

Original Article

Effects of isorhamnetin on protein expression of VEGF, MMP-2 and Endostatin in Lewis lung cancer mouse

Yingchao Zhu¹, Lining Sun¹, Huijun Zhang², Ying Li³, Songtao Lai¹

¹Department of Radiation Oncology, Fudan University Shanghai Cancer Center, Shanghai, China; ²Department of Cardiothoracic Surgery, Huashan Hospital of Fudan University of Shanghai, Shanghai, China; ³Department of Thoracic Surgery, Shanghai Pulmonary Hospital of Tongji University, Shanghai, China

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Abstract: Tumor angiogenesis is a marker for invasion, depending on the balance between angiogenesis suppressor and facilitating factors. This study established a Lewis lung cancer mouse model, on which the effect of isorhamnetin on the protein expression of vascular endothelial growth factor (VEGF), endostatin and matrix metalloproteinase-2 (MMP-2) was measured for investigating the possible functional mechanisms. SPF grade C57BL/6 mice were treated with 50 mg/kg isorhamnetin and/or 2 mg/kg cisplatin. All groups received subcutaneous inoculation of Lewis lung cancer cells except control group. Drugs were delivered via intraperitoneal injection starting from day 2 after inoculation for 14 days. Tumor volume and suppressor rate were measured. Number of metastatic lesions at pulmonary lobe surface was counted, followed by HE staining and ELISA analysis of serum levels of interleukin-2 (IL-2), interferon-gamma (IFN- γ), VEGF and endostatin, along with immunohistochemistry (IHC) staining for measuring those proteins in tumor tissues. Model group had a larger tumor volume, lower serum IL-2, IFN- γ and endostatin level, plus higher VEGF level ($P < 0.05$ compared with control group). In metastatic tumor tissues, VEGF and MMP-2 protein expressions were up-regulated, accompanied with lower endostatin ($P < 0.05$). Compared with model group, drug treatment decreased tumor volume, number of metastatic lesions and tumor cell density ($P < 0.05$). Co-treatment resulted in higher tumor suppressing rate, serum IL-2, IFN- γ and endostatin levels compared with single drug treatment, and lower serum VEGF, and VEGF/MMP-2 proteins in metastatic tumors ($P < 0.05$). In conclusion, isorhamnetin inhibits tumor progression, possibly related with down-regulation of VEGF or MMP-2, as well as up-regulation of endostatin.

Keywords: Isorhamnetin, lung cancer, VEGF, Endostatin, MMP-2

Introduction

Lung cancer has a relatively higher incidence and mortality rate, as more than 50% cases belong to pulmonary adenoma. Surgery and chemo-/radiotherapy are major approaches for lung cancer treatment. Due to high incidence of early phase metastasis, most patients are already not suitable for surgery. In recent years, patient population of lung cancer is becoming younger [1, 2]. Although short-term treatment efficacy has been improved with combined therapy of surgery, chemo-/radio-treatment and molecular targeting, long-term survival rate and life quality are still unsatisfactory for some patients [3, 4]. The pathogenesis of lung cancer has not been fully elucidated. Angiogenesis plays an important role in the

occurrence and progression of tumor cells, as well as the process of invasion or metastasis. The balance between angiogenesis factor and vascular inhibiting factor is critical for mediating angiogenesis. Tumor angiogenesis involves the formation of new basal membrane and vascular rings, dissolve of endothelial basal membrane, and migration/proliferation of endothelial cells, forming a complex process with the interaction between vascular endothelial cells and tumor cells [5, 6]. Initially step of angiogenesis is the degradation of extracellular matrix (ECM), during which matrix metalloproteinase-2 (MMP-2) is involved via interacting with ECM structural proteins. Vascular endothelial growth factor (VEGF) plays a significant role among vascular modulatory factors, and can specifically stimulate vascular endothelial cell prolifer-

