Anti-proliferative and anti-metastasis activity of Chinese medicinal plants and the investigation on the anti-lung cancer metastasis activity of the essential oil of Cangzhu

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Abstract:

75 herbal extracts from the most commonly used herbs in the clinic in China were screened the anti-proliferative activity against A549 cells, and the most effective extract (PC) among them was fatherly screened the anti-proliferative activity against A549, A2508, UACC 257, MDA-M231 in multiple dosages. As a result, PC displayed the better selectivity against melanoma cell line A2508 and UACC 257. We next evaluated the potential of PC on inhibiting the metastasis of melanoma cells by wound healing experiment and transwell chamber assay and results showed that PC had obvious in vitro anti-metastasis activity of UACC 257 cells. In the in vivo experimental lung metastasis model, PC could inhibit the metastatic lung colonization of UACC 257-luc cells significantly, which supplied a solid proof for the utilization of PC in treating melanoma and its metastasis.

Background:

Cancer has become the most common disease in the world, for example, individuals born in the US today have a 41% lifetime risk of being diagnosed with cancer. Even though we have found out lots of chemical agents like platinum, CPT-11, taxol, vincristine, ginsenoside Rg3 and etc in the recent centuries, the emergence of new problems seems to be always accompanied, like the drug-resistant problem, ignorable severe adverse effects and etc. Especially, cancer metastasis is largely incurable because of the resistance of disseminated tumor cells to existing therapeutic agents, which makes the development of new anticancer or anti-metastasis agents longterm and also urgent [1-3].

China has accumulated precious experiences in a long history by using the herbal treatment. We believed that these experiences provided us the best sources to treat the incurable disease like cancer and metastasis. In previous study, 600 prescriptions from five Eminent and Experience Chinese Medicine Practitioners in Beijing were analyzed to get the frequency of each drug

used in lung cancer metastasis treatment. All the applications of the herbal drugs or their combinations with the chemical therapy were reported to either improve the long-term survival rate of patients with malignant tumor, or change the bad status of their life [4]. In order to find the potential anti-cancer or metastasis materials, we investigated the anti-proliferative and anti-metastasis activities of these most common used herbs in cancer patients in clinic in present study and hope to get the candidates for treating cancers in further study.

Thus, in this study, we concerned about two questions: (1) We expect to screen new potential anticancer or metastasis medicinal plants from the mostly commonly used herbs; (2) We expect to choose one candidate to investigate the in vivo anti-metastasis activity and hope to evaluate its real anti-metastasis activity.

Materials and Methods:

Cell culture and cytotoxicity assay

Four cell lines including A549 (human lung adenocarcinoma), A2508 (human melanoma), UACC 257 (human melanoma cell line) and MDA-M231 (human breast cancer) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). 25 Medicinal herbs were purchased from Guoda Pharmacy in Shenyang and numbered from 1 to 25. Each one was extracted with methanol, methanol-water and water ultrasonically to get three kinds of extracts so that 75 tested samples were gotten.

These cell lines were maintained as monolayer cultures in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) in an atmosphere containing 5% CO_2/air at 37 ℃. The quantification of cell viability was performed using the cell proliferation reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) (DOJINDO, Kuma-moto, Japan). All the cell lines were plated in 96-well microplates in 5000 cells/wells and then incubated for 24 h. Sample-containing medium was added into each well, and cells were incubated for another 24 h. As last, 10 μl WST-1 reagents were added into each well, and the absorbance was measured at 450 nm.

Wound healing assay

UACC 257 cells were plated in a 24-well plate at a concentration of 5×10^5 and allowed to form a confluent monolayer for 24 h. The monolayer was then scratched with a sterile pipette tip (1000 μl), washed with medium to remove floated and detached cells with PBS, and photographed (time 0h). Cells were successively treated in medium in the presence of different concentrations of PC (40 μg/ml) along with the vehicle DMSO for 24 h. Scratched areas were photographed (magnification, × 40) at 0 h and then again 24 h later to assess the degree of wound healing. The percentage of wound closure was estimated by the following equation: wound closure % = 1– (wound area at t_{24}/ wound area at t_0) × 100%, where t_{24} is the time after wounding and t_0 is the time immediately after wounding.

