
### Rosmarinic acid inhibited the growth of OVCAR-3 cancer cells

The antiproliferative activity of rosmarinic acid (Figure 1) against OVCAR-3 cancer cells was evaluated by MTT cell viability assay which is a colorimetric assay for assessing cell metabolic activity. The results are shown in Figure 2 and reveal



**Figure 1.** Chemical structure of rosmarinic acid.

that rosmarinic acid induced time-dependent and concentration-dependent cytotoxic effects in these cells. The  $IC_{50}$  values at 48 and 72 hrs time intervals were 34.6 and 25.1  $\mu$ M, respectively.  $IC_{50}$  values give an indication about the potency of the drug and the lower its value, the higher the drug potency.



**Figure 2.** Inhibition of cell growth induced by different doses of rosmarinic acid in OVCAR-3 cancer cells. After treating cells with 0, 5, 10, 20, 40, 80 and 160  $\mu$ M of rosmarinic acid, the cells were incubated for 48 and 72-h time intervals. Data are shown as the mean  $\pm$  SD of three independent experiments. \**p* < 0.05, \*\**p* < 0.01 vs 0  $\mu$ M (control).

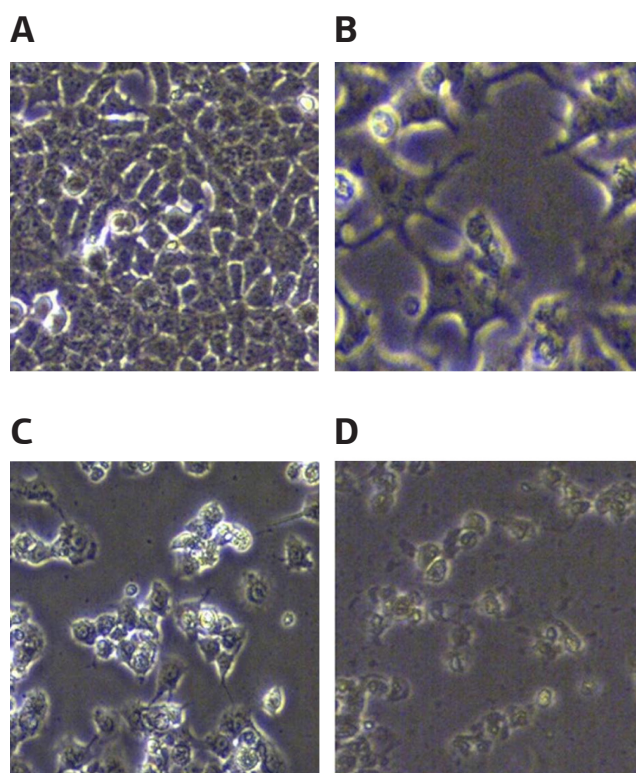
### Rosmarinic acid induced morphological changes in OVCAR-3 cells

Inverted phase contrast microscopy was used to assess the morphological changes induced by rosmarinic acid. The microscopy results indicated that, as compared to the untreated control cells which showed normal cellular morphology, rosmarinic acid-treated cells showed significant changes in cell morphology including cellular shrinkage and rounding of cells. The cells also lost attachment with the plate surface in addition to the decrease in the number of cells as the dose of rosmarinic acid increased (Figure 3).

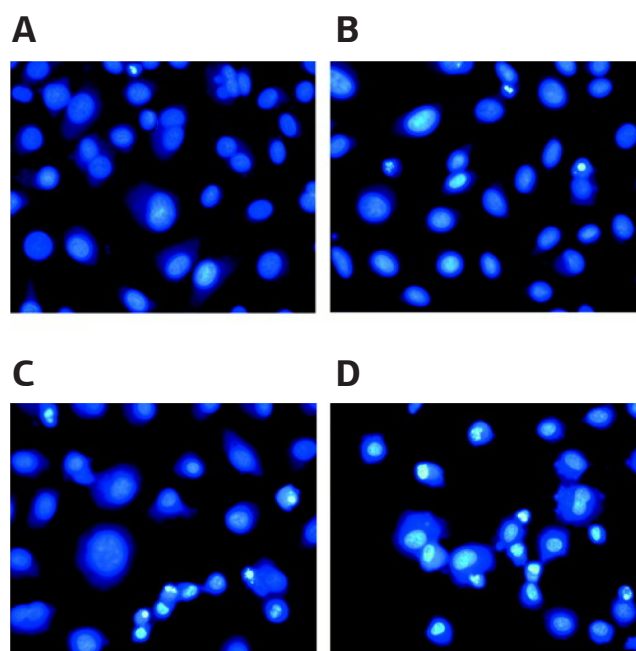
### Apoptotic induction by rosmarinic acid in OVCAR-3 cells as evaluated by fluorescence microscopy