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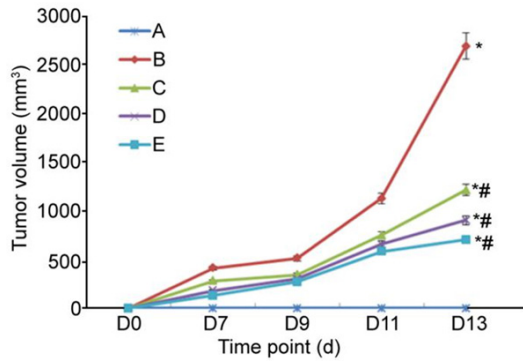


Figure 1. Tumor growth curve. A: Control group; B: Model group; C: Isorhamnetin group; D: Cisplatin group; E: Isorhamnetin + cisplatin group. *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with model group.

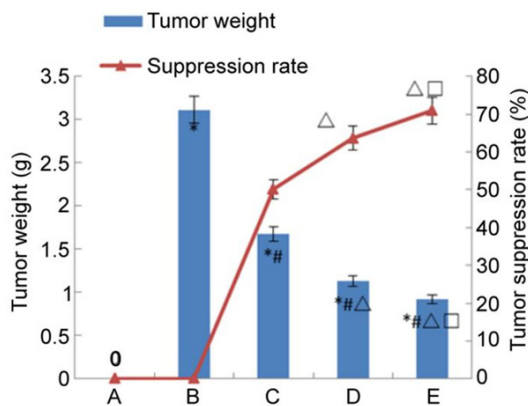


Figure 2. Comparison of tumor weight and suppression rate. A: Control group; B: Model group; C: Isorhamnetin group; D: Cisplatin group; E: Isorhamnetin + cisplatin group. *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with model group; Δ , $P < 0.05$ compared with group C; \square , $P < 0.05$ compared with group D.

eration. In various tumor tissues, tumor infiltration and metastasis are correlated with VEGF level, and endostatin can inhibit tumor angiogenesis via specifically inhibiting growth and migration of endothelial cells [7, 8].

Isorhamnetin is a flavonoid compound existing in multiple plants, and is the major active ingredient of sea buckthorn. It has various pharmaceutical roles against cardiac ischemia, and thus is widely applied in treating cardiovascular diseases. *In vitro* studies showed that isorhamnetin had significantly anti-tumor effects in gastric cancer, breast carcinoma or lung cancer cells. It can exert anti-tumor effects via induc-

ing tumor cell apoptosis and inhibiting telomerase activity, and can suppress the expression of P-glycoproteins for multi-drug resistance of tumors, and also can induce Lewis lung cancer cell apoptosis via suppressing Bcl-2 or PCNA protein expression and up-regulating expression of tumor suppressor genes P53 or Bax [9, 10]. This study constructed a Lewis lung cancer mouse model, on which the effect of isorhamnetin on the protein expression of VEGF and angiogenesis suppressing factor Endostatin or MMP-2 was analyzed, to investigate the possible mechanisms.

Materials and methods

Reagent and materials

Mouse Lewis lung cancer cell line was purchased from Cell Biology Institute, Chinese Academy of Science (Shanghai, China), and was kept in DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 18% fetal bovine serum (FBS) (Gibco, Waltham, MA, USA) in a 37°C incubator with 5% CO₂. Culture medium was changed every other day, and was passed every 5 days.

Experimental animals

Male SPF grade C57BL/6 mice (N=30, 4~6 weeks, body weight 18~20 g) were provided by Laboratory Animal Center of Tumor Institute, Chinese Medicine Academy, and were kept in an SPF grade animal facility with food and water *ad libitum*.

Mice were used for all experiments, and all procedures including the permission of animal use were approved by the Animal Ethics Committee of Huashan Hospital of Fudan University of Shanghai.

