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Anti-cancer Effect in Volatile Components of Hiba Essential Oil (*Thujopsis dolabrata*)

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Abstract

Objective: Although combinations of multiple cancer therapies have reduced the mortality, they often cause severe adverse reactions, and the new strategies with fewer adverse reactions are desired. Our present study demonstrated that an essential oil preparation from hiba (*Thujopsis dolabrata*) exerts an antitumor effect against gastric cancer cells.

Methods: MKN45 gastric cancer cells were incubated with whole of hiba essential oil (HEO) or volatile components of the HEO, followed by assessment of tumor growth inhibition by MTT assay. Apoptotic change of these cancer cells was also analyzed by TUNEL reaction. Nude mice were used to establish a model of gastric cancer tumor growth and peritoneal disseminated metastasis, in which the volume of tumor and the number of peritoneal disseminations were evaluated after inhalation of volatile components of HEO for 4 weeks. In addition, the antitumor effect of the hinokitiol, one of the anti-tumor ingredients of hiba, was compared.

Results: HEO treatment induced the inhibition of tumor growth and apoptosis in MKN45 gastric cancer cells. Volatile components of the HEO also inhibited the growth and induced the apoptosis in MKN45 cells, and significantly reduced the peritoneal dissemination and metastasis in *in vivo* mice model. The hinokitiol, an ingredient of HEO, exhibited the weaker tumor growth inhibition effect than the whole of HEO preparation.

Conclusion: Our study indicates that HEO, especially volatile components, have an anti-tumor activity of gastric cancer. We also reveal that not only hinokitiol, but also other components may play a role as antitumor factor.

Keywords: *Thujopsis dolabrata*; Hiba essential oil (HEO); Volatile components; Anti-tumor effect; Gastric cancer

Abbreviations:

HEO: Hiba essential oil; CM: Centimeter

Introduction

Cancer is the leading cause of death in Japan, and it is well known that the one in two people will develop cancer [1]. Cancer therapies include surgical procedures, anticancer agents, molecular-targeted agents, and radiation therapy. The advancement of individual therapies and bringing in the multimodal therapy, i.e., effective combination of multiple therapies, have improved the outcome of cancer therapy and reduced the mortality from cancer [1]. However, these therapies often cause severe adverse reactions, and this may prevent the completion of the treatment in some patients. And despite good initial responses, drug resistance and disease recurrence remain major issues for cancer treatments. Under these circumstances, safer and more effective new strategies are desired.

In traditional therapies such as traditional Japanese medicine (Kampo medicine), natural preparations are reported about their effectiveness for cancer treatment [2-4]. Combination therapy using anticancer drugs and Kampo preparation is revealed to improve therapeutic outcomes [5]. Essential oil constituents from plants are also used as alternative treatments for a wide range of illnesses including cancer prevention and treatment [6]. It will become very useful treatment tool for the patient with dysphagia or anorexia who has difficulty to take anti-cancer drugs. Such traditional preparations are expected to become a component of multimodal cancer therapies.

In the present study, we demonstrated that *Thujopsis dolabrata* (Hiba in Japan) essential oil had antitumor effect against gastric cancer cell. The volatile component of hiba essential oil (HEO) also showed the apoptosis inducing activity to the gastric cancer cell *in vitro*, and it was confirmed in mouse models. Our findings suggest that HEO could be a promising ingredient for treating gastric cancer patients.

Materials and Methods

HEO and hinokitiol preparations

In this study, HEO was mainly extracted from Noto-derived hiba by using a standard hydrodistillation technique, and the constituents were analyzed through GC-MS. The yield was 1.5-2.0% in HEO/raw material. Dimethyl sulfoxide (DMSO) was applied to the obtained HEO at a concentration of 2-10 g/mL. The solution was used for the following experiments. Hinokitiol was purchased from WAKO (Osaka, Japan), and dissolved in DMSO (2-10 g/mL) as a stock stored at −20°C.

Cell lines and culture conditions

Gastric cancer cells (MKN45) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). MKN45 cells were cultured in DMEM medium containing 5% FCS. All culture media were supplemented with antibiotics.

