Antitumor and anti-metastatic mechanisms of *Rhizoma paridis* saponins in Lewis mice

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1 INTRODUCTION

Lung cancer is one of the most common diagnosed malignancies worldwide. The major causes of death in lung cancer include tumor burden, infection, and metastasis.¹ Therefore, inhibition of metastasis could be regarded as one of the therapeutic strategies for lung cancer. Metastasis formation is correlated with initial primary tumor growth by angiogenesis and intravasation into the blood stream through degradation of matrix barriers and inducement of cellular motility, survival and transport at a distal site, and formation of a metastatic lesion based on cell-cell and cell-matrix adhesion.² Genes that regulate functions, such as unlimited growth potential, angiogenesis, migration, and so forth, are involved in giving lung cancer tumors invasive and metastatic competence.³

*Paris polyphylla* var. *yunnanensis* (Fr.) Hand-Mazz., mostly distributed in the Southwest of China, is a traditional Chinese medicinal herb that has been used in treating cancer for many years.⁴ *Rhizoma paridis* saponins (RPS) as the effective parts of *Paris polyphylla* showed strong anti-lung cancer and pulmonary metastasis in different models.⁵,⁶ According to the previous reports, the inhibition of RPS on pulmonary metastasis might be associated with the amelioration of inflammation responses, decrease in reactive oxygen species (ROS),⁷ upregulation of TIMP-2 and downregulation of MMP-2 and MMP-9 levels.⁵ Meanwhile, other researchers reported that some monomers isolated from RPS exhibited improving the body’s immune system by diosgenyl saponins⁸ and inhibition of angiogenesis by Paris saponin II⁹,¹⁰

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**Abbreviations:** CAT, catalase; DDP, cisplatin; Erk, extracellular signal-regulated kinase; IL, interleukin; JNK, c-Jun N-terminal protein kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa B; PI3K, phosphatidylinositol-3-kinase; RAGE, receptor for advanced glycation end-products; ROS, reactive oxygen species; RPS, *Rhizoma paridis* saponins; SOD, Superoxide dismutase; VEGF, vascular endothelial growth factor.
However, previous research was not focused on RPS influencing the comprehensive metastasis formation including angiogenesis, invasion, and adhesion involved in the inflammation, oxidation, immunity response, and so forth. For it is difficult to understand the antitumor mechanism of RPS from a holistic view, here we successfully established Lewis pulmonary adenoma mice to analyze the pathways involved in RPS intervening tumor formation and progression.

2 | MATERIALS AND METHODS

2.1 | Drugs

The dried rhizoma of *Paris polyphylla* var. *yunnanensis* was collected in September 2010 from Lijiang, Yunnan Province, China, and identified by Professor Gao. A voucher specimen (GWCL201009) was deposited at the School of Pharmaceutical Science and Technology at Tianjin University, Tianjin, China. RPS was prepared as previously described. In briefly, the dried crushed roots of *Paris polyphylla* were extracted with 70% ethanol. The combined 70% ethanol extracts were concentrated and then filtered and centrifuged. The supernatant dissolved in water was then eluted by 65% ethanol on macroporous adsorptive resin D101. The eluent was finally condensed with a vacuum rotary evaporator to give a gray, viscous extract, which was RPS. Cisplatin (DDP) was purchased from Qilu Pharmaceutical Co., Ltd. (Jinan, China).

2.2 | Animals

18 male C57BL/6 mice were purchased from the Laboratory Animal Center of academy of Military Medical Sciences (Beijing, China quality certification number: SCXK (Jun) 2014-0004). After 1 week of acclimatization, the mice were housed in a room maintained at 24 ± 1°C, relative humidity of 55 ± 5%, artificial lighting from 8:00 to 19:00 and air-exchange rate of 18 times per hour. The animals were inoculated with lung adenocarcinoma cells (5 × 10³) s.c. in the armpit area. Before carrying out the animal experiment, we measured the body weight of each mouse. Five days after injection of Lewis cells, C57BL/6 mice with tumor volume reaching 100 mm³ on 6th day were randomly divided into three groups:

- The RPS group (RPS) received an intragastric administration of 100 mg of RPS in 10 mL of 0.9% sodium chloride per kg of body weight every day for a month.
- The non-treated group (Model) received oral administration of saline solution every day.
- The cisplatin group (DDP) received an intraperitoneal injection of 20 mg of DDP in 10 mL of 0.9% sodium chloride per kg of body weight every other day for 1 week.