Reagents

Isorhamnetin was provided by Botany Institute, Chinese Academy of Science. DMEM culture medium and FBS (Gibco, Waltham, MA, USA). Cisplatin injection fluid (Qilu Pharm, Jinan, China). Rabbit anti-mouse VEGF, MMP-2 and Endostatin antibody, SP immunoassay kit, and protein quantification kit were provided by Boster (Wuhan, China). IL-2, IFN- γ , Endostatin, and VEGF ELISA kits were all purchased by Jiancheng Bio (Nanjing, China).

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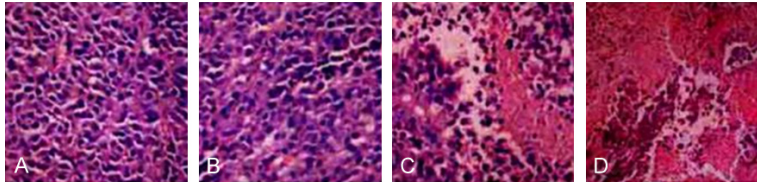


Figure 3. Morphology of Lewis lung cancer mouse tumor tissues with isorhamnetin treatment (HE, $\times 400$). (A) Model group; (B) Isorhamnetin group; (C) cisplatin group; (D) Isorhamnetin + cisplatin group. Model (A) group showed irregular cell shape with close proximity and platelet like distribution, plus pathological nuclear division, blurred mesenchymal-soma boundary and few necrosis. In group (B-D), sparsely distributed cells with nuclear lysis, condensation and cell necrosis lesion.

Subcutaneous transplantation of Lewis lung cancer cells

Lewis lung cancer cells at log-growth phase were prepared into single cell suspension, and were inoculated subcutaneously into left forearm pit under sterile conditions ($1 \times 10^6/L$, 0.2 mL for each animal). General conditions of mice were observed daily. Long axis diameter (a) and short axis diameter (b) were measured from subcutaneous tumors, whose volume was calculated as $V = \pi/6(a \times b^2)$ for plotting tumor growth curves.

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Animal grouping and drug delivery

Animals were randomly assigned into control group (A), model group (B), 50 mg/kg isorhamnetin group (C), 2 mg/kg cisplatin group (D) and 50 mg/kg isorhamnetin + 2 mg/kg cisplatin group (E, N=10 each). Lewis lung cancer cells were subcutaneously injected in all groups except group A. On day 2 after inoculation, 0.2 ml drug solution was injected intraperitoneally daily for 14 consecutive days. Control group (A) received equal volume of saline. Drug dosage was chosen based on pilot study.

Tumor suppression rate

All mice were sacrificed and dissected for tumor tissues. Subcutaneous metastatic tumor volume and weight were measured for calculating tumor suppression rate, which was equal to $(\text{tumor weight in control group} - \text{tumor weight of experimental group}) / \text{tumor weight of control group} \times 100\%$. Lung tissues were collected and fixed with BOUIN's buffer. The number of metastatic lesions on pulmonary lobular surface (presented as white spots) was counted under a microscope.

Serum index assay

Venous blood samples were centrifuged for collecting serum, in which levels of IL-2, IFN- γ , Endostatin and VEGF were measured by ELISA kits following the manual instructions. Absorbance (A) values were measured by a microplate reader.

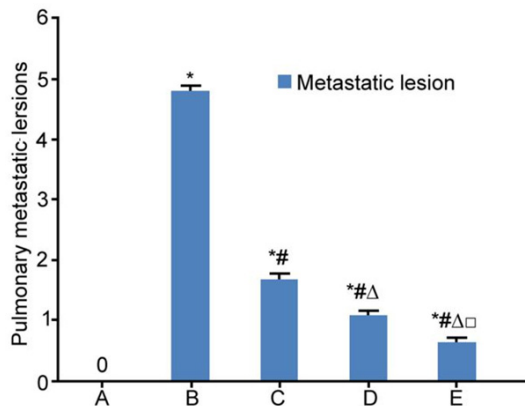


Figure 4. Effects of isorhamnetin on pulmonary metastatic lesion number in Lewis lung cancer mice. A: Control group; B: Model group; C: Isorhamnetin group; D: Cisplatin group; E: Isorhamnetin + cisplatin group. *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with model group; Δ , $P < 0.05$ compared with group C; \square , $P < 0.05$ compared with group D.