Fluorescence microscopy using Hoechst 33342 staining was used to evaluate the apoptotic induction by rosmarinic acid in OVCAR-3 cells. Hoechst 33342 is easily permeable within the cell and is usually used to indicate DNA fragmentation and chromatin condensation within the apoptotic cells. The results which are shown in Figure 4 indicate that the untreated control cells showed normal cell morphology with intact oval shaped cells and the cell nuclei displayed less bright blue





**Figure 3.** Rosmarinic acid induced morphological changes in OVCAR-3 cancer cells. The cells were treated with 0 (A), 10 (B), 40 (C) and 160 (D)  $\mu$ M of rosmarinic acid for 48 hrs and then analyzed by inverted phase contrast microscopy. The drug induced dose-dependent morphological changes in the cells, characteristic of apoptosis.



**Figure 4.** Fluorescence microscopy evaluation of the apoptosis induction in OVCAR-3 cancer cells by rosmarinic acid. The cells were treated with 0 (A), 10 (B), 40 (C) and 160 (D)  $\mu$ M of rosmarinic acid for 48 hrs, stained with Hoechst 33342 and then analyzed by fluorescence microscope at  $\times 200$  magnification. Increased drug dose led to dose-dependent chromatin condensation and DNA fragmentation.

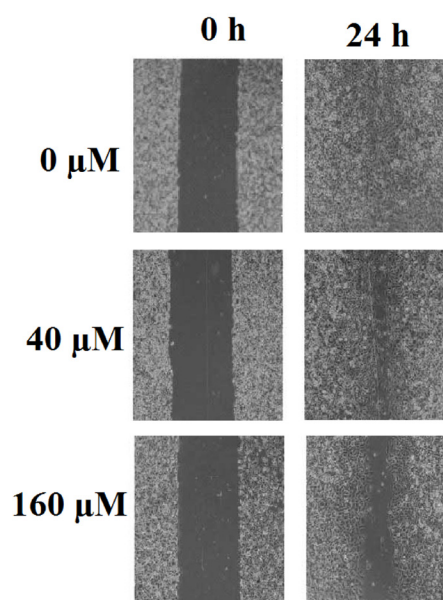
fluorescence. However, 10, 40 and 160  $\mu$ M dose of rosmarinic acid led to substantial alterations in cell morphology marked by an increased bright blue fluorescence which is a signpost of chromatin condensation and DNA fragmentation. The number of these apoptotic cells increased significantly with increase in rosmarinic acid concentration.

#### *Rosmarinic acid led to inhibition of cell migration in OVCAR-3 cells*

*In vitro* wound healing assay evaluated the effect of rosmarinic acid on the cell migration tendency in OVCAR-3 cells. The untreated control cells after making the scratch, completely filled up the space between the OVCAR-3 cells after a time interval of 24 hrs. However, treatment with 40 and 160  $\mu$ M dose of rosmarinic acid led to a significant suppression of cell migration corresponding to 46.5% and 86.2 % cell migration inhibition at 40 and 160  $\mu$ M doses, respectively. These results are shown in Figure 5, where images of the treated and untreated cells were captured by phase contrast microscope.

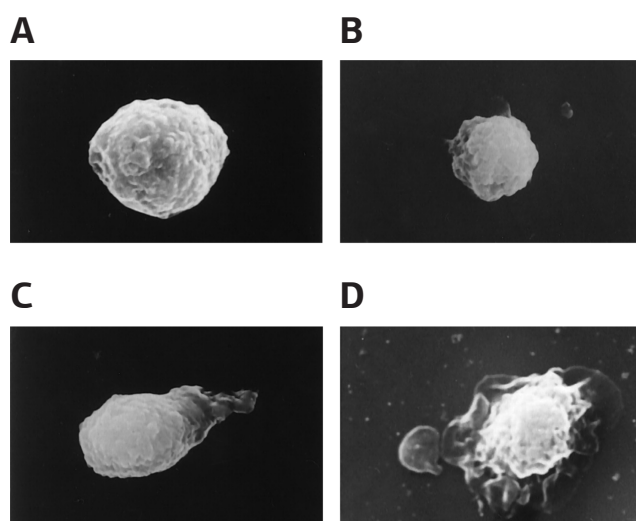
#### *Apoptosis evaluation by scanning electron microscopy (SEM)*

SEM can be used to monitor the alterations in cell surface morphology in apoptotic cells. These changes in cell surface morphology include cell



