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Continuous intake of the Chaga mushroom (*Inonotus obliquus*) aqueous extract suppresses cancer progression and maintains body temperature in mice

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Abstract

Aims: Cancer is a leading cause of morbidity and mortality worldwide; therefore, effective measures for cancer prevention and treatment are in constant demand. The extracts of *Inonotus obliquus* (Chaga mushroom) demonstrate potent anti-tumor activities and have been used to treat cancer in several countries; however, the actual effect and underlying mechanisms are still unclear. In the present study, we aimed to investigate the effects of continuous intake of aqueous extract from *I. obliquus* on tumor suppression.

Main methods: Anticancer activity of the *I. obliquus* extract was examined in mouse models of Lewis lung carcinoma growth and spontaneous metastasis after

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3 weeks of continuous extract intake at the dose of 6 mg/kg/day, which corresponded to that ingested daily with Chaga infusion in Japan.

Key findings: The extract of *I. obliquus* caused significant tumor suppressive effects in both models. Thus, in tumor-bearing mice, 60% tumor reduction was observed, while in metastatic mice, the number of nodules decreased by 25% compared to the control group. Moreover, *I. obliquus* extract-treated mice demonstrated the increase in tumor agglomeration and inhibition of vascularization. Interestingly, *I. obliquus* intake decreased body weight in middle-aged mice and increased body temperature in response to light-dark switching in mature adult mice. Furthermore, *I. obliquus* prevented temperature drop in mice after tumor implantation.

Significance: Our findings suggest that the *I. obliquus* extract could be used as a natural remedy for cancer suppression by promoting energy metabolism.

Keywords: Biochemistry, Biological sciences

1. Introduction

Cancer is one of the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer-related deaths documented in 2012 [1]. While discovering novel therapeutics and providing adequate care to cancer patients is very important for decreasing cancer burden, prevention is probably a more critical aspect which was somewhat neglected [2]. Cancer prevention through socioeconomic interventions, environmental changes, and lifestyle modifications could be a solution for reducing constantly increasing cancer incidence. Recent epidemiological data demonstrate that obesity increases the risk of cancer development [3]. Cao et al. [4, 5] have reported that mice housing with increased space, physical activity, and social interactions (enriched environment, EE) which stimulated physical and mental activities, demonstrated the suppression of both tumor growth and obesity via inhibition of leptin secretion from white adipose tissue. EE is also known to decrease adiposity, stimulate energy expenditure, and induce brown-like (beige) cells in white fat [6]. Furthermore, it has been reported that cancer patients are characterized with hypothermia and hyperglycemia [7] and that physiologic responses to high body temperature improve the tumor microenvironment [8]. These findings indicate that the induction of lipid metabolism and maintenance of proper body temperature could be important aspects in cancer prevention.

Medicinal mushrooms have an established history of use in traditional oriental therapy and nutritionally functional foods. *Inonotus obliquus* (Chaga mushroom) belonging to the family *Hymenochaetaceae* of *Basidiomycetes*, preferably grows on the trunks of mature live birch trees [9]. The extracts of *I. obliquus* have been used in China, Korea, Japan, Russia, and the Baltics for their favorable effects on

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lipid metabolism and cardiac function, as well as for anti-bacterial, anti-inflammatory, anti-oxidant, and anti-tumor activities [9]. *I. obliquus* extracts were found to inhibit hepatitis C virus [10] and human immunodeficiency virus [11, 12] and demonstrated strong anti-oxidant and immunostimulatory activities *in vitro* [13, 14]. At the same time, animal studies revealed that aqueous extracts of *I. obliquus* exhibited anti-inflammatory effects in experimental colitis [15, 16] and promoted lipid metabolism [17]. Several studies investigated the anti-tumor activity of the *I. obliquus* aqueous extract and found that it suppressed the proliferation [18] and induced apoptosis [19] of various carcinoma cell lines. Furthermore, the compounds isolated from *I. obliquus* extracts were shown to inhibit skin carcinogenesis [20] and tumor growth in Sarcoma-180 cell-bearing mice [21]. However, despite increasing evidence of anticancer activity exhibited by the *I. obliquus* extract and its individual components [9, 22], the underlying mechanisms are still unclear and the effects of *I. obliquus* on cancer prevention are not understood.

