Objective: To study the effect of Runing II (a Chinese herbal preparation for mammary cancer) on the growth and metastasis of transplanted tumor of mammary cancer MA-891-bearing TA2 mice and its mechanism. Methods: The model of mammary cancer MA-891 cell strain transplanted tumor of TA2 mice with lung metastasis were developed to observe the effect of Runing II on the growth and metastasis of the transplanted tumor. The immunohistochemical method and image analysis were adopted to detect the levels of vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor (VEGFR), and micro-vessel count (MVC) and micro-vessel area (MVA). Results: In the Runing II group, the tumor weight inhibition rate and the lung metastasis inhibition rate were 37.3% and 65.4% respectively, the tumor growth and lung metastasis were obviously inhibited; And the levels of VEGF and VEGFR, MVC and MVA were significantly decreased as compared with those in the tumor-bearing control group (P<0.05). Conclusion: The Chinese herbal preparation Runing II can inhibit the metastasis of tumor through inhibiting the angiogenesis, and the mechanism is possibly related with down-regulation of VEGF and VEGFR expression. Key words: mammary tumor; angiogenesis; vascular endothelial growth factor (VEGF); Runing II (a Chinese herbal preparation for mammary cancer)

Mammary cancer is a malignant tumor severely threatening female health and the disease incidence ranks the first in the female malignant tumors, with a tendency to increase year by year. Although its therapeutic methods have made some advances, the long-term survival rate for the patient of mammary cancer has not been obviously raised because of the invasive growth and metastasis of tumor. The angiogenesis of tumor is a basic condition for growth, invasion and metastasis of tumor. Therefore, inhibition of angiogenesis of tumor is an effective way for preventing production and development of tumor, and prolonging life of the patient.¹ In the present study, the mechanism of Chinese drug Runing II with functions of supplementing qi and nourishing yin, regulating thoroughfare and conception vessels, resolving mass and detoxicating in inhibiting tumor and anti-metastasis was studied from angiogenesis and its regulatory factors in the transplanted tumor of mammary cancer MA-891 of TA2 mice with lung metastasis.

MATERIALS
Sixty SPF female TA2 mice, weighing (20±2) g, aged 6-8 weeks, were purchased from the Department of Laboratory Animals, Tianjin University of Medical Sciences. Mammary cancer MA-891 cell strain of the mouse was supplied by Prof. Luo Liqin, the Pathological Laboratory, Institute of Tumor, Chinese Academy of Medical Sciences.

Runing II is composed of Sheng Huang Qi (Radix Astragali Mongolici), Shan Ci Gu (Pseudobulbus Cremastrae), Tai Zi Shen (Radix Pseudostellariae), Gou Qi Zi (Fructus Lycii), E Zhu (Rhizoma Curcumae Phaeocaulis), Yi Yi Ren (Semen Coicis), Yin Yang Huo (Herba Cryptotaeniae), and other Chinese herbs.
Herba Epimedii), Dang Gui (Radix Angelicae Sinensis), etc., with dose ratio of 1.67:1.33:1.33:1.33:1.33:1.33:1, which were prepared a decoction by the Section of Pharmaceutics, Longhua Hospital, according to traditional technique, and kept at 4°C for use; Cyclophosphamide (CTX, 200mg/ampule, branch number: 981104) and tamoxifen (TAM) were produced by Shanghai Hualian Pharmaceutical Co. Ltd.; Normal saline was produced by Shanghai Fumin Pharmaceutical Co.

Vascular endothelial growth factor (VEGF) polyclonal antibody (working dilutions 1:25–1:50), vascular endothelial growth factor receptor (VEGFR, working dilutions 1:30–1:50), and FVIIIAg polyclonal antibody (working dilution 1:50) were purchased from Santa Cruz Biotechnology Inc; EnVision second antibody, rabbit anti-rat VEGF antibody, goat anti-rabbit FVIII Ag antibody and goat anti-rabbit VEGFR antibody, and EnVision Kit were purchased from Dako Co., Denmark; 3,3-Diaminobenzidine (DAB) was purchased from Shanghai Huamei Bioengineering Factory.

METHODS

Developing of animal model
Animal model was developed with inference to the methods of Luo and Gao, et al.2,3. In vitro cultured mammary cancer MA-891 cells of the mouse at a logarithmic survive phase were selected and the concentration of cell was regulated as 1x10^7/ml. Under aseptic condition the mammalian cancer cells were inoculated into the TA2 mice subcutaneous part of the right axilla (0.2 ml/mouse, i.e. 2X10^6 cells/mouse).

