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The basic research of Kampo medicines in view of clinical application
Prevention of cancer metastasis by a Kampo medicine and evaluation of the safety of Kampo medicines used for menopausal symptoms

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It is important in the evolution of Oriental medicine that the basic research results of Kampo medicines are returned to clinical cure. Recently, we established some novel assay methods using both cell biology and molecular biology to screen Kampo medicines for the prevention of cancer relapses or to assess the safety of these medicines to treat menopausal symptoms. Our basic studies using these methods brought about some new findings, as follows: (1) maoto suppresses cancer metastasis through inhibition of cancer cell motility, and (2) the medicines used to treat menopausal symptoms have estrogen-like activities and bind directly to estrogen receptors. These studies are the first steps toward applying basic research findings about Kampo medicines to clinical cure. Further basic studies are needed to examine the use of maoto for patients with cancer or to propose the safety of the medicines used to treat menopausal symptoms. We are now preparing some new ex vivo or in vivo assays in view of the clinical application of Kampo medicines.

Key words basic research, cancer, motility, metastasis, estrogen, safety.

Introduction

The purposes of our studies are to apply Kampo medicines to modern diseases and to ensure the safety of treatments using these medicines. The medicines are composed of several herbal medicines containing various compounds. They are mainly administered orally, and expression of their activities is thought to be caused by mixtures of the original compounds with their metabolites. Thus, we considered the mixtures to be one medicine. We examined the phenomena induced by the medicines and established some new assays using both cell biology and molecular biology to analyze their functions and to evaluate their safety.

Cancer is representative of modern diseases and is the leading cause of death in Japan. Many cancer patients are treated with surgery, chemotherapy, and/or radiotherapy, but Western medicine has almost no therapies for the prevention of relapses of cancer. Many postoperative cancer patients have visited our Oriental Medicine Research Center in hope of preventative therapy by Oriental medicine. However, we had only little evidence for the preventative role of Kampo medicine on cancer relapses. To address the issue, we started to screen Kampo medicines for a medicine to prevent cancer metastasis, which is the main cause of relapses.

In Japan, Kampo medicines are used for the treatment of multiple menopausal symptoms. The medicines have been believed to be safer than hormone replacement therapy (HRT) and are also used to treat patients in whom HRT is contraindicated who have estrogen-dependent tumors such as breast, ovarian, and endometrial cancer. However, the level of estrogen-like activity in the medicines is not clear. To evaluate the safety of these Kampo medicines, we analyzed the estrogen-like activity in the medicines.

In this review, we summarize our current evidence for the role of Kampo medicines in prevention of cancer metastasis and for estrogen-like activity in Kampo medicines for the safety evaluation.

1. Prevention of cancer metastasis by a Kampo medicine

Kampo medicines have been used as an adjunctive therapy following surgery, radiation therapy, or chemotherapy of cancer in Japan. The Kampo medicines play a crucial role in recovery from surgery and control of the side effects of radiation therapy and chemotherapy.

Recently Kampo medicines have been reported to suppress cancer metastasis in mouse models. Juzentaihoto and shiimotsuto have been reported to prevent liver metastasis by murine colon carcinoma cells through activation of macrophages and T cells. Shichimotsukokato suppresses pulmonary metastasis of B16 melanoma cells by elevating nitric oxide production from macrophages. These Kampo medicines suppress cancer metastasis through activation of the immune system.

Metastasis involves numerous different biological processes including dissociation of cancer cells from

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primary tumor, invasion into surrounding stroma, intravasation, extravasation and generation of secondary tumors (Fig. 1). It remains to be clarified whether Kampo medicines are directly involved in the metastatic process. Thus we examined the direct suppressive effect of Kampo medicines on the metastatic processes.

1-1. Screening of candidacy of Kampo medicines for an inhibitor of metastasis using our novel assay system (13)

Cancer cell motility is thought to be closely associated with invasion into the surrounding stroma, intravasation, and extravasation in metastatic processes (Fig. 1), and acceleration of motility of cancer cells is associated with an enhancement of their metastatic ability. (14,15) Therefore, we tried to screen Kampo medicines for an inhibitor of cancer cell motility. First, we established a novel cancer cell motility assay system using mouse serum, because the serum was reported to contain some migration-stimulating factors. (16,17) We confirmed that mouse serum induced cancer cell motility. The mouse serum induced the motility of mouse osteosarcoma cell line, highly metastatic FBJ-LL cells, in a serum concentration-dependent manner (Fig. 2). If a Kampo medicine has migration inhibitory activities, we predicted that cancer cell motility would be reduced by the serum from a mouse given the medicine because the serum from a mouse given the medicine is assumed to contain the original compounds, their metabolites of the medicine, and migration-stimulating factors (Fig. 3).