Individual body weight was measured and recorded at least once a week. Individual food/water consumption was measured and recorded every other day. The mice were sacrificed at the conclusion of the 4-week treatment, and autopsies were harvested. Major organs, such as lung, spleen, and tumor mass, were then dissected and their weight was measured. Portions of each tissue were fixed in 10% formalin (pH 7.4) for histology, snap frozen in and maintained at −80°C until analysis.

2.3 | Histopathological examination

For the histopathological examination, portions of lung tissue were fixed in 10% formalin. After proper dehydration, the lung tissues were embedded in paraffin wax. Five-μm-thick sections were prepared and stained with hematoxylin and eosin. Every organ was randomly cut into three histological sections. Histopathology examination was completed using Nikon eclipse TE2000-U Microscope and performed by a pathologist who was unaware of whether tissues were treated.

2.4 | Mouse cytokine antibody microarray

The RayBio Custom Mouse Mouse Cytokines Antibody Array kit was purchased from RayBio (RayBio, Shanghai, China) and used according to the manufacturer’s instructions. Briefly, total proteins from tumor tissues were isolated using RayBio Cell Lysis Buffer (RayBio, Shanghai, China). After blocking with 1 × blocking buffer (provided by the manufacturer), membranes were incubated for 2 hours with 80 μL of each sample. Following wash steps, each array was incubated with 70 μL of biotin-conjugated antibodies for 2 hours. Then the membranes were washed again and incubated with 70 μL of 1500-fold diluted streptavidin-conjugated fluorescent dye for 2 hours. Subsequently, cover the incubation chamber with Adhesive film. Cover the plate with aluminum foil to avoid exposure to light and incubate in dark room temperature for 2 hours. Soon, incubation chambers were discarded and slides were aspirated, washed, and dried completely in air at least 20 minutes. Dried slides were immediately scanned and imaged using a GenePix 4000B microarray scanner and GenePix 5.0 software (Axon Instruments, Union City, CA). All fluorescence intensities in scanned images were reported as background corrected mean fluorescence intensities of the pixels within the spot ellipse.

2.5 | RT-PCR

Total RNA was isolated from tumor tissues using TRIzol (Life Technologies, Inc. Shanghai, China) according to the manufacturer’s instruction. The concentration and purity of RNA was determined by using Nano Drop spectrophotometer (ND-1000, Wilmington, Delaware). cDNA was synthesized by two-step RT-PCR Kit (TIANGEN BIOTECH, Shanghai, China). Resulting reverse transcription products were stored at −80°C until analysis. Polymerase chain reactions were performed in a final volume of 50 μL, which contained 2 × Taq Master Mix (25 μL), 2 μL of cDNA, 2 μL each of the forward and reverse primers and RNase-Free water. Polymerase chain reaction products were electrophoresized on 3.0% agarose gel and visualized after ethidium bromide staining.

2.6 | Western blot analysis

Total proteins from tumor tissues were isolated using the tissue protein extraction kit (Sangon Biotech, Shanghai, China), and the obtained protein was quantified by the Bradford Assay Kit (Biyuntian, Shanghai, China). The protein samples (20–100 μg) were separated on 12% SDS-
Proteins were transferred to PVDF membranes (Millipore Corp. Burlington, Massachusetts, USA) and probed with β-actin (1:1000, Bio-world AP0060), p38 (1:200, santa sc-7972), P-p38MAPK (1:1000, Cell Signaling #4511), JNK(1:100, Boster BA1219), P-JNK (1:1000, Cell Signaling #9251), Erk(1:100, Boster BM4326) P-Erk (1/2) (1:1000, Cell Signaling #4370S), NF-kB (1:500, Bioworld BS1253), AKT (1:100, Boster BM612), P-AKT (1:500, Bioworld BS4007), PI3K (1:1000, Cell Signaling #4228) antibodies followed by appropriate secondary antibody. Equal protein loading was checked by quantifying β-actin. The relative optical densities of the bands were quantified using Odyssey infrared imaging system (LI-COR Biotechnology Lincoln, Nebraska, USA).

2.7 | SOD and catalase activity

SOD and catalase activity was determined using the superoxide dismutase assay kit and Catalase assay kit (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China) following the manufacturer’s instructions.

2.8 | Statistic processing

SPSS 17.0 for Windows (SPSS, Inc. New York, USA) was used to analyze the data. Data were expressed as the mean ± standard error (SE). Multi-group comparisons were performed through One-way ANOVA test, while couple comparisons were performed through q test. Differences were considered statistically significant if P values were less than 0.05.

3 | RESULTS

3.1 | General observation of RPS and DDP on Lewis mice

Final body weight did not vary among the control mice and the treated ones. However, for 1-week treatment, the body weight of DDP group significantly dropped compared with the model group within the second and the third weeks. DDP also decreased the food and water consumption during DDP-treated period. In contrast, RPS did not show any toxicity to mice (Figure 1).