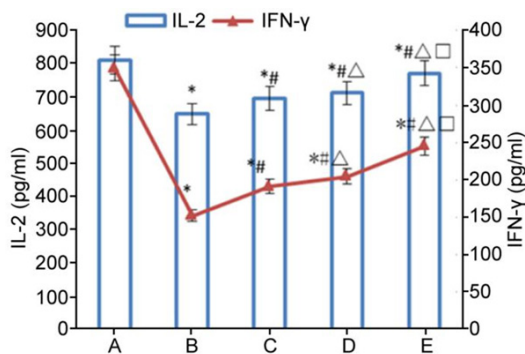


Figure 5. Effects of isorhamnetin on serum IL-2 and IFN- γ levels. A: Control group; B: Model group; C: Isorhamnetin group; D: Cisplatin group; E: Isorhamnetin + cisplatin group. *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with model group; Δ , $P < 0.05$ compared with group C; \square , $P < 0.05$ compared with group D.

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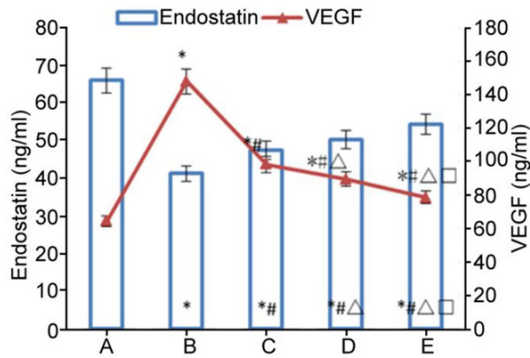


Figure 6. Effects of isorhamnetin on serum Endostatin and VEGF levels in Lewis lung cancer mice. A: Control group; B: Model group; C: Isorhamnetin group; D: Cisplatin group; E: Isorhamnetin + cisplatin group. *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with model group; Δ , $P < 0.05$ compared to group C; \square , $P < 0.05$ compared with group D.

HE staining

Mice were sacrificed and observed for general morphology of transplanted tumors. Tumor tissues were fixed in paraformaldehyde and embedded in paraffin for preparing 5 μm slices for hematoxylin-eosin staining. After mounting coverslips, morphology of tumors was observed by a light field microscopy.

Endostatin, VEGF and MMP-2 protein expression in tumor tissues

Tumor tissues were collected for measuring Endostatin, VEGF or MMP-2 protein expression by immunohistochemistry SP approach, by the aid of microscopic observation following manual instruction of SP test kit. Tumor tissues were prepared for 4 μm consecutive slices for IHC staining using antibodies against Endostatin (1:50), VEGF (1:100) and MMP-2 (1:100). Paraffin slices were de-waxed and treated for antigen-retrieval by microwaves. DAB substrate was then added for development, followed by hematoxylin counter-staining and coverslip mounting. Slices were examined under a light field microscope. Results deduction: Yellow or brown granules in cytoplasm or membrane were identified as positive staining. Under high mag ($\times 400$) lens, number of positive cells and staining intensity were analyzed. Quantitative analysis was performed for measuring averaged absorbance values of positive staining area.

Statistical analysis

SPSS 19.0 software was used for analysis. Those measurement data fitted normal distribution were presented as mean \pm standard deviation (SD), and were tested by analysis of variance (ANOVA), and LSD test. A statistical significance was defined when $P < 0.05$.