![Fig. 1 Metastatic processes.](image)

Cancer cells detach from the primary tumor and invade the surrounding stroma, and the stroma is degraded by MMPs secreted from the cancer cells. The cells migrate to blood vessels and then enter the circulatory system. The blood flow carries the cells into distant organs, where they extravasate and enter the surrounding microenvironment. Finally, they grow at the metastatic site.

![Fig. 2 Induction of motility of FBJ-LL cells by MS.](image)

Cells (5 x 10^6 cells) were suspended in 100 µl of RPMI 1640 medium containing 0, 0.1, 0.5, 1, or 5 % MS and placed in the upper well of the Transwell. The lower well contained 600 µl of RPMI 1640 medium containing 0, 0.1, 0.5, 1, or 5 % MS, respectively. At 24 hr, cells migrating into the lower well were counted. Each assay was performed in triplicate, and the error bars represent the standard deviation. The significant difference was determined by the Dunnett's test. *P<0.01 vs. 0% MS.

![Fig. 3 Normal mouse serum and serum from mouse given Kampo medicine.](image)

Normal mouse serum induces cancer cell motility, because it contains migration-stimulating factors. Serum from mouse given Kampo medicine involves migration-stimulating factors, ingredients in the medicine, its metabolites. If the medicine has migration inhibitory activity, the cell motility will be reduced by addition of the serum from mouse given it.

We chose three Kampo medicines, juzentaihoto, hochuekkito, and maoto. Juzentaihoto and hochuekkito are classified as “Hozai”, which is a group of formulations with protective and tonic effects that has been used as an adjunct cancer therapy in Japan. These Kampo medicines have been
reported to suppress cancer metastasis through activation of the immune system.\textsuperscript{10,18,19} Maoto is, on the contrary, a representative of "Shazai", which is a group of formulations with eliminative and purgative effects. Maoto is generally used to treat acute febrile conditions such as fever, chills, lumbago, headache, acute influenza, and arthritis. Maoto, juzentaihoto, hochuekkito, or water as a control was administered orally to two mice per group, and the whole blood was collected in 4 days. Mouse sera were prepared from the whole blood. These sera were used for our motility assay system. The number of migrated cells was significantly reduced by sera obtained from mice given maoto (Fig. 4). There was no significant difference in the number of migrated cells between control serum and sera obtained from mice after the administration of juzentaihoto or hochuekkito (Fig. 4). These results indicate that the serum obtained from mice given maoto exhibits an inhibitory effect on cancer cell motility.

To clarify whether the inhibitory activity of migration is present in the serum from mice given maoto or in maoto, we investigated the effect of the addition of maoto to mouse serum on cancer cell motility. The motility was decreased dependent on the concentration of maoto, suggesting that primary compound (s) in maoto inhibit cell motility (Fig. 5). Juzentaihoto was used as a negative control. The motility was unchanged by the addition of 0.1 to 100 μg/ml of juzentaihoto. A high concentration, 1000 μg/ml, of juzentaihoto suppressed the motility (Fig. 5).

**Fig. 4** Effect of serum obtained from mice given Kampo medicines on FBJ-LL cell motility.

Cells (5 x 10^4 cells) were suspended in 100 μl of RPMI 1640 medium containing 0.5% MS obtained from mice given water (C), Maoto (M), Juzentaihoto (J) or Hochuekkito (H) for 3 d. The lower well of Transwell contained 600 μl of RPMI 1640 medium containing 0.5% MS obtained from mice given water or the Kampo medicines respectively. At 24 h, cells migrating into the lower well were counted. Each assay was performed in triplicate and repeated two times. The error bars represent the standard deviation. Statistical significance was determined by the Scheffe’s test.