Grouping of animals and methods of administration
Two days after inoculation, the mice were randomly divided into 4 groups, 15 mice in each group. 1) Tumor-bearing control group: 0.4 ml normal saline was administrated intragastrically, once each day, five times each week. 2) Runing II group: Runing II was diluted with normal saline and 0.4 ml of this dilution corresponding to 48 g·kg^-1·d^-1 which was calculated according to the attached table “Conversion Table between Human and Animal Body Surface Areas” in “Methodology of Studies on pharmacology of Chinese Drugs”.4 was given intragastrically, once daily, five times each week. 3) CTX control group: In reference with the dosage used by Jiang Bo,5 CTX was diluted with normal saline and 0.4 ml corresponding to 50 mg·kg^-1·d^-1 was given intra-gastrically, once every other day, thrice each week. 4) TAM control group: TAM was diluted with normal saline and 0.4 ml corresponding to 2.7 mg·kg^-1·d^-1 was administrated intragastrically, once daily, five times each week. The drugs were given for 3 weeks in all the four groups.

Sampling
At the 22nd day after administration, the mice were sacrificed by dislocation of cervical vertebrae, and weighed. Then the tumor mass and the lung were taken and weighed respectively. The real body weight of the mouse = the body weight weighted – the tumor weight. Grey nodes on the lung surface, i.e., metastasis focuses, were counted by naked eyes. The tumor mass was placed into 10% neutral formaldehyde solution for fixation, followed by paraffin embedding, ultrathin sectioning, immunohistochemical Envision two-step straining, and determination of VEGF, VEGFR, microvessel count (MVC) and microvessel area (MVA).

The tumor weight inhibition rate
The tumor inhibition rate = (mean tumor weight of the control group after treatment – mean tumor weight of the medication group after treatment)/mean tumor weight of the control group after treatment x 100%.

The lung metastasis inhibition rate
The lung metastasis inhibition rate = (mean lung metastasis node number of the control group after treatment – mean lung metastasis node number of the medication group)/mean lung metastasis node number of the control group x 100%.

Immunohistochemical Envision two-step staining
The paraffin section of 4 μm was taken, followed by routine deparaffin with xylene, gradient dehydration, washing with PBS, 5 min x 3 times; the section was
Immerged in 0.01 mol/L, pH 6.0 citrate buffer, put in a microwave oven of 98°C for 10 min for antigenic repair and naturally cooled at room temperature after taking from the microwave oven; dripping the first antibody (above-mentioned antibody and dilution of corresponding antibody respectively), 37°C, 90 min, washing with PBS, 5 min x 3 times; dripping Envasion second antibody, at room temperature for 60 min, washing with PBS, 5 min x 3 times; dripping newly prepared DAB color-developing solution, controlling developing color for 3–10 min under a microscope, counterstaining for 45 s with campeachy, rinsing with tap water, drying with a blower, mounting with neutral gum. The cell with the cytoplasm of brown-yellow or black brown granules was regarded as positive. The known positive section of mammary cancer was used as positive control, and that in which PBS buffer substituted for the first antibody was used as negative control.

Image analysis
ACE video camera (resolving power 930 lines) and LEICAQ500W image analyzer and LEICA full-automatic image analysis system were used. Under a microscope x 200, 2 vision fields in each section were randomly selected for determination of VEGF or 3 vision fields for determination of VEGFR and MVA, and the percentage of positive area to whole vision field area were calculated in all of the groups. For determination of MVC, 5 regions with the richest blood vessels were selected in each section under a microscope x 100, and then under x 400 the micro-vessels in each vision field of 5 no-overlapping regions were counted, and the mean was used as average micro-vessel counter (MVC) of the tumor.

Statistical method
Software SPSS8.0 was used and chi-square test was used for comparison between the rates, and t-test or one-way ANOVA was used for comparison of means.

RESULTS
Tumor-inhibiting and anti-metastasis effects of Running II on the transplanted tumor of mammary cancer MA-891-bearing TA2 mice with lung metastasis

It was showed in Table 1 that the tumor weight inhibition rate was 37.3% in the Running II group and 86.2% in the CTX control group, both tumor inhibition rates being >30%, with significant difference (both \( P<0.05 \)) as compared with both tumor-bearing control group and TAM control group, indicating that both Running II and CTX have a certain tumor-inhibiting action. And there was a significant difference (\( P<0.05 \)) between Running II group and CTX control group in the tumor inhibition rate, indicating that the tumor-inhibiting effect of Running II was lower than that of CTX. The lung metastasis inhibition rate was 65.4% in the Running II group and 43.9% in the CTX control group, with significant difference (both \( P<0.05 \)) as compared with tumor-bearing control group and TAM control group, indicating that both Running II and CTX have a certain tumor metastasis-inhibiting action. And there was a significant difference between Running II group and CTX control group (\( P<0.05 \)) in the lung metastasis inhibition rate, suggesting that the anti-metastasis action of Running II is superior to that of CTX.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Tumor weight (g)</th>
<th>Tumor inhibition rate (%)</th>
<th>Number of lung metastasis focus</th>
<th>Metastasis inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor-bearing control</td>
<td>10</td>
<td>3.45±1.27</td>
<td>–</td>
<td>12.70±2.41</td>
<td>–</td>
</tr>
<tr>
<td>Running II</td>
<td>10</td>
<td>2.16±1.50*</td>
<td>37.3%*</td>
<td>4.40±1.58*</td>
<td>65.4%*</td>
</tr>
<tr>
<td>CTX control</td>
<td>8</td>
<td>0.48±0.22*</td>
<td>86.2%*</td>
<td>7.13±3.23*</td>
<td>43.9%*</td>
</tr>
<tr>
<td>TAM control</td>
<td>9</td>
<td>5.47±2.20</td>
<td>–</td>
<td>12.22±3.15</td>
<td>–</td>
</tr>
</tbody>
</table>