In this study, we examined anti-cancer effects of the continuous intake of the *I. obliquus* extract using mouse models of tumorigenesis and spontaneous metastasis. The dose of *I. obliquus* extract (6 mg/kg/day) was calculated based on the daily intake of the extract as tea infusion in Japan. We also tested the real time body temperature using an implanted nano-thermometer. This is the first study showing that continuous intake of the *I. obliquus* aqueous extract suppresses cancer progression and maintains body temperature.

2. Material and methods

2.1. Animals

C57BL/6 mice obtained from Japan SLC Inc. (Shizuoka, Japan) at the age of 8 weeks were kept in the room with controlled temperature (23 ± 2 °C) and a 12-h light/dark cycle (lights on at 8 am). All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Showa University (Permit Number: 55019), which operates in accordance with the Japanese Government for the care and use of laboratory animals.

2.2. Preparation of aqueous extract from *I. obliquus*

I. obliquus sclerotia were collected from birch trees in Fujiyoshida city, Yamanashi prefecture, Japan, and identified by Drs. Mashasi Osawa and Hisasi Shibata of Yamanashi Forest Research Institute, Japan. The material was powdered and 32.0 g was suspended in 1 L of water, boiled for 90 min to be concentrated to 200 ~ 300 ml. The extract was filtered through filter paper (No. 101, Advantec, Tokyo, Japan) and freeze-dried, yielding 1.3 g (4.1% of raw

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material). It was re-dissolved in sterilized distilled water to the concentration of 2.4 mg/ml and stored at −80 °C as stock solution.

2.3. Cancer cell culture

Lewis lung carcinoma cell line (3LL) was obtained from National Institutes of Biomedical Innovation (Osaka, Japan) and maintained in RPMI 1640 medium supplemented with glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μ g/mL), and 10% (v/v) heat-inactivated FBS (Thermo Fisher, Waltham, MA, USA).

2.4. *I. obliquus* anticancer activity in mouse models of carcinoma and spontaneous metastasis

Mice were given water with or without 24 μ g/mL extracts from *I. obliquus* (1% of stock solution) for 3 weeks before tumor inoculation and throughout the experimental period; approximately 6 mg/kg of *I. obliquus* extract was ingested in 5 ml of drinking water per day. Cancer models were established as described previously [23]. Briefly, 5×10^5 live 3LL cells were suspended in 0.2 ml of serum-free MEM and injected subcutaneously in the right flanks of mice to develop solid intra-abdominal tumors (tumor-bearing model) or into the tail vein to produce colonies of metastatic cells in the lung (spontaneous metastasis model). In the tumor-bearing model, tumor size was measured with calipers every day and tumor volume was calculated as $(\text{width})^2 \times \text{length} \times 0.52$. Mice were sacrificed at day 16 after tumor cell inoculation, and solid tumors were collected and weighted. For histological examination, some mice were anesthetized at day 7 of cancer cell implantation by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and perfused transcardially with saline, followed by 4% paraformaldehyde in 50 mM phosphate buffer (pH 7.2). Pulmonary nodules in the metastasis model were counted using 3D lung images acquired by micro-CT and visually confirmed in the lungs fixed with neutral formalin and observed under a stereomicroscope. For micro-CT scanning, animals were anesthetized with isoflurane at day 9 after cancer cell inoculation and analyzed using an R_mCT2 micro-CT scanner (Rigaku Corporation, Tokyo, Japan) under the following conditions: FOV24, ϕ24 mm × H19 mm; tube voltage, 90 kV; tube current, 160 μA. Some mice were sacrificed 2 weeks after cancer cell injection, and the extracted lung tissues were fixed with neutral formalin and used for histology.