**Fig. 5** Effect of mouse serum with the addition of maoto or juzentaihoto on FBJ-LL cell motility.

Cells (5 x 10^4 cells) were suspended in 100 μl of RPMI 1640 medium containing 0.5% MS with the addition of 0, 0.1, 1, 10, 100, or 1000 μg/ml of maoto (straight line) or juzentaihoto (broken line). The lower well of Transwell contained 600 μl of RPMI 1640 medium containing 0.5% MS with 0, 0.1, 1, 10, 100, or 1000 μg/ml of the Kampo medicines. At 24 h, cells migrating into the lower well were counted. Each assay was performed in triplicate. Motility is expressed as the ratio number of cells migrating with the addition of Kampo medicine / number of cells migrating with 0.5% mouse serum only. The number of cells migrating with the mouse serum were 1451 ± 316 (maoto) and 1352 ± 288 (juzentaihoto). The error bars represent the standard deviation. The significant difference was determined by the Dunnett's test. *P<0.01 when compared with the number of cells migrating with 0.5% mouse serum.
We also analyzed the effect of maoto or juzentaihoto on cell growth under the same conditions as those of the motility assay. Neither maoto nor juzentaihoto had any effect on FBJ-LL cell growth at 0.1 to 100 μg/ml. The cell growth was decreased significantly by 1 mg/ml of both maoto and juzentaihoto (Fig. 6), each of which caused a remarkable reduction in cell motility at that concentration (Fig. 5). The reduction of cell motility by 1 mg/ml of either of these Kampo medicines may have been caused by their cytotoxicity. Maoto suppressed cancer cell motility at low concentrations (~100 μg/ml) without cytotoxicity, which suggests that original compound(s) in maoto play a crucial role in the inhibition of cell motility and that the inhibitory activity is specific to maoto.

**Fig. 6** Effect of maoto and juzentaihoto on growth of FBJ-LL cells. Cells (1 x 10^6 cells) were suspended in 100 μl of RPMI 1640 medium containing 10 % FCS with the addition of 0, 10, 100, or 1000 μg/ml of maoto (A) or juzentaihoto (B) in 96 well plate. After 0, 24, 48 or 72 h of incubation, each well was added 10 μl of Cell Counting Kit-8 and incubated at 37 ℃ for 2 h. The absorbance (450 nm) of formazan generated in the well was measured with a microplate reader. Each assay was performed in triplicate. The error bars represent the standard deviation. The significant difference was determined by the Dunnett's test. *P<0.01 when compared with 0 μg/ml of Kampo medicines at each time.

**1-2. Effect of maoto on the spontaneous metastasis of cancer**

To elucidate whether the spontaneous metastasis that arises from a subcutaneous primary tumor was suppressed by oral administration of maoto, we transplanted 2 x 10^6 of the FBJ-LL cells subcutaneously into the left thigh of Balb/c mice (5 mice per group) with free access to drinking water or maoto. Mice were killed at 4 to 5 weeks. There was no significant difference between the primary tumor weight in the mice given maoto and that in the mice given water, suggesting that maoto had no effects on the primary tumor.

**Fig. 7** Effects of oral administration of maoto on spontaneous metastasis. Five mice per group were inoculated subcutaneously with FBJ-LL cells and were administered with free-drinking of water or maoto. After 4-5 weeks, mice were killed, the liver was weighed (A), and the number of liver metastatic nodules was counted (B). Dot bar, normal mouse; open bar, the mouse given water; closed bar, the mouse given maoto, respectively. Each bar represents the average of 5 mice ± S.D. The significant difference was determined by Dunnett's test. *P<0.05 vs normal mouse (A) or the mouse given water (B).
growth in vivo (data not shown). The liver weight in mice given water was significantly heavier than that in the normal mice, but no significant difference was observed between the liver weight in the mice given maoto and that in the normal mice (Fig. 7A). As the number of metastatic nodules in the liver was predicted to correlate with the liver weight, metastatic nodules in the livers were counted visually. The average number of metastatic nodules in the liver of the mice given maoto was 1.5 ± 1.3, and that of the mice given water was 20 ± 12 (Fig. 7B). These results indicate that maoto significantly reduced the rate of spontaneous liver metastasis of FBJ-LL cells.