Results

Tumor growth and general conditions of mice

6 days after inoculation, tactile swelling can be found in the injection site. One week after inoculation, tumor growth was further accelerated with 100% tumor formation rate. With elongated treatment time, tumor growth velocity was highest in model group, and was lower in group C, D and E in sequential (**Figure 1**). Few animals died in model and isorhamnetin treatment group: one mouse in model group died on D12 due to tumor compression of pulmonary tissues and respiratory distress, and one mouse from isorhamnetin group died due to necrosis of subcutaneous tumor on D13. Model group mice showed loss of fur shininess and decreased food/water intake, and slow movement or unresponsiveness after inoculation. All drug treatment groups showed improvement of mental status.

Tumor suppression rates

Model group showed a relatively larger tumor volume. All drug treatment groups showed decreased tumor weight ($P < 0.05$ compared with model group). Tumor suppression rate was highest in group E, and was lower in group D and group C in sequential ($P < 0.05$, **Figure 2**).

Effects of isorhamnetin on tumor pathomorphology of Lewis cancer mice

Model group mice had more tumor cells with fruitfully growth, in a platelet distribution. Drug treatment decreased the number of tumor cells, which were sparsely distributed in a string-like pattern with necrotic spots (**Figure 3**).

Effects of isorhamnetin on serum IL-2, IFN- γ , Endostatin and VEGF levels

Compared with control group, model group mice showed decreased serum IL-2, IFN- γ and

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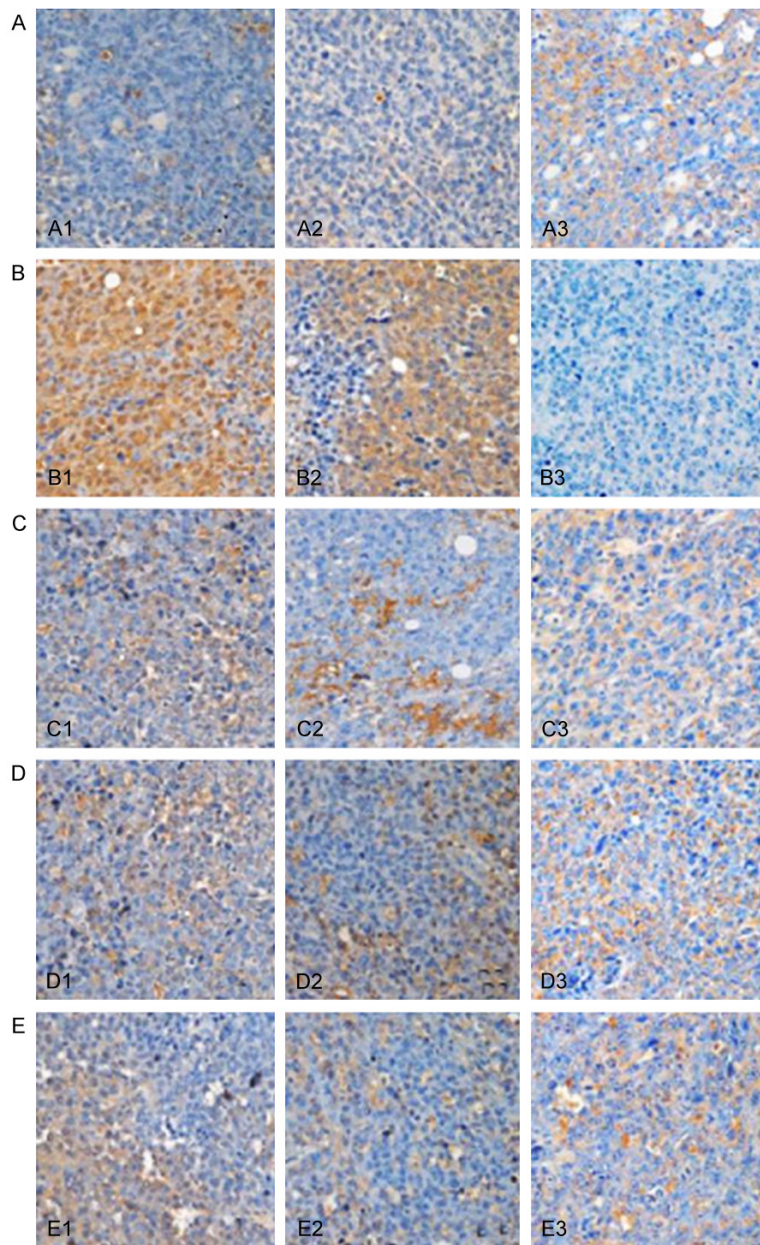