Cell proliferation assay

The antitumor effects of whole HEO or volatile components in HEO were quantitatively analyzed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cancer cells were plated on 96-well plates at a density of 1 × 104 per 100 L. The cells were added with whole HEO at a concentration of 0.00006 ~2 μg/mL, and incubated at 37°C for 1-72 hours. For experiments of volatile components, 0.001 g in 0.1 mL of HEO was applied only in the well of the right lower edge, and incubated at 37°C for 60 hours. After incubation, 10 L of Solution I from the MTT cell proliferation assay Kit (Roche Diagnostics, Tokyo, Japan) was added to each well. After incubation at 37°C for 4 hours, visualization solution (100 L) was added to each well, and the plate was incubated at 37°C overnight. The absorbance at 450 nm was measured in each well using a multi-function plate reader (Filter Max F5; Molecular Devices, Wokingham-Berkshire, UK).

Morphological observation and detection of apoptosis

The antitumor effect of whole HEO or volatile components in HEO on MKN45 gastric cancer cells was assessed by observation of individual cell morphology by polarization microscopy. Additionally, apoptosis inducing ability of whole or volatile components of HEO were analyzed with TUNEL reaction (In Situ Cell Death Detection Kit, Fluorescein, Roche Diagnostics, Tokyo, Japan). Briefly, pelleted cells (2 × 106) were suspended with 100 μL of Fixation solution (60 min, 25°C). After wash with PBS, cells were resuspended with 100 μL of Permeabilisation solution for 2 min on ice. Induction of apoptosis was analyzed by flow cytometry (BD AccuriTM C6, BD Biosciences, USA).

Animal experiments

Female nude mice at 4 weeks old were purchased from SLC Inc. (Hamamatsu, Japan) and used for the study. 2 × 106 of MKN45 cells were injected under the back skin of 10 mice to establish a tumor growth model. MKN45 cells were also injected into the peritoneal cavity of other 10 mice at an amount of 2 × 106 per animal to establish a model of gastric cancer peritoneal disseminated metastasis, as reported previously [7]. HIBA group and control were set, and each group was consisted of five animals, which were kept in individual cages. Starting on the following day, the HEO (0.001 g in 0.1 mL) applied dish was set in the cage of the HIBA group. The dish was separated from mice, and changed to the fresh oil once in every 24 hours. Mice in the control group were kept in the room air. Flooring tips were replaced once weekly in each cage. Mice were weighed once weekly to evaluate weight variation.

Statistical methods

All data were expressed as mean ± S.D. Comparisons between multiple groups were made by the student's t-test. P values less than 0.05 were assumed to indicate significance. All analyses were done with JMP11.0 software.

Results

Inhibition of MKN45 gastric cancer cell growth by whole HEO

MKN45 gastric cancer cells were treated with whole HEO and the activity of tumor growth inhibition was assessed by MTT assay. The results indicated that whole HEO inhibited the growth of gastric cancer cells in a time-and concentration-dependent manner (Figures 1a and 1b).