Juzentaihoto has been reported to prevent the experimental metastasis of murine colon carcinoma cells through activation of macrophages and T cells.\(^{18,19}\) We examined whether the spontaneous metastasis of FBJ-LL cells was suppressed by free-drinking of juzentaihoto as compared with the effect of maoto. FBJ-LL cells (2 × 10⁶ cells/mouse) were transplanted subcutaneously into the left thigh of Balb/c mice (5 mice per group) with free access to drinking water or juzentaihoto. Mice were killed at 4 to 5 weeks. There was no significant difference between the weight of the primary tumor in the mice given juzentaihoto and that in the mice given water. The liver of the mice given water or juzentaihoto was significantly heavier than that of the normal mice. The average number of metastatic nodules in the liver of the mice given juzentaihoto was 23 ± 13, and that in the liver of mice given water was 20 ± 12. There was no significant difference between the number of metastatic nodules in the liver of mice given juzentaihoto and that in the liver of mice given water. These results indicated that juzentaihoto had no effect on the spontaneous metastasis of FBJ-LL cells.\(^{20}\)

Our data demonstrated that maoto suppresses the spontaneous metastasis of FBJ-LL cells. On the other hand, juzentaihoto had no effect on the spontaneous metastasis of FBJ-LL cells, although the medicine has been reported to suppress the experimental metastasis of murine colon carcinoma cells.\(^{18,19}\) Spontaneous metastasis arises from a subcutaneous primary tumor, and experimental metastasis develops following intravascular injection of cancer cells in the absence of a primary tumor. Hence it is possible that the mechanism of metastasis inhibition by maoto is different from that by juzentaihoto.

1-3. Target molecules of maoto\(^{20}\)

Determining the target molecules of maoto is very important, because the action mechanism of maoto is not yet clear, and it is also possible that these target molecules could act as indexes of cancer cells that would be sensitive to maoto. We examined the effects of maoto on matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP), which are thought to be associated with cancer cell invasion into the surrounding stroma in metastatic processes.

MMPs degrade extracellular matrix proteins and accelerate cancer metastasis. The MMPs are secreted as zymogens and are activated by proteolytic cleavage of the N-terminal peptides. The active forms of these MMPs without the N-terminal propeptide are believed to act in vivo. The FBJ-LL cells secreted MMP2, MMP9, and its active forms (Fig. 8). The amount of active forms of MMPs was markedly decreased with the addition of maoto, whereas that of latent forms of MMPs was unchanged by the addition of maoto. Thus the activation of both MMP2 and MMP9 was suppressed by maoto (Fig. 8).

TIMP-1 inhibits the activity of MMPs. The levels of TIMP-1 were increased in serum from the tumor-bearing mice given maoto,\(^{20}\) and expressions of TIMP-1 and TIMP-2 genes in cancer cells were induced by serum from mice given maoto (unpublished data). These results showed that maoto may suppress cancer metastasis by regulating the balance between MMPs and TIMPs.

1-4. The application of maoto for the prevention of cancer relapse

In the present review, we showed that maoto suppresses the motility of mouse cancer cells and spontaneous metastasis. These results suggest that maoto prevents cancer metastasis through the direct suppression of the metastatic processes (Fig. 9) and that this medicine may be a novel inhibitor of metastasis.

We think that maoto may be useful for cancer patients with a robust constitution (jitu-sho) after surgery or during chemotherapy and radiotherapy to prevent cancer relapses. However, maoto is not a good treatment for cancer patients.
2. Evaluation of the safety of Kampo medicines used for menopausal symptoms

Multiple menopausal symptoms such as hot flashes, sleeping disturbance, and depression often reduce the quality of daily life of women. Hormone replacement therapy (HRT) plays a crucial role in the treatment of these symptoms that arise due to estrogen insufficiency. However, recent reports of both the Million Women Study and the Women’s Health Initiative indicated that HRT is not successful in all patients and is contraindicated in patients with a high risk of estrogen-dependent tumours such as breast and endometrial cancers. In Japan, some Kampo medicines have been used to effectively treat a variety of menopausal symptoms. The medicines have been believed to be safer than HRT and are also used to treat the patients in whom HRT is contraindicated. However, the mechanisms of the pharmacological effect of Kampo medicines have been poorly identified, and the level of estrogen-like activity in the medicines is not clear. Accordingly, it was very important to analyze the estrogen-like activity in these Kampo medicines for the safety evaluation. We tried to analyze the estrogen-like activity in the medicines and examined whether they act directly on estrogen receptors (ERs) α and β.