Figure 7. Effects of isorhamnetin on VEGF, MMP-2 and Endostatin protein expression in Lewis lung cancer mice ($\times 400$). A: Control group; B: Model group; C: Isorhamnetin group; D: Cisplatin group; E: Isorhamnetin + cisplatin group. 1, VEGF positive expression mainly locates in cytoplasm or membrane as shown by brown yellow granules; 2, MMP-2 positive expression in cytoplasm; 3, Endostatin in cytoplasm.

Endostatin levels, plus elevated pulmonary metastatic nodule number and higher VEGF levels ($P < 0.05$). All drug treatment groups showed significantly lowered pulmonary metastatic nodules ($P < 0.05$). Serum IL-2, IFN- γ and Endostatin levels were highest in group E, and lower in group D and group C in sequential

($P < 0.05$). Serum VEGF level was lowest in group E, and higher in group D and C in sequential ($P < 0.05$, **Figures 4-6**).

Effects of isorhamnetin on VEGF, MMP-2 and Endostatin protein expression in Lewis lung cancer mice

Compared with control group, model group had elevated VEGF and MMP-2 protein expression in metastatic tumor tissues, with lowered Endostatin protein expression ($P < 0.05$). Compared with group B, combined treatment group (E) had the highest Endostatin level and lowest VEGF or MMP-2 protein levels in metastatic tumor tissues, followed by group D and group C in sequential ($P < 0.05$, **Figures 7 and 8**).

Discussion

Pathogenesis, occurrence, invasion and migration of lung cancer or other malignant tumors all depend on tumor angiogenesis, which involves ECM degradation and cell migration. The ratio between angiogenesis inhibitory and facilitating factors affects such process. Endostatin is a highly specific endogenous angiogenesis inhibitor and can suppress tumor angiogenesis via suppressing eNOS activation and subsequent blockade of VEGF-induced signal transduction pathway [11, 12]. VEGF can facilitate vascular endothelial cell division, and facilitate angiogenesis and regulate endothelial proliferation via specific binding to membrane receptor. Previous studies showed elevated expression of VEGF in various tumor tissues or cell lines, which was correlated with tumor infiltration metastasis and prognosis [13, 14]. Isorhamnetin exists in

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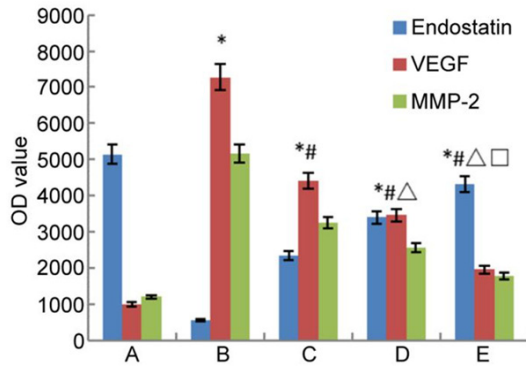


Figure 8. Expression intensity of VEGF, MMP-2 and Endostatin protein in Lewis lung cancer mice. A: Control group; B: Model group; C: Isorhamnetin group; D: Cisplatin group; E: Isorhamnetin + cisplatin group. *, $P < 0.05$ compared with control group; #, $P < 0.05$ compared with model group; Δ, $P < 0.05$ compared to group C; □, $P < 0.05$ compared with group D.