2.5. Histochemistry

Tissues were immersed in 20% sucrose in 0.1 M phosphate buffer (pH 7.2) at 4 °C and embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan). Frozen sections were cut with a microtome at the thickness of 8 μm, washed in PBS (pH 7.2), and stained with Gill's hematoxylin and eosin (HE). For immunostaining,

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tissue sections were treated with 0.3% H_2O_2 in PBS to quench endogenous peroxidase activity, blocked in 5% normal horse serum in PBS for 60 min, and incubated overnight with rat anti-CD31 polyclonal antibodies (1:500; 550274, BD Biosciences, East Rutherford, NJ, USA) at 4 °C. Sections were washed with PBS and incubated with biotinylated goat anti-rat IgG (1:200; BA-4001, Vector, Burlingame, CA, USA) for 2 h at room temperature, followed by the reaction with avidin-biotin complex solution (Vector) and chromogen diaminobenzidine (Vector). Images were captured using an AX70 microscope (Olympus, Tokyo, Japan).

2.6. Body temperature measurement

Mouse body temperature was monitored using the DST nano-T temperature logger (17 mm × 6 mm, 1 g; STAR ODDI, Gardabaer, Iceland). The loggers were implanted in the abdomen and body temperature was continuously measured at 30-min intervals.

2.7. Statistical analysis

Unpaired T-test was performed to assess the significance of independent experiments after statistical outliers were removed using the Smirnoff-Grubbs rejection test. Three groups (Fig. 5B and C) were compared using one-way ANOVA with a Tukey multiple comparison test. The Ekuseru-Toukei software (Social Survey Research Information Co. Ltd., Tokyo, Japan) was used for statistical analysis. P values < 0.05 were considered statistically significant.

3. Results

3.1. *I. obliquus* extract promoted a decrease of body weight in old mice

First, we examined the effect of continuous intake of the *I. obliquus* aqueous extract on body weight of mature adult mice (12–15 weeks) and middle-aged mice (30 weeks). The results indicated that middle-aged mice lost about 8% of weight after 17 days of drinking *I. obliquus* extracts compared to the control group (Fig. 1A); there was no difference in water intake between the two groups (Fig. 1B). Interestingly, the *I. obliquus* extract caused no changes in body weight or the amount of consumed water in mature adult mice (Fig. 1C and D). These data suggest that the intake of the *I. obliquus* extract promoted lipolysis in developed fatty tissue; therefore, in further experiments we used mature adult mice (12–15 weeks) to avoid the influence of body weight difference between control and treated mice observed prior to tumor implantation on subsequent tumor growth.

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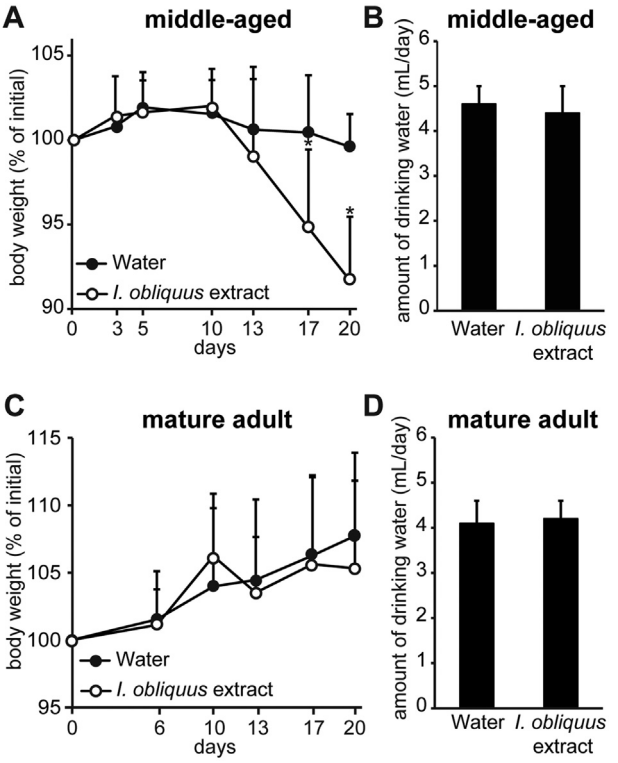