2-1. Measurement of estrogen-like activity in Kampo medicines using the luciferase assay system

The estrogen-like activity of Kampo medicines can't be detected by existing methods such as immunoassay, because the medicines are thought to contain various estrogen-like compounds but not estrogen molecules. Therefore, we used the luciferase assay system to measure the intensity of estrogen-like activity of the estrogen receptor binding molecules (Fig. 10). The intensity of luciferase activity increased according to the concentration of 17β-estradiol and plateaued in the terminal stage who have a deficient constitution (kyo-sho) and many metastatic foci in their tissues, because maoto has no effect on proliferation of metastasized cancer cells. Hozai medicines such as juzentaiho and hochuekkito are appropriate for these patients.

As the first step in establishing the clinical application of maoto for the prevention of cancer relapses, we are now investigating whether the motility of human cancer cells is inhibited by human serum from healthy volunteers given maoto.

Fig. 9 Target sites of maoto in metastatic processes.

Estrogen-like molecules in Kampo medicine

Nucleus

Luciferase Gene

Expression

Measuring the luminescence intensity

Fig. 10 Detection of estrogen-like activity in Kampo medicines by luciferase assay.

In this process, cells from the estrogen receptor (ER)-positive breast cancer cell line MCF-7 are transiently transfected with pGV-ERE vector, which inserts sequences of estrogen-responsive element (ERE) upstream of the luciferase gene. If the Kampo medicine contains estrogen-like molecules, the molecules bind to ERs and the conformation changes of ERs are induced. The ERs bind to ERE, and the luciferase gene is expressed. The luminescence is detected with a Luminometer.
at 100 pM. The detection limit of the estrogen concentration was 6.25 pM (Fig. 11). This assay system was hence shown to have a high sensitivity, and we tried to analyze the estrogen-like activity in Kampo medicines.

We selected twenty-four kinds of Kampo medicines used to treat a variety of menopausal signs and gynecological diseases as follows: (1) hachimijiogon (TJ-7), daisiakoto (TJ-8), shosaikoto (TJ-9), saikokeishito (TJ-10), saikokeishihakankyo (TJ-11), saikokaryukotsuboreito (TJ-12), ohregedokuto (TJ-15), tokishakuyakusan (TJ-23), kamishoyosan (TJ-24), keishibukuryogon (TJ-25), hochoyokuroto (TJ-41), juzentaihoto (TJ-48), yokukansan (TJ-54), tokakujokito (TJ-61), boshushosan (TJ-62), nyoshinsan (TJ-67), kossan (TJ-70), shiromokuto (TJ-71), unketo (TJ-106), ninjin'yoetito (TJ-108), san'oshashinto (TJ-113), and saireito (TJ-114) are used for a variety of perimenopausal complaints, (2) kakkonto (TJ-1) is used for the treatment of mastitis, and (3) kakkonokokato was also selected because this prescription contains kakkon ((Pueraria lobata) and koka (Carthamus tinctorius)), which have long been known to be effective for some gynecological symptoms.

The intensity of luciferase activity induced by each of the Kampo medicines was converted into the daily dose of

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**Table 1** Estrogen-like activity of Kampo medicines