Notes: Compared with the tumor-bearing group, *\( P<0.05 \); Compared with the CTX control group, \( ^{a}P<0.05 \); Compared with the TAM control group, \( ^{b}P<0.05 \).
Effects of Runing II on VEGF and VEGFR expressions in the transplanted tumor of MA-891-bearing TA2 mice

As shown in Table 2, the expressions of VEGF and VEGFR in both Runing II group and CTX control group were significantly lower than those in the tumor-bearing control group ($P<0.05$), with significant difference between the two. In the Runing II group, the VEGF and VEGFR expressions were not significantly different from those in the CTX control group ($P>0.05$), the VEGF expression was significantly lower than that in the TAM control group ($P<0.05$), and the VEGFR expression was similar to that in the TAM control group ($P>0.05$). In the CTX control group, the VEGF and VEGFR expression was lower than those in the TAM control group. In the TAM control group, the VEGF expressions were similar to that in the tumor-bearing control group ($P>0.05$), and the VEGFR expression was lower than that in the tumor-bearing control group ($P<0.05$).

Effect of Runing II on MVA and MVC of the transplanted tumor in MA-891-bearing TA2 mice

As shown in Table 3, the levels of MVA and MVC in the transplanted cancer in the medication groups were significantly lower than those in the tumor-bearing control group ($P<0.05$); in the Runing II group, the levels of MVA and MVC were significantly higher than those in the CTX control group ($P<0.05$), the level of MVC was lower than that in the TAM control group ($P<0.05$), and the MVA level was similar to that in the TAM control group ($P>0.05$).

Table 2. Comparison of VEGF and VEGFR expressions in the transplanted tumor of MA-891-bearing TA2 mice in the groups (%,$\bar{x}\pm s$)

<table>
<thead>
<tr>
<th>Group</th>
<th>VEGF (%)</th>
<th>VEGFR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor-bearing control</td>
<td>20.3±7.9 (7)</td>
<td>14.64±3.67 (8)</td>
</tr>
<tr>
<td>Runing II</td>
<td>13.7±6.4 (8) $^*\Delta$</td>
<td>7.90±3.88 (8) $^\Delta$</td>
</tr>
<tr>
<td>CTX control</td>
<td>13.6±5.4 (7) $^*\Delta$</td>
<td>5.93±5.23 (6) $^*\Delta$</td>
</tr>
<tr>
<td>CTM control</td>
<td>25.7±10.0 (9) $^\Delta$</td>
<td>10.19±4.16 (7) $^\Delta$</td>
</tr>
</tbody>
</table>

Notes: Compared with the tumor-bearing group, $^*P<0.05$; Compared with the CTX control group, $^\Delta P<0.05$; Compared with the TAM control group, $^\Delta P<0.05$. Sample number showed in the brackets.

Table 3. Comparison of MVC and MVA in transplanted tumor of MA-891-bearing mice in the groups (%,$\bar{x}\pm s$)

<table>
<thead>
<tr>
<th>Groups</th>
<th>MVC (%)</th>
<th>MVA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor-carrying control</td>
<td>37.73±10.26 (8)</td>
<td>15.08±1.12 (8)</td>
</tr>
<tr>
<td>Runing II</td>
<td>16.00±2.78 (9) $^*\Delta \times$</td>
<td>6.68±2.13 (8) $^*\Delta \times$</td>
</tr>
<tr>
<td>CTX control</td>
<td>9.21±3.53 (5) $^\Delta$</td>
<td>4.87±2.23 (6) $^\Delta$</td>
</tr>
<tr>
<td>TAM control</td>
<td>26.83±11.77 (8) $^\Delta \times$</td>
<td>10.59±2.93 (7) $^\Delta$</td>
</tr>
</tbody>
</table>

Notes: Compared with the tumor-bearing group, $^\Delta P<0.05$; Compared with the CTX control group, $^\Delta P<0.05$; Compared with the TAM control group, $^\Delta P<0.05$. Sample number showed in the brackets.