Fig. 1. The intake of the *I. obliquus* extract promoted body weight loss in middle-aged mice. Middle-aged and mature adult mice received water without (control) or with the *I. obliquus* extract for the indicated times and were analyzed for body weight. (A) Body weight of middle-aged mice (weight at day 0: 37.3 ± 3.5 g and 37.9 ± 5.1 g for the water and *I. obliquus* group, respectively; n = 4–5). (B) Amount of water drunk by middle-aged mice. (C) Body weight of mature adult mice (weight at day 0: 22.7 ± 1.8 g and 23.3 ± 2.1 g for the water and *I. obliquus* group, respectively; n = 8–9). (D) Amount of water drunk by mature adult mice. The data were normalized to the body weight at day 0 and expressed as the mean ± SD; *P < 0.05.

3.2. Continuous intake of the *I. obliquus* extract slowed tumor progression in mice with implanted Lewis lung carcinoma cells

To explore whether *I. obliquus* had the ability to suppress tumor growth, mice received water supplemented with *I. obliquus* extract for 3 weeks prior to and 16 days after the implantation of 3LL cells for the development of solid intra-abdominal tumors. The images of carcinomas from the treated and control mice showed that the *I. obliquus* extract suppressed tumor growth (Fig. 2A). Furthermore, quantitative analysis revealed significant retardation of tumor development in the *I. obliquus* group starting from day 14 after cancer cell implantation (Fig. 2B); at day 16, the average tumor size in the treatment group was 60.3% less than in the control group (Fig. 2C). These results indicate that continuous uptake of the *I. obliquus* extract produced a strong anti-tumor effect.

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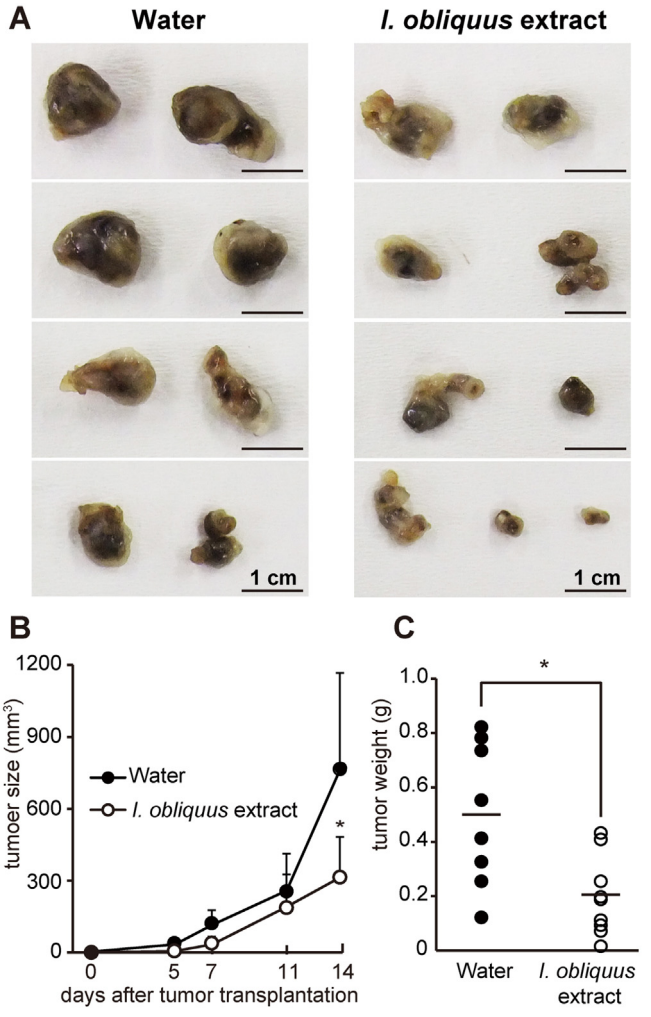

Fig. 2. The intake of the *I. obliquus* extract suppressed tumor development in mice implanted Lewis lung carcinoma cells. Mice drinking water with or without the *I. obliquus* aqueous extract were injected 3LL cells subcutaneously in the right flanks and analyzed for tumor size. (A) Representative images of carcinomas. (B) Quantification of tumor size at the indicated times after 3LL cell implantation. (C) The weight of solid tumors at day 16 after 3LL cell implantation. Each dot represents a single mouse and lines show mean values (n = 8 per group; *P < 0.05).