<table>
<thead>
<tr>
<th>Kampo medicines</th>
<th>Daily dose</th>
<th>Concentration of Kampo medicines</th>
<th>Estrogen-like activity in Kampo medicines</th>
<th>Daily dose converted into E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TJ-7</td>
<td>4</td>
<td>1000</td>
<td>4.03 ± 0.16 (pg/ml)</td>
<td>1098 ± 42 (ng/day)</td>
</tr>
<tr>
<td>TJ-23</td>
<td>4</td>
<td>1000</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>TJ-24</td>
<td>4</td>
<td>500</td>
<td>24.5 ± 2.95 (pg/ml)</td>
<td>13336 ± 1606 (ng/day)</td>
</tr>
<tr>
<td>TJ-54</td>
<td>3.25</td>
<td>500</td>
<td>19.86 ± 3.02 (pg/ml)</td>
<td>10817 ± 1645 (ng/day)</td>
</tr>
<tr>
<td>TJ-70</td>
<td>2</td>
<td>500</td>
<td>37.34 ± 6.13 (pg/ml)</td>
<td>20342 ± 3337 (ng/day)</td>
</tr>
<tr>
<td>TJ-71</td>
<td>2.75</td>
<td>1000</td>
<td>50.13 ± 13.43 (pg/ml)</td>
<td>13654 ± 3658 (ng/day)</td>
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<tr>
<td>TJ-106</td>
<td>5</td>
<td>500</td>
<td>17.36 ± 1.19 (pg/ml)</td>
<td>9457 ± 647 (ng/day)</td>
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<tr>
<td>TJ-15</td>
<td>1.5</td>
<td>1000</td>
<td>30.94 ± 9.98 (pg/ml)</td>
<td>8428 ± 2720 (ng/day)</td>
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<tr>
<td>TJ-25</td>
<td>1.75</td>
<td>1000</td>
<td>7.77 ± 1.32 (pg/ml)</td>
<td>217 ± 360 (ng/day)</td>
</tr>
<tr>
<td>TJ-61</td>
<td>3</td>
<td>500</td>
<td>47.04 ± 1 (pg/ml)</td>
<td>25624 ± 550 (ng/day)</td>
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<tr>
<td>TJ-62</td>
<td>4.5</td>
<td>300</td>
<td>30.39 ± 2.29 (pg/ml)</td>
<td>27593 ± 2080 (ng/day)</td>
</tr>
<tr>
<td>TJ-67</td>
<td>4.5</td>
<td>250</td>
<td>19.98 ± 3.24 (pg/ml)</td>
<td>21769 ± 3531 (ng/day)</td>
</tr>
<tr>
<td>TJ-113</td>
<td>1.75</td>
<td>600</td>
<td>29.11 ± 2.3 (pg/ml)</td>
<td>13217 ± 1045 (ng/day)</td>
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<td>TJ-8</td>
<td>4.5</td>
<td>500</td>
<td>43.62 ± 9.90 (pg/ml)</td>
<td>23765 ± 5393 (ng/day)</td>
</tr>
<tr>
<td>TJ-9</td>
<td>4.5</td>
<td>1000</td>
<td>50.23 ± 2.17 (pg/ml)</td>
<td>13680 ± 591 (ng/day)</td>
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<tr>
<td>TJ-10</td>
<td>4</td>
<td>250</td>
<td>34.7 ± 4.45 (pg/ml)</td>
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<tr>
<td>TJ-11</td>
<td>3.5</td>
<td>300</td>
<td>30.83 ± 3.43 (pg/ml)</td>
<td>27988 ± 3114 (ng/day)</td>
</tr>
<tr>
<td>TJ-12</td>
<td>3.5</td>
<td>600</td>
<td>6.60 ± 3.62 (pg/ml)</td>
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<td>TJ-114</td>
<td>6</td>
<td>500</td>
<td>14.6 ± 1.55 (pg/ml)</td>
<td>11653 ± 23 (ng/day)</td>
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<tr>
<td>TJ-41</td>
<td>5</td>
<td>100</td>
<td>2.14 ± 0.04 (pg/ml)</td>
<td>24978 ± 1409 (ng/day)</td>
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<tr>
<td>TJ-48</td>
<td>5</td>
<td>100</td>
<td>9.17 ± 0.52 (pg/ml)</td>
<td>3588 ± 836 (ng/day)</td>
</tr>
<tr>
<td>TJ-108</td>
<td>6</td>
<td>500</td>
<td>10.22 ± 1.54 (pg/ml)</td>
<td>394112 ± 115869 (ng/day)</td>
</tr>
<tr>
<td>Kakkonokokato</td>
<td>4.74</td>
<td>100</td>
<td>78.43 ± 25.74 (pg/ml)</td>
<td>213645 ± 70112 (ng/day)</td>
</tr>
</tbody>
</table>

*The concentration of Kampo medicine was used for the luciferase assay.

*1 The concentration of Kampo medicine was used for the luciferase assay.

*2 (pg/M) : calculated by the standard curve

*3 (pg/ml) = \(2 \times 0.27239\)

*4 (pg/ml) = \(3 \times 100000000 / \) concentration of Kampo medicines (µg/ml)

*5 (ng/day) = \(4 \times \) daily dose (g) / 1000

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**Fig. 11** Luciferase activity induced by 17β-estradiol.