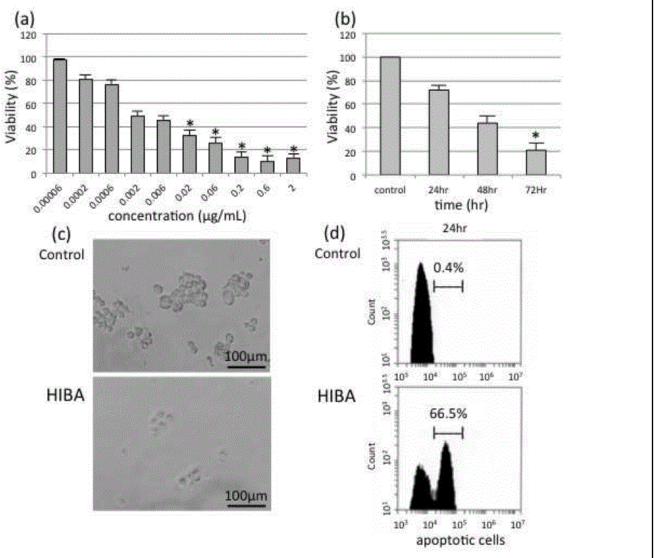

Figure 1: Inhibition of MKN45 gastric cancer cell growth by HEO. (a) Variation of antitumor effect by HEO concentration. 1 × 104 of MKN45 cells were incubated for 60 hours with each concentration of HEO. (b) HEO-mediated change in tumor growth as a function of time. 1 × 104 of MKN45 gastric cancer cells were incubated with 20 μg/mL of HEO within each time periods. Viability (%): Data represent the percentage relative to the control value. *p<0.05 (student's t-test). (c) Morphological changes of MKN45 gastric cancer cells after 60 hours of HEO treatment (20 μg/mL). Control; no treated cells, HIBA; HEO treated cells. (d) The ratio of apoptotic cells with TUNEL reaction of MKN45 gastric cancer cells that was induced with HEO (HIBA) or control medium (control) for 24 hours.

The morphological changes of MKN45 gastric cancer cells were analyzed by polarization microscopy and compared to control cells. After 60 hours treatment, the living cell was obviously decreased in

whole HEO treated MKN45 cell culture, and it was contained some cells exhibiting of apoptotic characteristics whose nuclei were more shrank than control culture (Figure 1c). Furthermore, MKN45 cell culture was analyzed with TUNEL reaction to investigate apoptotic cells. After 24 hours treatment, it was indicated that 66.5% of apoptotic cell was exhibited in HEO treated culture, and it was clearly more than control (0.4%) (Figure 1d). It was shown that the apoptosis inducing effect was existed in the HEO.

Tumor growth inhibition by volatile component of HEO

In order to identify tumor growth inhibitory effects in volatile components of the HEO, MKN45 gastric cancer cells were incubated in 96-well plates and the HEO (0.001 g in 0.1 mL) was applied only in the well of the right lower edge, and the tumor growth inhibition was compared using MTT assay (Figure 2a).

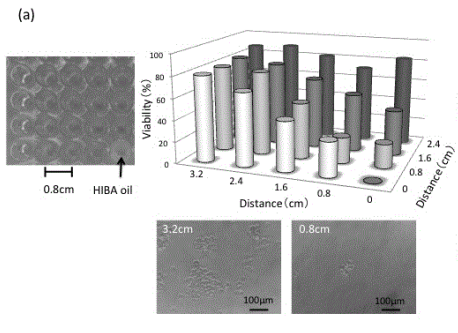

Figure 2: Inhibition of MKN45 cell growths by volatile components of HEO. (a) Upper figure: Tumor growth inhibition activity of volatile components in HEO was investigated by MTT assay. 1 × 104 of MKN45 cells were incubated for 60 hours in each well with 5% FCS contained DMEM medium. 0.001 g in 0.1 mL of HEO was applied only in the well of the right lower edge. Viability (%): Data represent the percentage relative to the control value. Lower figure: Morphological changes of MKN45 gastric cancer cells after 60 hours of HEO treatment. Cancer cells in the well of 0.8 cm (left) and 3.2 cm (right) separated from HEO applied well were shown. (b) The ratio of apoptotic cells with TUNEL reaction of MKN45 gastric cancer cells that was induced in 1.6 cm separated from HEO (HIBA) or control medium (control) for 24 hours.

The results indicated that volatile components of the HEO inhibited the growth of gastric cancer cells in a distance-dependent manner. In wells in proximity to the HEO (0.8 cm), the number of viable cells was decreased than remote wells (3.2 cm). Then, MKN45 cell culture was analyzed with TUNEL reaction to investigate apoptotic cells. After 24 hours treatment, 12.9% of apoptotic cell was exhibited in the well of 1.6 cm away from HEO, and the apoptotic ratio was much more than control (0.1%) (Figure 2b). It was shown that the apoptosis inducing effect was contained in volatile components of the HEO.