3.3. *I. obliquus* extract decreased tumor vascularization

Next, we analyzed the effect of the *I. obliquus* extract by histochemistry. HE staining revealed agglomerated tumor morphology and decreased tumor size in the *I. obliquus* group (Fig. 3A). Furthermore, immunostaining for CD31, a marker of vascular endothelial cells, revealed that the presence of CD31-positive cells in *I. obliquus*-treated mice tended to decrease compared to the control group, indicating that tumor vascularization could be suppressed by the intake of the *I. obliquus* extract (Fig. 3B). These results suggest that the continuous intake of the *I. obliquus* extract could decrease tumor vascularization and, consequently, suppress cancer progression.

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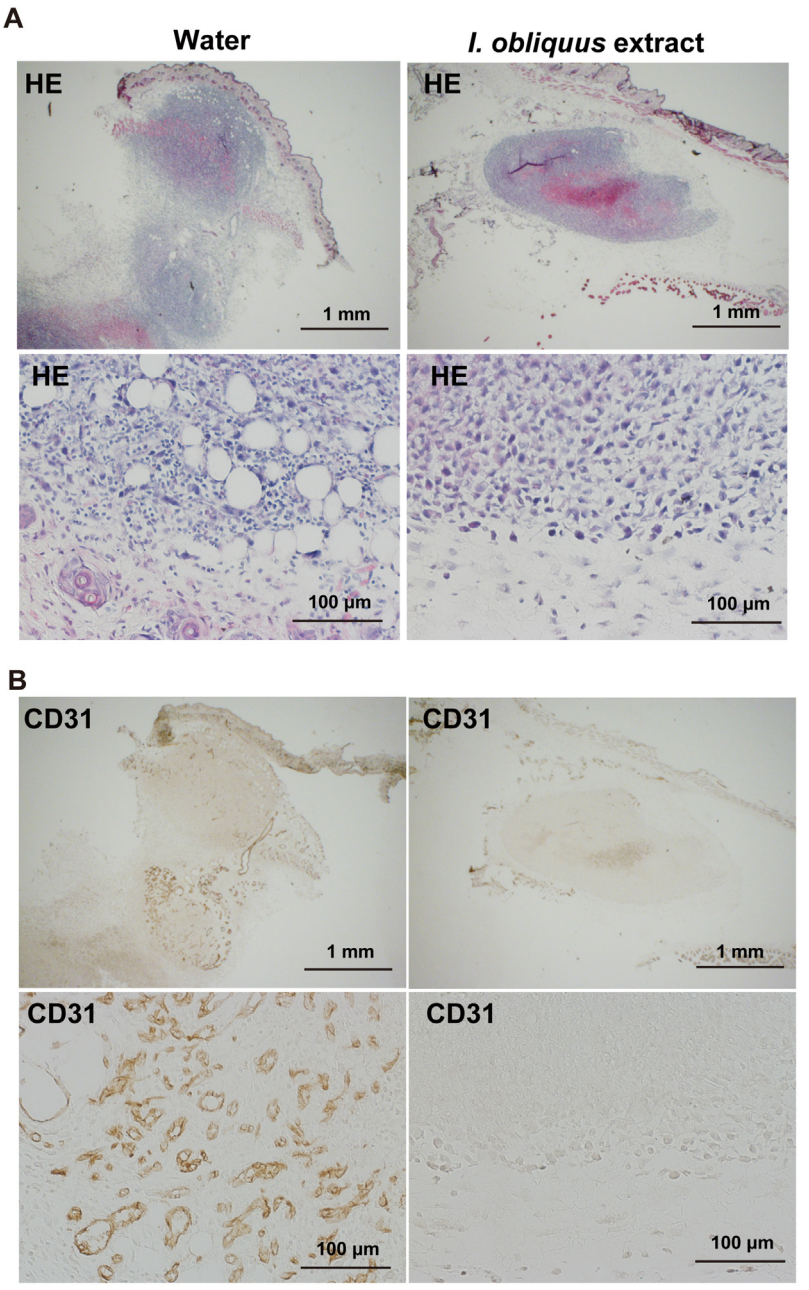