**Figure 5.** Rosmarinic acid treatment led to the suppression of the cancer cell migration in OVCAR-3 cancer cells. The cells were treated with 0, 40 and 160  $\mu$ M dose of rosmarinic acid for 48 hrs. The number of cells that migrated into the scratched area were photographed ( $\times 200$  magnification) at 0 h and 24-h time intervals and calculated as a percentage of migration.

shrinkage, membrane blebbing and disappearance of microvillous structures. The results of the current study are shown in Figure 6 and reveal that untreated OVCAR-3 cancer cells showed normal cellular morphology with no indications of any cell surface morphological alterations. In contrast, OVCAR-3 cells treated with 10, 40 and 160  $\mu$ M dose of rosmarinic acid led to a substantial changes in cell surface morphology comprising the appearance of several cell surface projections, plasma membrane blebbing and formation of condensed apoptotic bodies.



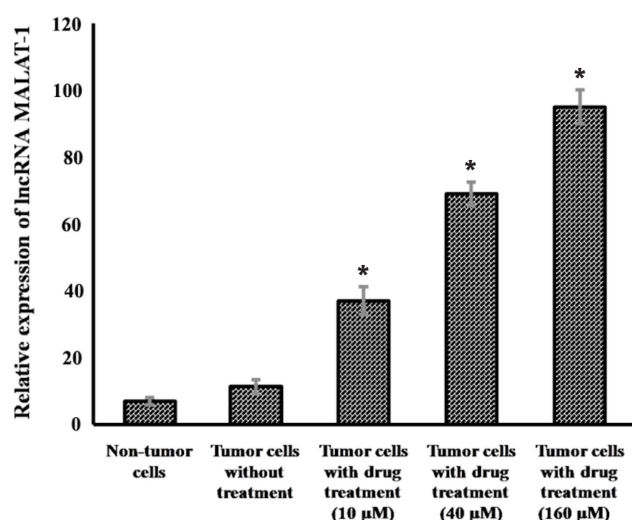
**Figure 6.** Scanning electron micrographs of the rosmarinic acid-treated and untreated OVCAR-3 cancer cells. The cells were treated with 0 (A), 10 (B), 40 (C) and 160 (D)  $\mu$ M dose of rosmarinic acid for 48 hrs and then analyzed using scanning electron microscope. The untreated cells showed normal cell surface morphology. Increased rosmarinic acid dose led to increased plasma membrane blebbing and cellular surface projections.

#### *Rosmarinic acid induced upregulation of lncRNA MALAT-1 expression*

In a further experiment, the changes in the Malat-1 expression in OVCAR-3 cancer cells was evaluated using qRT-PCR. The results which are shown in Figure 7 indicate that the expression levels of lncRNA Malat-1 in OVCAR-3 cells treated with different doses of rosmarinic acid were considerably higher than that in adjacent non-tumor tissues as well as cancer cells without any drug treatment. GAPDH was used as a vehicle control.

## Discussion

Plant-based compounds include a wide spectrum of naturally occurring molecules belonging to different classes including flavonoids, sesquiterpenes, alkaloids, diterpenoids, and polyphenolics



**Figure 7.** Effect of rosmarinic acid on the expression of lncRNA MALAT-1 in OVCAR-3 cancer cells and surrounding non-cancer cells. The bands were detected by qRT-PCR; \* $p < 0.01$ .

and most of these compounds exhibit anticancer properties against a wide variety of cancer cells. Herbal medicinal agents have been shown to offer a huge pharmaceutical potential and various herbal agents have been especially used to treat different forms of human malignancies [10,11]. The fact that natural products are so important in public health care worldwide is reflected by the fact that around 70-80% of the world population still banks on plant-based medicines. Additionally, it has also been documented that about 50-55% of all drugs that are being used clinically are either pure natural products, their synthetic or semisynthetic derivatives or their analogs. It has also been reported that more than 3,000 plant species have been reported to be used in cancer treatment [12-14]. The main treatment option for treating solid and hematological malignancies is chemotherapy. But this treatment is not without serious side-effects coupled with the problem of multidrug resistance. In order to minimize such inadvertent effects, there is an urgent need to design and develop novel anticancer treatment modalities which are more effective and tolerable to patients [15]. Apoptosis involves a programmed cell death process and any disturbance in this process plays a key role in cancer development. Various plant-derived compounds have been isolated which specifically induce apoptosis in cancer cells leaving normal cells intact. Apoptosis induction is regarded as a new and promising biochemical target for innovative mechanism-based drug discovery [16,17]. Keeping in mind the role of natural products in cancer treatment and apoptosis induction, the main purpose of the current study