Migration and invasion assay

The filters of a Transwell cell culture insert (8 μm pore size; Whatman Japan KK, Tokyo, Japan) were pre-coated with fibronectin (Iwaki, Tokyo, Japan, 1 μg/filter) on the lower surfaces. For

the invasion assay, the upper surface of the filter was coated with Matrigel (Becton-Dickinson, Bedford, MA, 2 μg/filter). Cells were pre-incubated with or without PC (40 μg/ml) for 24 h. After trypsinization, cells in 0.1% (v/v) BSA medium (3×10^5) were placed in the upper chamber of Transwells. After the subsequent incubation at 37 ℃, the residual cells were removed from the upper surface of the membrance and the migrated cells on the lower surface of the membrane were fixed in 100% methanol and stained with hematoxylin and eosin. Migration was determined by counting the cells with a microscope at 100 × magnification. Five visual fields were chosen randomly and the average number of migrating cells in the five fields was taken for each group.

Experimental lung metastasis model

Female scid mice (six weeks, 14-19 g) were purchaced from Japan SLC, Inc. (Hamamatsu, Japan) and maintained in a temperature- controlled, pathogen-free room. All animals were handled according to the approved protocols and guidelines of the Animal Committee of Toyama University. For the experimental metastasis model, UACC 257-luc cells were inoculated intravenously (i.v., 1×10^6) with or without pre-treatment with PC (40 μg/ml, 24 h) for each mouse. For the lung metastasis imaging, mice were injected with D-luciferin the day after tumor inoculation, then the lungs were removed to measure luminescence using in vivo imaging system (IVIS Lumina Ⅱ, Caliper Life Sciences, MA, USA).

Statistical analysis

All the data are expressed as the mean ± SD of at least two or three independent experiments unless otherwise stated. Statistical significance was analyzed using Student's t-test. $P< 0.05$ was considered significant.

Result and Discussion

1. The screening of 75 herbal extracts from the mostly commonly used herbs in clinic

Due to lung cancer is one of the most lethal cancers with the highest incidence in the worldwide, we referred to the literatures to get the most commonly used traditional Chinese medicines in treating lung cancers. As a result, 25 herbal medicines were selected and extracted with water, methanol-water and methanol to get 75 kinds of extracts. And then, they were screened the anti-lung cancer activity by using A549 cells at the concentration of 100 μg/mL. As shown in Table 1, among the 75 medicinal herbal extracts, the methanol extract and methanol- water extract of the sample NO. 15 (named PC for short) showed the best anti proliferative activity against A549 cells with the inhibition rate over 90%. To further confirm its effects on other cell lines, we evaluated the cytotoxicity with more cell lines at multiple concentrations. The results were shown in Fig. 1. It could be found out that PC inhibited the proliferation of A549, A2508, UACC 257 and MDA-M231 cells in dose dependent manner. Especially for the melanoma cells A2508 and UACC 257, PC displayed obviously better inhibition activity compared with other cell lines at 80μg/ml, which illustrated that PC might be more sensitive to the melanoma cells. However, at 40μg/ml, the selectivity disappeared and PC displayed mild inhibition activity against all the cell lines. PC is a

traditional kidney enriching agent and its anti metastasis activity haven't been reproted before. In further study, we focused on the anti-metastasis potential of PC against melanoma cells at the non-cytotoxic dosage 40μg/ml.

Table 1 The anti-proliferative activities of 75 herbal extracts against A549 cell line