Fig. 3. *I. obliquus* extract caused tumor agglomeration and suppressed vascularization. 3LL cells were injected subcutaneously at the left abdomen of mice drinking water with or without the *I. obliquus* aqueous and tumors were analyzed by histology 7 days after cells implantation. (A) HE staining. (B) Immunostaining for CD31.

3.4. Continuous intake of the *I. obliquus* extract suppressed metastasis

To further explore anti-tumor effects of the *I. obliquus* extract, it was tested in a model of spontaneous metastasis to lung tissue induced by intravenous injection

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of 3LL cells. First, we analyzed lung metastasis under a stereomicroscope 14 days after tumor implantation. As shown in Fig. 4A and B, lung nodules in *I. obliquus*-treated mice tended to decrease compared to the control group. Then, the lungs from animals anesthetized at day 9 after cancer cell inoculation were analyzed for the number of pulmonary nodules using micro-CT. The results indicate that the intake of the *I. obliquus* extract significantly decreased the number of tumor nodules in the lungs (Fig. 4C and D). Histology analysis (HE staining) of tumor nodules demonstrated that *I. obliquus* intake decreased the size

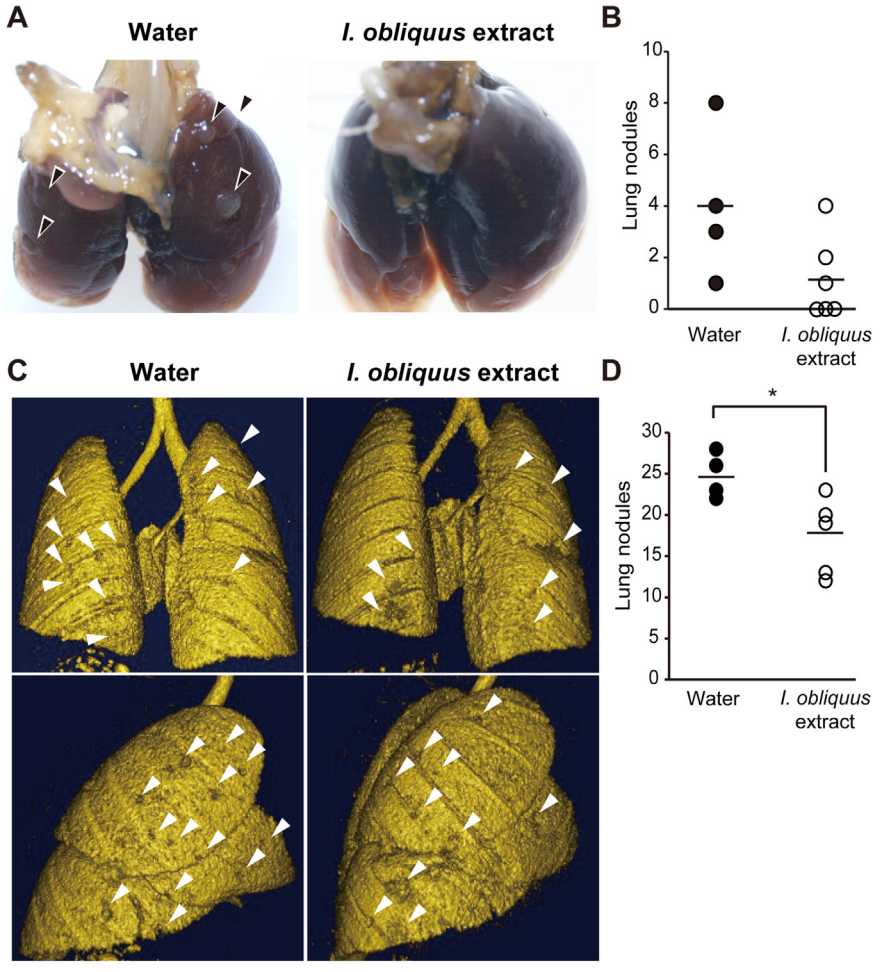

Fig. 4. *I. obliquus* extract suppressed metastasis in mice injected with Lewis lung carcinoma cells. Mice drinking water with or without the *I. obliquus* aqueous extract received intravenous injection of 3LL cells. (A) Representative stereomicroscopic images of fixed mouse lungs containing carcinoma nodules. Arrowheads mark 3LL nodules on the lung. (B) The number of metastatic nodules counted under a stereomicroscope. Each dot represents a single mouse and lines show mean values (n = 4–6 per group). (C) Representative CT images of mouse lungs containing carcinoma nodules. Arrowheads mark 3LL nodules visible as dents on the lung. (D) The number of nodules measured in CT images of the lungs extracted at day 9 after cancer cell injection. Each dot represents a single mouse and lines show mean values (n = 4–5 per group; *P $<$ 0.05).