was to investigate the anticancer effects of rosmarinic acid in OVCAR-3 cancer cells along with evaluating its effects on apoptosis, cell migration as well as on the expression of lncRNA MALAT-1. Chemically, rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. The compound has been reported to exhibit a wide range of biological activities including antibacterial, anti-oxidant, antiviral and anti-inflammatory activities [18]. The current study indicated that rosmarinic acid induces time-dependent and concentration-dependent cytotoxic effects in the ovarian cancer cells. The IC<sub>50</sub> values at 48 and 72-h time intervals were found to be 34.6 and 25.1  $\mu$ M, respectively. Rosmarinic acid-treated cells also revealed significant changes in cell morphology including cellular shrinkage and rounding of cells. Fluorescence microscopy revealed that 10, 40 and 160  $\mu$ M dose of rosmarinic acid led to substantial alterations in cell morphology marked by an increased bright blue fluorescence which is a signpost of chromatin condensation and DNA fragmentation. Treatment with 40 and 160  $\mu$ M dose of rosmarinic acid led to a significant suppression of cell migration corresponding to 46.5% and 86.2 % cell migration inhibition, respectively. SEM results revealed that OVCAR-3 cells treated with 10, 40 and 160  $\mu$ M dose of

rosmarinic acid led to substantial changes in cell surface morphology comprising the appearance of several cell surface projections, plasma membrane blebbing and formation of condensed apoptotic bodies. The expression levels of lncRNA Malat-1 in OVCAR-3 cells treated with different doses of rosmarinic acid was considerably higher than that in adjacent non-tumor tissues.

## Conclusion

In conclusion, the current study has shown that rosmarinic acid induced potent anticancer effects in OVCAR-3 human ovarian cancer cells by inducing apoptosis, inhibiting cell migration and modulating lncRNA Malat-1 expression.

## Acknowledgement

This study was supported by Key Projects in Pillar Program of Health Planning Commission of Hubei Province (Grant no.WJ2017Z002).

## Conflict of interests

The authors declare no conflict of interests.

## References

1. Jemal A, Siegel R, Ward E et al. Cancer statistics, 2006. *CA Cancer J Clin* 2006;56:106-30.
2. Cannistra SA. Cancer of the ovary. *N Engl J Med* 2004;351:2519-29.
3. Wei K, Li Y, Zheng R et al. Ovary cancer incidence and mortality in China, 2011. *Chin J Cancer Res* 2015;27: 38-43.
4. National Cancer Centre, Bureau for Disease Control and Prevention of Health Ministry. Chinese Cancer Incidence and Mortality, 2003-2007. Beijing: Military Medical Science Press 2012;17:251-8.
5. See HT, Kavanagh JJ, Hu W et al. Targeted therapy for epithelial ovarian cancer: Current status and future prospects. *Int J Gynecol Cancer* 2003;13:701-34.
6. Vergote I, Amant F, Kristensen G, Ehlen T, Reed NS, Casado A. Primary surgery or neoadjuvant chemotherapy followed by interval debulking surgery in advanced ovarian cancer. *Eur J Cancer* 2011;47 (Suppl 3): S88-92.
7. Ho JW, Leung YK, Chan CP. Herbal medicine in the treatment of cancer. *Curr Med Chem Anti-Cancer Agents* 2002;2:209-214.
8. Setzer WN, Setzer MC. Plant-derived triterpenoids potential antineoplastic agents. *Mini Rev Med Chem* 2003;3:540-56.
9. Liang CC, Park AY, Guan JL. In vitro scratch assay: A convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2007;2:329-33.
10. Christen P, Cuendet M. Plants as a source of therapeutic and health products. *Chimia* 2012;66:320-3.
11. Amin AR, Kucuk O, Khuri FR, Shin DM. Perspectives for cancer prevention with natural compounds. *J Clin Oncol* 2009;27:2712-25.
12. Fulda S. Evasion of apoptosis as a cellular stress response in cancer. *Int J Cell Biol* 2010: 370835.
13. Hsu CL, Yu YS, Yen GC. Anticancer effects of *Alpinia pricei* Hayata roots. *J Agric Food Chem* 2010;58:2201-8.
14. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. *J Ethnopharmacol* 2005;100:72-9.
15. Cragg GM, Newman DJ. Discovery and development of antineoplastic agents from natural sources. *Cancer Invest* 1999;17:153-63.
16. Hirano T, Abe K, Gotoh M, Oka K. Citrus flavone tangeretin inhibits leukaemic HL-60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. *Br J Cancer* 1995;72:1380-8.
17. Jiang MC, Yang-Yen HF, Yen JJ, Lin JK. Curcumin induces apoptosis in immortalized NIH 3T3 and malignant cancer cell lines. *Nutr Cancer* 1996;26:111-20.
18. Petersen M, Simmonds MS. Rosmarinic acid. *Phytochemistry* 2003;62:121-5.