NO.	Water		Methanol		Water-Methanol (1:1)	
	yields	Inhibition rate	yields	Inhibition rate	yields	Inhibition rate
1	74.78%	20.85%	7.34%	23.59%	15.02%	21.55%
2	16.05%	-1.83%	4.89%	-29.09%	19.90%	-26.40%
3	46.18%	-2.05%	17.98%	6.885%	48.06%	7.53%
4	68.63%	-12.05%	5.88%	-4.17%	51.31%	-14.15%
5	31.68%	7.45%	2.96%	7.75%	16.56%	4.99%
6	12.17%	10.53%	2.09%	7.15%	5.59%	16.09%
7	18.23%	22.68%	0.82%	23.5%	9.34%	28.77%
8	35.88%	2.01%	10.62%	-18.17%	35.19%	-13.03%
9	11.65%	10.90%	5.04%	6.98%	15.43%	0.35%
10	14.48%	11.29%	7.69%	3.52%	23.07%	7.25%
11	13.66%	14.67%	3.22%	-24.76%	10.42%	-15.42%
12	18.90%	14.40%	4.83%	6.91%	42.34%	2.64%
13	5.39%	-13.69%	21.79%	-30.5%	6.97%	-13.61%
14	7.57%	2.97%	3.62%	-11.23%	12.25%	0.335%
15	8.90%	-8.27%	18.03%	92.69%	10.51%	93.46%
16	6.32%	19.29%	3.91%	12.16%	10.58%	13.82%
17	11.40%	5.16%	2.72%	-5.07%	19.50%	-17.84%
18	40.38%	11.46%	1.20%	22.2%	35.91%	34.46%
19	21.07%	17.52%	5.56%	14.45%	22.41%	5.77%
20	29.67%	5.42%	16.85%	7.48%	22.43%	-11.29%
21	11.75%	-39.20%	1.24%	-23.26%	12.21%	-31.46%
22	18.73%	21.34%	3.90%	-4%	15.70%	-31.59%
23	25.74%	15.49%	5.16%	19.59%	24.66%	16.16%
24	17.60%	-11%	6.17%	-4.90%	27.08%	-5.69%
25	0.36%	21.6%	0.51%	20%	3.51%	25.2%
Taxol	Inhibition rate					
	44.75%					

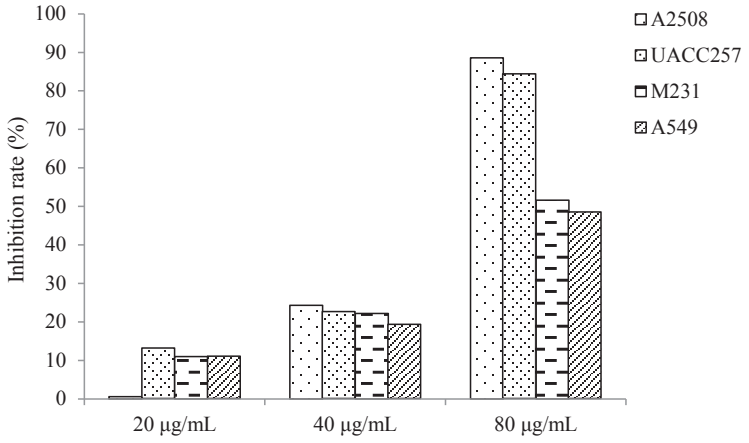

Figure 1 The anti-proliferative activities of PC against A549, A2508, UACC 257 and MDA-M231 cell lines at three different dosages

(Cells were seeded into 96 well plate and incubated for 24 h. Then sample in different concentrations were added into each well and incubate for 24 h. After that, WST-1 reagent was added into each well and the inhibition rates were calculated according to the formula: 1-(optical density of the sample well / optical density of the blank control well)

2. The potential evaluation of PC in inhibiting the metastasis of melanoma cells in vitro

In order to evaluate the anti-metastasis potential of PC, wound healing experiment, migration and invasion assay were performed in our research. As shown in Fig. 2, PC could inhibit the cell migration of UACC257 cells, with the inhibition rate 29%, as determined by the wound closure assay. In addition, PC pre-treatment showed significant inhibition of migration activity (Fig.