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and induced agglomerated morphology of tumor nodules (Fig. 5). These data suggest that continuous intake of the *I. obliquus* extract could suppress tumor progression.

3.5. Continuous intake of the *I. obliquus* extract prevented body temperature decrease after tumor implantation

We hypothesized that the maintenance of the body temperature could be a key factor in suppressing tumor development by the *I. obliquus* extract. To test this hypothesis, we subcutaneously implanted nano-temperature loggers in the abdomen and measured the body temperature in a real-time format. Interestingly, tumor-free mice continuously drinking the *I. obliquus* extract showed higher body temperatures at the switch from darkness to light (Fig. 6A). As there was no difference in water intake between the *I. obliquus* and control groups (Fig. 1D), these data suggest that *I. obliquus* could upregulate energy metabolism. While body temperature gradually decreased after tumor implantation in the control group at the light-to-dark switching (Fig. 6B), it was not the case in the group taking the *I. obliquus* extract. These data suggest that *I. obliquus* may suppress tumor growth by regulating energy metabolism.

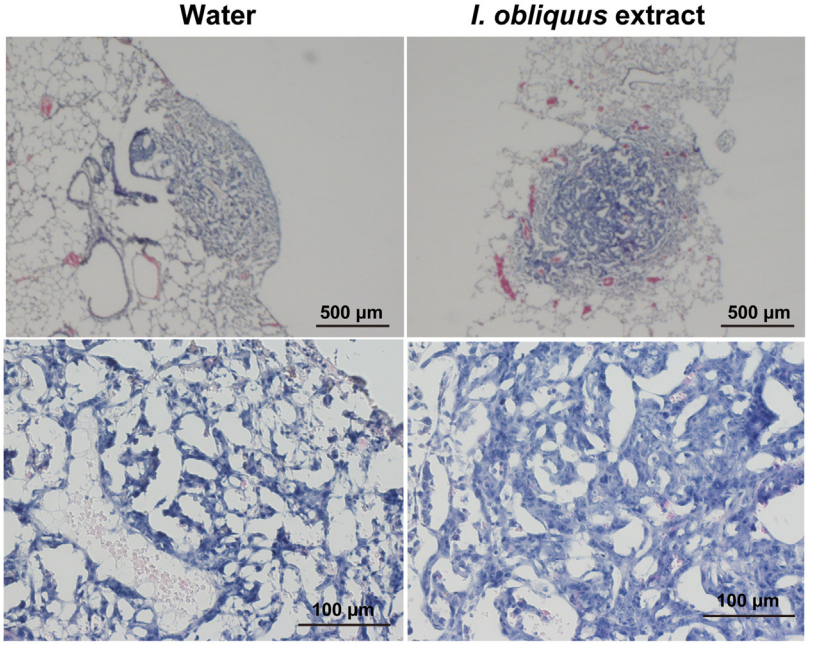

Fig. 5. The intake of the *I. obliquus* extract agglomerated tumor cells in the mouse model of spontaneous metastasis. Mice drinking water with or without the *I. obliquus* aqueous extract received intravenous injection of 3LL cells. Representative HE-stained images of lung sections 2 weeks after cancer cell injection are shown.