MCF-7 cells were transiently cotransfected with pRL-CMV vector and pG5-ERE vector and were treated with 5 % dextran-coated charcoal-treated FCS phenol-red-free DMEM containing vehicle or various concentrations of 17β-estradiol (6.25, 12.5, 25, 50 and 100 pM) for 18 h. Luciferase activity was measured with Luminometer. Each experiment was performed in triplicate, and repeated twice. The error bars represent the standard deviation.
17β-estradiol using the standard curve (Fig. 11). These data are shown in Table 1. Estrogen-like activities were detected in twenty-three kinds of Kampo medicines, but not in tokishakuyakusan (TJ-23). The activities in twenty-one kinds of Kampo medicines, not including kakkonto (TJ-1) and kakkonkokato, were under 150 ng/day and were lower than that in HRT (625 µg/day). In kakkonto (TJ-1) and kakkonkokato, estrogen-like activities of above 1 µg/day were detected (Table 1). These Kampo medicines have a common plant, kakkon (Pueraria lobata), and natural isoflavonoid phytoestrogens such as genistein and daizein are reported to be present in the root of kakkon (Pueraria lobata).25 These phytoestrogens, which are plant-derived compounds of nonsteroidal structure, can mimic the effect of estrogen. Therefore, the active ingredients in these Kampo medicines are suggested to be these phytoestrogens.26

2-2. Agonistic or antagonistic action of Kampo medicines used for menopausal symptoms on estrogen receptor subtypes ERα and ERβ27

We showed that twenty-three kinds of Kampo medicines used to treat gynecological diseases have estrogen-like activities. However, we did not clarify whether these medicines act directly on estrogen receptor α and β. We selected nine kinds of Kampo medicines as follows: hachimijijogon (TJ-7), orengedokuto (TJ-15), tokishakuyakusan (TJ-23), kamishoyosan (TJ-24), keishibukuryogon (TJ-25), tokakujkito (TJ-61), nyoshinsan (TJ-67) and unkeito (TJ-106) are frequently used to treat a variety of menopausal symptoms, and kakkonto (TJ-1), which exhibited a strong estrogen-like activity. We investigated whether these medicines act as agonists or antagonists on the estrogen receptors.

Five kinds of Kampo medicines, TJ-1, TJ-7, TJ-24, TJ-25, and TJ-61, had agonistic effects on ERα or ERβ (Fig. 12). Of these, TJ-1 showed the highest binding ability to the ERs. This result is in agreement with the result from Luciferase assay.24 TJ-1 includes Puerariae Radix, which contains genistein, daizein, and biochanin A.28 An et al. reported that these phytoestrogens are the triggers of the transcriptional pathways of ERβ.29 Thus, the phytoestrogens in TJ-1 may act agonistically on ERα. TJ-7, TJ-24, and TJ-25 acted agonistically on ERβ. Recently, studies on ERβ provided new insights into the mechanism underlying estrogen signaling. Numerous clinical and in vitro studies showed that compared with benign tumors and normal tissue, cancers such as breast, ovary, prostate, or colon cancer showed a decreased ERβ expression, whereas ERα expression increased or persisted.30 Hence, the reduction in ERβ expression may be responsible for the progression of these cancers. Moreover, Paruthiyil et al. reported that the proliferation of human breast cancer was suppressed and its cell cycle was arrested in the G2 phase by transfection with the ERβ gene.31 Therefore, it is possible that TJ-7, TJ-24, and TJ-25 can be used as alternatives to HRT for the treatment of women with breast cancer. The binding ability of TJ-61 to ERα and ERβ increased at 0.5 mg/ml and decreased at 5.0 mg/ml (Fig. 12). TJ-61 contains multiple kinds of bioactive substances; therefore, the antagonist-like molecules in the medicine may show activity at 5.0 mg/ml.