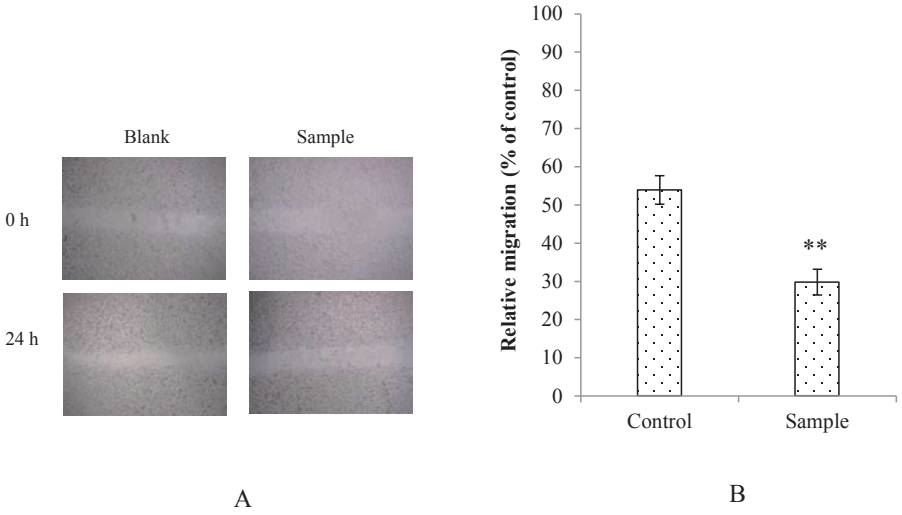

Figure 2 Effects of PC on cellular migration of UACC cells in wound healing experiment

(Cells were treated with PC for 24hs in wound healing assay. Briefly, UACC 257 cell monolayers were scraped with a sterile micropipette tip, and the cells were treated for 24hs with PC at the dosage of 40μg/mL or with verhicle. Scratched areas were photographed (magnification, × 40) at 0h and then again 24h later to assess the degree of wound healing. The data are representative results of two independent experiments. **p<0.01 compared with the blank control group.)

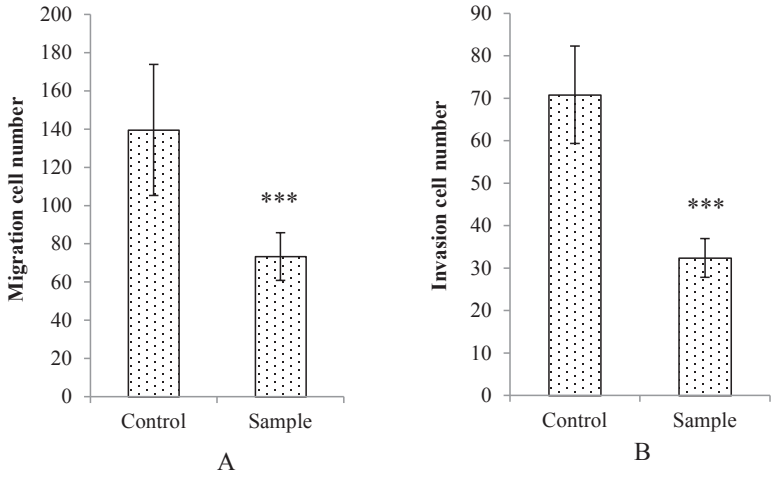

Figure 3 Effect of PC on migration or invasion of UACC257 cells in Transwell chamber assay

(For the migration assay (A) and invasion assay (B), cells were pretreated with PC for 12 h. Then an aliquot of cells (3×10^5) was transferred to each upper well. After incubation, migratory and invasive cells on the bottom of the insert membrane were fixed in 100% methanol and stained with hematoxylin and erosin. Migration and invasion were determined by counting the cells with a microscope at × 100 magnification. Five visual fields were chosen randomly and the average number of invasive cells in the five fields was taken for each group. Each experiment was performed in triplicate. The data are presented as the mean ± SD of three replicate experiments. ***p<0.001 compared with the untreated blank control group.)

3A) and invasion activity of UACC 257 cells (Fig. 3B) in the Transwell chamber assay by comparing the cell numbers on the lower side of the membrane between the control group and the sample group. Collectively, these results clearly indicated that PC could reduce the metastatic potential of UACC 257 cells, which provided the proofs for the further evaluation of its in vivo anti-metastasis activity.

3. The potential evaluation of PC in inhibiting the metastasis of melanoma cells in vivo

Based on the results of the in vitro experiments, we performed the in vivo activity evaluation of PC fatherly. UACC 257-luc cells were inoculated intravenously (i.v., 1 × 106) with or without pre-treatment with PC (40 μg/ml, 24 h) for each mouse. One day after, the mice were sacrificed and the lungs were removed to measure luminescence using in vivo imaging system. Results showed that pre-treatment with PC could inhibit the metastatic lung colonization of UACC 257-luc cells in this experimental lung metastasis model of UACC 257 melanoma (Fig.4).