Inhibition of peritoneal dissemination and metastasis by HEO

A model of tumor growth analysis model was created by injecting MKN45 cells at an amount of 2 × 106 per animal under the back skin of 4-week-old mice. The model mice were divided into two groups of each five animals, i.e., HIBA and control groups, and inhalation of HEO gas for 4 weeks in HIBA group. Body weight variation was not significantly different between these groups. Mice in the HIBA group had feces with similar appearance and consistency as that in the control group, and the HEO gas-treated mice did not exhibit diarrhea, decreased appetite, reduced body movement, or dirty change of hair. After 4 weeks, the mice were sacrificed, all tumors were resected, and the volume of resected tumors was measured. The result was shown that there was no obvious tumor in two-fifths mice of HIBA group, and the average of tumor volume in HIBA group mice was smaller than control group, but there was no significant difference (p=0.1329) (Figure 3a).

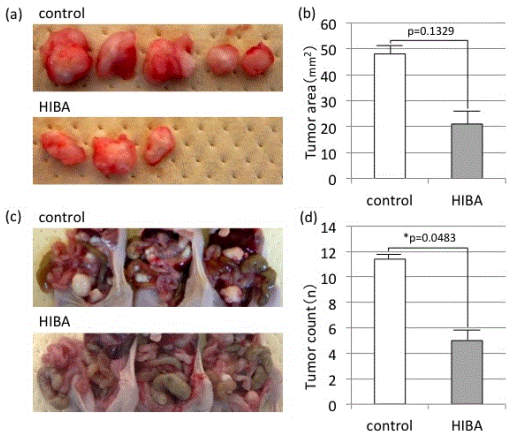

Figure 3: Inhibition of peritoneal dissemination metastasis by HEO. (a) Rejected tumors from the back of the HEO (HIBA) and control group mice. (b) Mean volume (mm^2) of rejected tumors in each group. (c) Inhibition of peritoneal dissemination metastasis by volatile components of HEO treatment. Sacrificed each group nude mice were subjected to laparotomy for comparison of the number of disseminations in the mesenterium. (d) Number of peritoneal disseminations in the HIBA and control groups. $^{*}p<0.05$ relative to control.

To elucidate the tumor suppression activity of HEO more clearly, a model of gastric cancer peritoneal dissemination metastasis was created by injecting 2 × 106 of MKN45 cells into the peritoneal cavity of 4-week-old mice. These mice were also divided into HIBA and control groups. From the next day, HEO gas was administered to HIBA group mice for 4 weeks as mentioned as above. After 4 weeks, the mice were sacrificed and subjected to laparotomy and counting of peritoneal disseminations. The results demonstrated a significant reduction in the number of peritoneal disseminations in the HIBA group compared with the control (p=0.0483) (Figure 3b). From the above findings, inhalation of HEO gas had a tendency to suppress the growth of gastric cancer and showed to suppress the metastasis of gastric cancer without apparent adverse reactions in mice.

Comparison of tumor growth inhibition by hinokitiol

Hinokitiol is one of the important ingredients of HEO, because of the anti-cancer effect. We examined the tumor growth inhibition effect of liquid and volatile component of hinokitiol by MTT assay. The

results showed that the liquid hinokitiol had the weaker tumor growth inhibition activity than the whole of HEO preparation (Figure 4a). The volatile component of hinokitiol was incubated with MKN45 gastric cancer cells, and 60 hours later, the variation in cell growth capacity was analyzed by MTT assay. The results demonstrated that the volatile component of hinokitiol had only a little activity of tumor growth inhibition (Figure 4b).

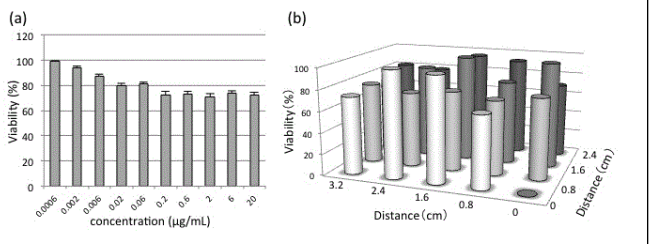

Figure 4: Tumor growth inhibitory activity in Aomori HEO and hinokichiol. (a) Antitumor effect by hinokichiol concentration. 1 × 104 of MKN45 cells were incubated for 60 hours with each concentration of hinokichiol. The result of MTT assay was shown. (b) Tumor growth inhibition activity of volatile components in hinokichiol was investigated by MTT assay. 1 × 104 of MKN45 cells were incubated for 60 hours in each well. 0.001 g in 0.1 mL of hinokichiol was applied in the well of the edge. Viability (%): Data represent the percentage relative to the control value.