![Fig. 12 Agonistic actions of Kampo medicines on ERα and ERβ were analyzed by the Receptor/Coactivator Ligand Assay. The Kampo medicines (0.005, 0.05, 0.5 or 5.0 mg/ml) were incubated with ERα (A) or ERβ (B) in a coactivator peptide-coated plate. After washing the plate, the complexes of the estrogen-like molecules in the medicine, the ER and the coactivator on the plate were detected by using an HRP-conjugated anti-ER antibody. In order to determine the HRP activity, the absorbance (450 nm) in the well was measured with a microtiter plate spectrophotometer. The binding ability (%) to ERα is expressed as (absorbance of the well containing the complexes of estrogen-like molecules in the Kampo medicine, ERα and the coactivator / absorbance of the well containing the complexes of 20 nM 17β-estradiol, the ERα and the coactivator) × 100. The binding ability (%) to ERβ is expressed as (absorbance of the well containing the complexes of estrogen-like molecules in the Kampo medicine, ERβ and the coactivator / absorbance of the well containing the complexes of 20 nM 17β-estradiol, the ERβ and the coactivator) × 100). Each experiment was performed in duplicate. The error bars represent the standard deviation.](image-url)
Fig. 13  Antagonistic actions of Kampo medicines on ERα and ERβ were analyzed by the Receptor/Coactivator Ligand Assay. The Kampo medicines (0.005, 0.05, 0.5 or 5.0 mg/ml) were incubated with both β-estradiol and ERα (A) or ERβ (B) in a coactivator peptide-coated plate. The binding ability (%) to ER is expressed as ((absorbance of the well containing the complexes of β-estradiol, estrogen-like molecules in the Kampo medicine, ERα and the coactivator/absorbance of the well containing the complexes of 40 nM 17β-estradiol, the ERα and the coactivator) × 100). The binding ability (%) to ERβ is expressed as (absorbance of the well containing the complexes of β-estradiol, estrogen-like molecules in the Kampo medicine, ERβ and the coactivator/absorbance of the well containing the complexes of 400 nM 17β-estradiol, the ERβ and the coactivator) × 100). Each experiment was performed in duplicate. The error bars represent the standard deviation.

We examined the antagonistic activity of four kinds of Kampo medicines-TJ-15, TJ-23, TJ-67, and TJ-106-that did not show any agonistic activity. TJ-15 and TJ-67 acted antagonistically on both ERα and ERβ (Fig. 13), while TJ-23 and TJ-106 acted antagonistically on ERβ (Fig. 13B). We have found that TJ-23 had no estrogen-like activity. These results also suggested that TJ-23 may be a specific ERβ antagonist. On the other hand, we found previously that TJ-15, TJ-67, and TJ-106 have low levels of estrogen-like activities. Thus, it is possible that these medicines exert an estrogen-like activity through mechanisms other than those involving any action on ERs.

We found for the first time that Kampo medicines bound directly to the ERs and regulated their functions. Further studies are needed to determine whether a physiological change of the uterus or growth of a hormone-dependent tumor will be induced by Kampo medicines. We are currently analyzing the effects of oral administration of Kampo medicines on both uterine weight and estrogen value in the serum of ovariectomized mice.

Conclusion

We reviewed the basic studies of Kampo medicines designed to examine their effect on the prevention of metastasis, and revealed for the first time that maoto suppresses cancer metastasis through the inhibition of cancer cell motility. Furthermore, we tried to evaluate the safety of Kampo medicines used to treat menopausal symptoms. The medicines have estrogen-like activities and bind directly to estrogen receptors, suggesting that further in vivo studies are needed to assess the effects of the medicines on physiological changes of the uterus and the growth of hormone-dependent tumors.

These basic studies are the first steps to lead the research results of Kampo medicines to the clinical application. We are now preparing some new ex vivo and in vivo assays in the hope that our basic research findings will promote the use of Oriental medicine in the clinical setting in the future.

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Japanese abstract

私は、漢方薬についての基礎研究の成果を臨床へ還元していくことが、東洋医学の発展のために重要であると考えている。最近、私は細胞生物学や分子生物学を応用して、かんの再発を予防する漢方薬をスクリーニングするための新しいアッセイ方法や、更年期調用漢方処方の安全性評価のための新しい方法を確立した。これらの方法を用いた私たちの基礎研究は、このような新しい見解をもたらした。すなわち、漢方薬は、がん細胞の運動を抑制することにより抑制を抑制すること、更年期調用漢方処方が、エストロゲン活性を有し、エストロゲン受容体に直接結合すること。これらの研究は、漢方薬の基礎研究成果を臨床へ応用するための第一歩となるものである。麻薬ががん患者に用いるためには、あるいは、更年期調用漢方処方の安全性をtram托するには、さらなる基礎研究が必要である。現在、私はこれらの成果を臨床応用するために、いくつかの新しいex vivoあるいはin vivoのアッセイ方法を検討しているところである。

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