3.2 | Histopathological examination

Drug treatment did not only reduce the tumor volume, but also make tumor masses grow much slower than the model ones (Figure 2B). Under the general observations and light microscope, tumor tissues were centered within dermis with irregular and asymmetric outlines. There were various degrees of inflammatory infiltration, pulmonary congestion, stromal invasion, and vascular invasion in Lewis mice lungs. RPS significantly decreased the tumor embolus in lung tissues and relieved infiltration of inflammatory cells and alveolar septal thickening (Figure 2).

3.3 | Screening of the content of differential cytokines between RPS and model groups

To determine the possible mechanism that RPS inhibited pulmonary metastasis, we evaluated the levels of pro-inflammatory cytokines and chemokines in the tumor tissues of the Lewis mice, which may increase pulmonary metastasis.12 As shown in Table 1, RPS significantly decreased the levels of CXCL16, RAGE, IL-17B R, VEGF R3, and VEGF D in tumors. All these cytokines or receptors as initiators induced tumor cell proliferation, adhesion, angiogenesis, and invasion.

3.4 | RPS inhibiting migration-related genes in Lewis mice

To understand the antitumor and anti-metastatic mechanisms of RPS in Lewis pulmonary adenoma mice, genomic analyses were used to deduce the pathways involved in drug intervening tumor formation and progression. As shown in Table 2, RPS significantly decreased mRNA levels of invasion enzymes (MMP2, MMP8, MMP9, MMP10, MMP12, and MMP13), angiogenic factor (VEGFA), adhesion factors (VCAM-1 and CTNNB1), lymphatic hyaluronan transport (LYVE-1), and transcription factors (HIF1A, NF-xB, Jun, and STAT3), which would induce tumor cell proliferation, adhesion, angiogenesis, and invasion. Meanwhile, RPS remarkably increased tissue inhibitor of MMPs such as TIMP-1 and TIMP-2. DDP influenced parts of genes in Lewis mice.
3.5 Treatment with RPS inhibiting PI3K/AKT, NF-κB, MAPK pathways and inducing antioxidant stress in Lewis mice

According to the above research, RPS and DDP influenced the formation of pulmonary metastasis through inhibition of angiogenesis, invasion, adhesion, and cancer-related inflammation. Then the downstream signaling proteins of the phosphatidylinositol-3-kinase (PI3K)/Akt, NF-κB, and mitogen-activated protein kinase (MAPK) pathways were analyzed by western blot assays. As a result, DDP and RPS significantly inhibited phosphorylation of Akt, PI3K, p38, Erk1/2, and JNK, and decreased the level of NF-κB in Lewis tumor tissues (p < .05) (Figure 3A,B). Considering our previous results that redox regulation might be altered within the tumor microenvironment, the content of SOD and catalase enzymes was determined in the tumors tissues. As a result, RPS significantly increased antioxidant index of SOD and catalase (Figure 3C) which reduced concentration of ROS in tumor tissues.

4 DISCUSSION

Tumor metastasis happened with a series of steps including vessel formation, cell attachment, invasion, proliferation, and so forth. In this research, RPS treatment did not only make tumor masses grow much slower than the model ones, but also inhibit the pulmonary metastasis in Lewis mice (Figures 1 and 2). According to the previous reports, the inhibition of RPS on pulmonary metastases might be associated with the amelioration of inflammation responses, decrease in ROS,\textsuperscript{7} upregulation of TIMP-2 and downregulation of MMP-2 and MMP-9 levels.\textsuperscript{5} However, these researches did not reveal the antitumor mechanism of RPS from a holistic view. Therefore, we analyzed the pathway involved in RPS intervening tumor formation and progression in this study.

As we known, activation of VEGF, AGE, and inflammatory cytokines such as chemokine, TNF, and IL receptor could mediate a series of physiological and pathological events, especially the recruitment of lymphocyte as well as tumor growth and metastatic spread. In this experiment, RPS containing pleiotropic molecules capable of interacting with several major molecular targeted including VEGFD/VEGFR3, RAGE, IL6R, IL17BR, and CXCL16 which were involved in inflammation and cancer in tumor tissues (Table 1). Among these cytokines/receptors, RPS downregulated VEGF-D/VEGFR-3 to suppress lymphangiogenesis and lymph node metastasis,\textsuperscript{13} and reduced the level of RAGE to restrain the inflammation-related cancer.\textsuperscript{14} Meanwhile, RPS neutralized CXCL16\textsuperscript{15} and decreased levels of IL-17BR\textsuperscript{16} and IL-6R\textsuperscript{17} to inhibit the proliferation and invasiveness of lung cancer.