Discussion

Essential oil treatments are characterized as milder therapies to correct imbalances throughout the body using careful observation of the body's condition while placing greater emphasis on natural healing potential and the patient's defense system [8]. For example, exposure to lavender oil alleviated cancer pain and associated anxiety [9], and lavender and rosemary essential oils enhanced free radical–scavenging activity and decreased stress hormones such as cortisol [10]. The potential of anti-cancer activity of essential oil has also been investigated [6]. In this study, we assessed the effects of hiba essential oil (HEO) and found that it had an apoptosis-inducing activity in gastric cancer cells.

Hiba (*Thujopsis dolabrata*) is a coniferous tree species endemic to Aomori and Noto in Japan [11] and is used as a building material for castles, temples and shrines where high personages were lived. The strong antimicrobial activity and healing effects of HEO are well known, and could be attributed to thujopsene ($C_{15}H_{24}$), β-dolabrin ($C_{10}H_{10}O_2$), and hinokitiol ($C_{10}H_{12}O_2$), which are the major components of HEO. Hinokitiol, which is also known as β-thujaplicin, has been reported to have applications in regulating several biological activities, including anti-inflammatory [12], anti-bacterial [13], anti-fungal [14], and anti-viral [15] activities. It has also been shown to have anti-proliferative effects against various cancer cell lines, including melanoma [16], prostate cancer [17], lung cancer [18], oral cancer [19], and colon cancer [20]; however, no developmental toxicity or carcinogenic effects have been observed in hinokitiol [21,22]. About the mechanism of tumor-growth inhibitory effects, it was reported that hinokitiol induced S-phase arrest and apoptosis by activation of caspase-9 and -3 in colon cancer cells [20]. In melanoma cells, hinokichiol was reported to induce G1 arrest and tumor growth inhibition [16]. Beta-dolabrin, thujaplicin, and 4-accetyltropolone, the components of hiba, also showed cytotoxic effects against leukemia cells [23], and gastric cancer cell [24]. In this study, it was shown that the activity of whole HEO was stronger than hinokitiol alone (Figures 4a and 4b). This indicated that not only the hinokitiol, but also other components of HEO, i.e., β-dolabrin, γ-thujaplicin, and/or 4-accetyltropolone, might be important for the anti-tumor activity of HEO.

We elucidated for the first time, that the volatile components of HEO had the anti-tumor activity against gastric cancer cells (MKN45) *in vitro* (Figures 1 and 2). We also showed that the inhalation of the volatile components of HEO suppressed the tumor growth and metastasis of gastric cancer in *in vivo* mouse models (Figure 3). Li et al. revealed the anti-tumor effect of hinokitiol by injection into the abdomen of lung cancer mouse model (18). Lee et al. administered the hinokitiol orally to colon cancer intradermally implanted mouse model, and also revealed the anti-tumor activity [20]. These data indicate that the hinokitiol inhibit the growth of tumor, situated away from where the drug was administrated. Matsuura et al. showed that HEO inhalation reduced stress-induced growth inhibition and stress-related anxiety, in restrained stressed rat model [25]. Otsu et al. also elucidated that HEO inhalation induced the up-regulation of chromogranin A (CgA) and Immunoglobulin A (IgA) [26]. These data suggested that some volatile components of HEO might be absorbed into the blood flow from membranes of nose and/or mouth, reached to target cells throughout the body, including the brain, inhibited the tumor cell growth and stress-related symptoms. Taken together, our data support the idea that the inhalation of volatile components of HEO could be used as a novel and safe strategy for the treatment of gastric cancer.

Conclusion

The present study demonstrated antitumor and apoptosis inducing effect of HEO against gastric cancer cell line. We also revealed that inhalation of volatile components of HEO induced antitumor effects in mouse models. In conclusion, inhalation of volatile components of HEO is expected to be effective against gastric cancers.

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