various plant cells and has similar anti-tumor activity and similar structure to quercetin, which is related with anti-oxidative response [15, 16]. Both *in vivo* and *in vitro* studies showed that isorhamnetin could inhibit or kill various tumor cells: *in vitro* study showed that it significantly inhibited nasopharyngeal CNE-2 cell growth or proliferation, kill mouse derived tumor cell lines Lewis or C6, inhibited breast cancer cell line MCF7 or BT549 proliferation, inhibited gastric tumor cell and skin cancer cell proliferation via modulating peroxidase activation receptor and MEK1 or PI3K activity, and facilitated tumor cell apoptosis by activating mitochondrial apoptosis pathway [17, 18]. Isorhamnetin can affect tumor cell proliferation via arresting cell cycle, and induce tumor cell apoptosis via facilitating Bax expression, inhibiting apoptosis protection factor Bcl-2 expression, and activating Caspase-3 [19, 20]. Currently, most *in vitro* studies investigating tumor suppressing effects of isorhamnetin mainly focused on tumor suppressor gene, oncogene and relate protein expression, or cell proliferation, with little knowledge about its anti-tumor effects on lung cancer animal model or anti-tumor angiogenesis. This study established Lewis lung cancer mouse model, on which the effect of isorhamnetin on Lewis lung cancer mouse and related mechanisms were studied. Results showed that isorhamnetin could inhibit Lewis lung cancer mouse tumor growth or tumor angiogenesis, which were consistent with a previous study conducted by Li et

al [13], and indicating significantly suppressing function against lung cancer by isorhamnetin.

During vessel-free phase, tumor growth is mainly dependent on diffusion of peripheral tissues for supplying nutrients, thus limiting tumor invasion. During vascular phase, tumor growth depends on angiogenesis of micro-vessels, which can significantly accelerate tumor growth velocity and metastasis. During tumor vascular invasion and metastasis, MMP-2 plays an important role [21, 22]. This study showed decreased serum Endostatin level, elevated VEGF expression, higher VEGF and MMP-2 protein expression in metastatic tumors as well as lower Endostatin protein expression in control group. Drug treatment groups showed significantly decreased tumor volume or pulmonary metastatic nodules number, plus higher serum Endostatin level and lower VEGF level. In metastatic tumor, VEGF and MMP-2 proteins were down-regulated, along with higher Endostatin protein expression. Combined treatment group had higher tumor suppressor rate than cisplatin group, which had a higher rate than that of isorhamnetin, indicating that isorhamnetin might suppress metastatic tumor growth on Lewis lung cancer mice, via suppressing VEGF or MMP-2 expression in lung cancer tissues, and facilitating Endostatin expression for inhibiting tumor angiogenesis and resisting tumors with synergistic effects after combined treatment with cisplatin. In immune treatment for tumors, cell immunity is the major way as IL-2 can reflect T cell function and exert tumor killing effects via potentiating NK cell killing activity, inducing LAK cell production and facilitating T cell proliferation. As an immune cytokine, IFN- γ has both *in vivo* and *in vitro* effects on suppressing tumor angiogenesis via down-regulating tumor angiogenesis factors expression. During lung cancer pathogenesis and progression, both IFN- γ and IL-2 play important roles and can reflect tumor cell growth activity as well as the immune status of lung cancer patient at certain extents. Results of our study showed significantly lowered IFN- γ and IL-2 levels in model group, whilst drug treatment groups had elevated cytokine levels, indicating that isorhamnetin could exert anti-tumor effects possibly through regulating IFN- γ and IL-2 levels. This study, however, only investigated the anti-angiogenesis effects of isorhamnetin, but the functional mechanism at genetic level

was not investigated, which requires further studies.

Conclusion

Isorhamnetin can inhibit progression of Lewis lung cancer on mice, probably via suppressing VEGF or MMP-2 expression and up-regulating Endostatin expression.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Songtao Lai, Department of Radiation Oncology, Fudan University Shanghai Cancer Center, No. 270, Andong Road, Xuhui District, Shanghai, China. Tel: +86-021-64175590; Fax: +86-021-64175590; E-mail: songtaolai9@yeah.net

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