Moreover, inflammatory cytokines and oxidative stress were two critical mediators in cancer. RPS did not only decrease levels of inflammatory cytokines, but also increased antioxidant index of SOD and catalase.

| TABLE 1 | Representative lung adenocarcinoma biomarkers from Lewis mice after drug treatment |

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Fold change (RPS/Model)</th>
<th>Biomarkers</th>
<th>Fold change (RPS/Model)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL16</td>
<td>↓</td>
<td>RAGE</td>
<td>↓</td>
</tr>
<tr>
<td>IL-6</td>
<td>-</td>
<td>VEGF</td>
<td>-</td>
</tr>
<tr>
<td>IL-6 R</td>
<td>↓</td>
<td>VEGF R1</td>
<td>-</td>
</tr>
<tr>
<td>IL-17B</td>
<td>-</td>
<td>VEGF R2</td>
<td>-</td>
</tr>
<tr>
<td>IL-17B R</td>
<td>↓</td>
<td>VEGF R3</td>
<td>↓</td>
</tr>
<tr>
<td>TNF-α</td>
<td>-</td>
<td>VEGF D</td>
<td>↓</td>
</tr>
</tbody>
</table>

\textsuperscript{<} Mean without significant difference between two groups.
antioxidant enzymes, RPS significantly inhibited NF-
CXCL16,21 VEGF-D/VEGFR-3,13 RAGE,14 and so forth would regulate
catalase (Figure 3C) which descend ROS affecting the angiogenesis sig-
TABLE 2
Representative metastasis-related gene in Lewis mice after drug treatment

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Compared between</th>
<th>Known or proposed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catenin (cadherin associated protein)1</td>
<td>CTNNB1</td>
<td>[*]</td>
<td>[*] Mediate cell-cell adhesion and recognition</td>
</tr>
<tr>
<td>Vascular cell adhesion protein 1</td>
<td>VCAM-1</td>
<td>[*]</td>
<td>Important in cell-cell recognition. Function in</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>leukocyte-endothelial cell adhesion</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular endothelial growth factor A</td>
<td>VEGFA</td>
<td>[*]</td>
<td>[*] Stimulates vasculogenesis and angiogenesis</td>
</tr>
<tr>
<td>Lymphangiogenesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lympathic vessel endothelial hyaluronan receptor-1</td>
<td>LYVE-1</td>
<td>[*]</td>
<td>[*] Function in lymphatic hyaluronan transport and have a role in tumor metastasis</td>
</tr>
<tr>
<td>Invasive enzyme</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrix metallo-proteinase-2</td>
<td>MMP-2</td>
<td>[*]</td>
<td>[*] Substrates include gelatin, Col I, II, III, IV, VII, X</td>
</tr>
<tr>
<td>Matrix metallo-proteinase-8</td>
<td>MMP-8</td>
<td>[*]</td>
<td>[*] Substrates include Col I, II, III, VII, VIII, X, aggrecan, gelatin</td>
</tr>
<tr>
<td>Matrix metallo-proteinase-9</td>
<td>MMP-9</td>
<td>[*]</td>
<td>[*] Substrates include gelatin, Col IV, V</td>
</tr>
<tr>
<td>Matrix metallo-proteinase-10</td>
<td>MMP-10</td>
<td>[*]</td>
<td>[*] Substrates include Col IV, laminin, fibronectin, elastin</td>
</tr>
<tr>
<td>Matrix metallo-proteinase-12</td>
<td>MMP-12</td>
<td>[*]</td>
<td>[*] Substrates include elastin, fibronectin, Col IV</td>
</tr>
<tr>
<td>Matrix metallo-proteinase-13</td>
<td>MMP-13</td>
<td>[*]</td>
<td>[*] Substrates include Col I, II, III, IV, IX, X, XIV, gelatin</td>
</tr>
<tr>
<td>Matrix metallo-proteinase-14</td>
<td>MMP-14</td>
<td>[*/-]</td>
<td>[*] Type-I transmembrane MMP; substrates include gelatin, fibronectin, laminin</td>
</tr>
<tr>
<td>Tissue inhibitor of metalloprotease 1</td>
<td>TIMP-1</td>
<td>[*]</td>
<td>[*] Collagenase inhibitor</td>
</tr>
<tr>
<td>Tissue inhibitor of metalloprotease 2</td>
<td>TIMP-2</td>
<td>[*]</td>
<td>[*] Interaction inhibits MMP2 activity</td>
</tr>
<tr>
<td>Transcription factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia inducible Factor 1, Alpha Subunit</td>
<td>HIF1A</td>
<td>[*]</td>
<td>[*] Regulator of cellular and systemic homeostatic response to hypoxia by activating transcription of many genes, including those involved in energy metabolism, angiogenesis, apoptosis, and other genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia</td>
</tr>
<tr>
<td>Jun proto-oncogene</td>
<td>Jun</td>
<td>[*]</td>
<td>[*] Involved in angiogenesis; axon regeneration; cellular process</td>
</tr>
<tr>
<td>NF-Kappa beta</td>
<td>NF-κB</td>
<td>[*]</td>
<td>[*] It’s associated with a number of inflammatory diseases, while its persistent inhibition leads to inappropriate immune cell development or delays cell growth</td>
</tr>
<tr>
<td>Signal transducer and activator of transcription 3</td>
<td>STAT3</td>
<td>[*]</td>
<td>[*] Mediates the expression of a variety of genes in response to cell stimuli, such as cell growth and apoptosis</td>
</tr>
</tbody>
</table>