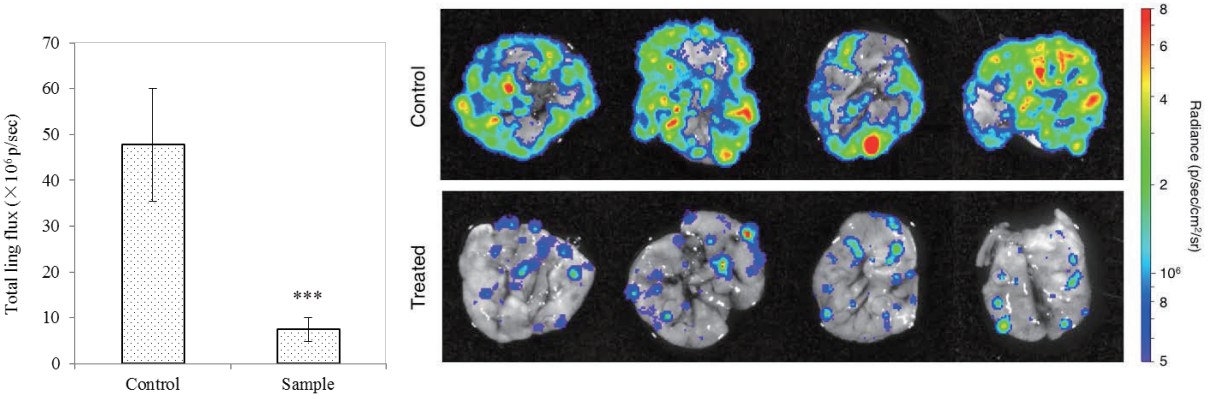

Figure 4 Anti-metastasis of PC in experimental melanoma metastasis model.

(Mice were given an intravenous injection of 1 × 10^6 UACC 257-luc cells pre-incubated with or without 40 μg/ml for 24h. One days after tumor inoculation, mice were sacrificed and lung metastasis were measured by the IVIS system. The data are representative results of two independent experiments and are presented as the mean luminescence ± SD (n=4). ***p<0.001.)

In our research, we found a promising candidate owning both the anticancer and antimetastasis activity from 75 herbal extracts in China. Even though the route of administration in our experimental animal model is different with the clinic ones such as intravenous injection or oral administration and the pharmacokinetics factors were excluded in this experiments, we still could get the conclusion that PC might be very potential because the micro metastasis experiment was mimic completely, which means that if only the active components of PC could act on the melanoma cells, it would definitely reduce the migration and invasion characters of cells, supplying a solid proof for the ultilization of PC in treating melanoma and its metastasis.

Besides, in our experiment, we also have some negative results: (1) In the screening of the anti-metastasis activity, we also investigated the potential of PC on inhibiting lung cancer metastasis, but PC displayed poor activity not only in the wound healing experiment but also in the

Transwell chamber assay. Therefore, we terminated further in vivo activity evaluation. (2) Even though PC displayed the obvious in vivo anti-metastasis activity, the molecular mechanism is undiscovered, we tried to detect the key protein in the metastasis pathway of melanoma cells including ERK, AKT, MMP-9 and SLUG by western blotting, but all the proteins have no changes except AKT after PC pretreatment. Unexpectedly, AKT displayed obvious down regulation. Thus, the specific mechanism of PC is still unclear and should be clarified fatherly in the future.

Reference:

[1] Scott V, Robert A. Tumor Metastasis: Molecular Insights and Evolving Paradigms, cell, 2011, 147, 275-292.

[2] Pan SY, Zhou SF. New Perspectives on How to Discover Drugs from Herbal Medicines: CAM's Outstanding Contribution to Modern Therapeutics, Evidence-Based Complementary and Alternative Medicine, 2013, Article ID: http://dx.doi.org/10.1155/2013/627375

[3] Heather G., Natural Products for Cancer Prevention, Semin Oncol Nurs. 2012, 28(1): 29-44.

[4] Rong ZH, The anti-lung cancer experience and clinical research results from famous TCM doctors in Beijing hospitals. 2013, Doctoral Dissertation of Beijing University of Chinese medicine.