DISCUSSION

Traditional Chinese medicine holds that genesis and development of tumor result from comprehensive effects of endopathic and exopathic factors but with the stress on the former. In previous clinical study, Prof. Lu and Tang held that vital-qi deficiency in the interior and disharmony of thoroughfare and conception vessels are main causes of tumor genesis, and internal invasion, moving and spreading of cancer pathogens are main conditions of growth and metastasis of mammary cancer. Therefore, in treatment, they advocate that nourishing vital qi and removing masses. Runing II is a Chinese herbal formula which is summed up through clinical practice of many years according to the treatment principle of strengthening the body resistance first
and eliminating pathogenic factors second. In the formula, Sheng Huang Qi (Radix Astragali Mongolici), Tai Zi Shen (Radix Pseudostellariae), Gou Qi Zi (Fructus Lycii), Yin Yang Huo (Herba Epimedii) are sovereign ingredients, with effects of supplementing qi and nourishing yin, regulating thoroughfare and conception vessels, which conforms to commonly-seen characteristics of mammary cancer such as both qi and yin deficiency and disharmony of thoroughfare and conception vessels. Clinically, this formula achieves obvious effect of prolonging survival time, increasing quality of life, stabilizing tumor focus and preventing metastasis of cancer.

The present study established the model of mammary cancer MA-891 cell strain transplanted tumor of TA2 mice with lung metastasis and observed the effect of Runing II on solid tumor growth and lung metastasis, and found that there were significant differences in the tumor inhibition rate and the lung metastasis inhibition rate between the Runing II group and the tumor-bearing group, and between the Runing II group and the CTX control group, showing that Runing II has obvious functions of inhibiting tumor growth and anti-metastasis. Additionally, tamoxifen (TAM) does not have obvious function of inhibiting tumor growth and anti-metastasis in the model mice, being similar to the result that mammary cancer MA-891 is ER-cell strain and the report about direct action of low concentration of TAM abroad.

Tumor is a typical blood vessel dependent disease, and its growth, infiltration and metastasis depend on angiogenesis. At the same time, the neovascularization again supply a metastasis channel for tumor cells, creating the favorable conditions for growth, metastasis and diffusion of tumor. VEGF is a pro-angiogenic factor with the strongest, unique and specific action on vascular endothelial cells, and many tumor cells can secrete VEGF, which increases vascular permeability and directly act on vascular endothelial cells through binding to VEGF receptor, stimulating its division and proliferation, inducing angiogenesis of tumor and influencing micro-vessel number, so as to promote growth and metastasis of tumor. Most tumor cells have high VEGF expression, while in the normal tissues, only a small number of organs such as kidney, ovary, etc., have higher VEGF expression. Therefore, some scholars hold that VEGF can be used as markers of tumor metabolism and metastasis and more ideal target part of blocking neoangiogenesis in tumor, and interfering vascular regulatory factor of tumor and its acting link, regulating expression of angiogenesis factors, and inhibiting angiogenesis possibly control growth and metastasis of tumor.

The results in the present study indicate that Runing II can down-regulate expressions of VEGF and VEGFR to possibly block signal conduction of VEGF-induced migration and proliferation of endothelial cells, and further inhibit angiogenesis in tumor, hence exerting antineoplastic and anti-metastasizing effects.

Previous studies showed that in the growth course of malignant tumor, over fast growth of the tissue inevitably induced serious anoxia of local tissue, and anoxia could stimulate synthesis of VEGF mRNA and slow down degradation of VEGF mRNA to induce genesis of a great number of local new capillary vessels. In observation of immune-histochemical positive staining cells, the authors found obvious VEGF expression in the cytoplasm of dying necrotic tumor cells, on the contrary, less or no expression of VEGF in tumor cells with active proliferation, which may be related with higher level of anoxia inducing factor in tumor cells, conforming to the report of Talks KL, et al. Runing II contains Dang Gui (Radix Angelicae Sinensis), E Zhu (Rhizoma Curcumae Phaeocaulis), Sheng Yi Yi Ren (Semen Coicis), Shan Ci Gu (Pseudobulbus Cremastrae), etc., which function activating blood circulation, resolving phlegm and removing turbid. They inhibit synthesis and secretion of VEGF possibly through improving local microcirculation of tumor, increasing oxygen supply
to tumor cells, hence inhibiting growth of tumor to perform anti-metastasis.

REFERENCES

1. 颜春红, 韩瑞. 肿瘤血管生成和抗转移研究. 肿瘤学新理论与新技术. 上海: 上海科技教育出版社 1997; 477-481.
5. 姜泊, 张亚历, 周敬元. 分子生物学常用试验方法. 北京: 人民军医出版社 1996; 57.
7. 王俊杰, 申文江. 三苯氧胺抗肿瘤的分子生物学机制. 国外医学肿瘤学分册 1997; 24: 335-338.

(Translated by WANG You-jing 王友京)