*P < .05, compared with the model group; */- *represents no significance.

catalase (Figure 3C) which descend ROS affecting the angiogenesis signals to downstream targets such as AKT, Erk1/2, HIF-1, and VEGF.18

Through the interaction with the above cytokines/receptors and antioxidant enzymes, RPS significantly inhibited NF-κB, and phospho-
ylation of PI3K/Akt and MAPK (including p38, Erk1/2, and JNK) (P < .05) (Figure 3). Among these pathways, decrease in cytokines such as IL-1β, CXCL16,19 and RAGE14,20 could mediated NF-κB pathway. Suppression of IL-6,17 CXCL16,19 VEGF-D/VEGFR-3,13 and ROS18 might influence PI3K/Akt signaling pathway. Inhibition of CXCL16,21 VEGF-D/VEGFR-3,13 RAGE,16 and so forth would regulate Erk1/2, JNK, and p38-MAPK signaling pathways.

RPS sequentially changed mRNA expression of nuclear factors including NF-κB, HIF-1A, STAT3, and Jun (Table 2), and consequently suppressed the expression of angiogenesis, lymphangiogenesis, adhesion, inflammatory factors, and invasive enzymes (Figure 4). For example, suppression of STAT3 activation decreased the levels of c-Myc, Cyclin D1, Bcl-xL, Survivin, and VEGF which were identified as Stat3 target genes that promoted Stat3-mediated tumor cell migration and invasion.22 RPS also downregulated the mRNA level of HIF-1A, which activated the transcription of target genes encoding proteins, promoted angiogenesis, lymphangiogenesis, and remodeled extracellu-
lar matrix in the lungs through increasing the product of secreted factors, such as VEGF, LYVE-1, MMPs, and so forth.23 In addition, RPS decreased levels of NF-κB and Jun nuclear transcriptional factors to regulate expression of multiple genes involved in tumor growth, metastasis, and angiogenesis containing MMP-2,24 MMP-9,25 VEGF, ILs, adhesion molecules, and so forth.26

Taken together, RPS treatment did not only make tumor masses grow much slower, but also inhibit the pulmonary metastasis in Lewis mice. For tumor metastasis happened with a series of steps including
vessel formation, cell attachment, invasion, proliferation, and so forth. RPS containing multiple compounds interacted with several major molecular targets including VEGFD, VEGFR3, RAGE, IL6R, IL17BR, and CXCL16, and raised the content of SOD and catalase enzymes. Soon after, RPS significantly inhibited the aberrantly active NF-κB, and phosphorylation of PI3K/Akt and MAPK (including p38, Erk1/2, and JNK) signaling pathways, sequentially changed mRNA expression of nuclear factors including NF-κB, HIF-1A, STAT3, and Jun, and consequentially activated the antioxidant and signaling pathways.

**FIGURE 3** The effects of RPS and DDP on the antioxidant and signaling pathways. A, MAPK pathway; B, PI3K; and NF-κB pathway. C, SOD and catalase enzymes in tumor tissues. *P < .05, **P < .01, compared with the model group [Color figure can be viewed at wileyonlinelibrary.com]

**FIGURE 4** Proposed signaling pathways for RPS-mediated pulmonary metastasis [Color figure can be viewed at wileyonlinelibrary.com]
suppressed the expression of angiogenesis, lymphangiogenesis, adhesion, inflammation, and invasion enzymes, which played important roles in many critical aspects of lung cancer growth and metastasis. All in all, this research provided a holistic view to understand the multi-target antitumor mechanisms of RPS which promoted the application of RPS in the future.

ACKNOWLEDGMENTS

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