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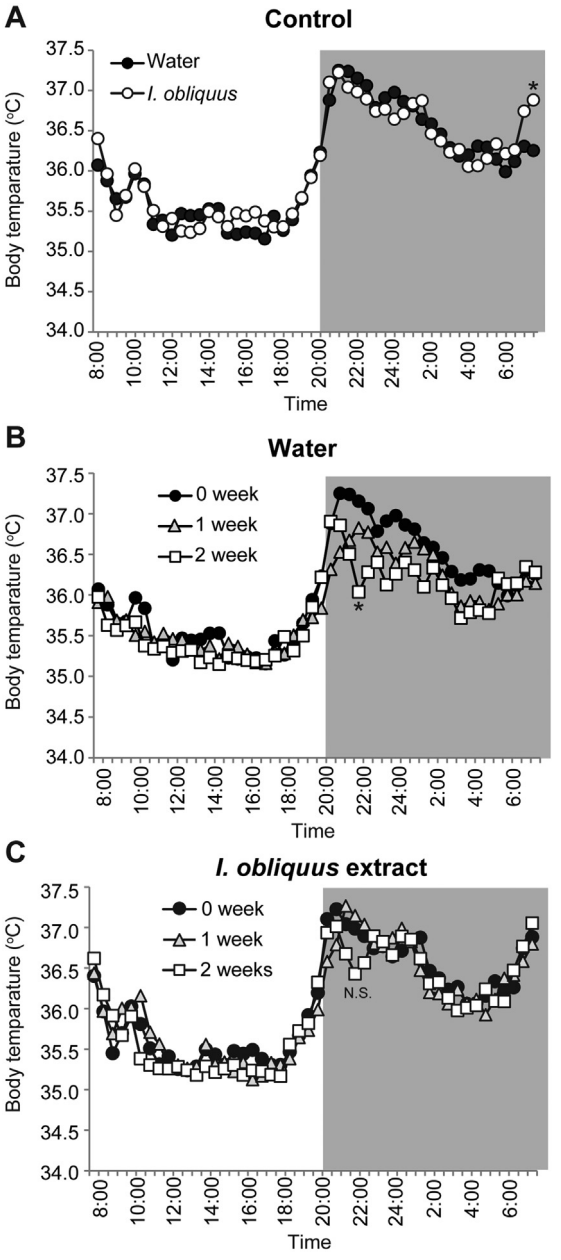

Fig. 6. Continuous intake of the *I. obliquus* extract prevented body temperature decrease in mice implanted Lewis lung carcinoma cells. (A) Average body temperature of mice measured from week 2 to 3 after the intake of drinking water without or with the aqueous extract of *I. obliquus*. (B, C) Average body temperature of mice receiving water (B) or *I. obliquus* extract (C) measured 1 week before (0 week), and 1 week and 2 weeks after cancer cell injection. The data are expressed as the mean (n = 3 per group); *P < 0.05 versus water (A) or 0 week (B, C).

4. Discussion

The results of this study indicate that daily intake of the *I. obliquus* extract has anti-cancer effects. We also showed, for the first time, that *I. obliquus* maintains the body temperature in a mouse model of tumorigenesis. Although previous studies

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have reported anti-tumor effects of *I. obliquus* [9, 22], most of them analyzed cancer treatment and not cancer prevention, as *I. obliquus* extracts were administered immediately before or after tumor transplantation [19, 24, 25]. We hypothesized that long-term continuous intake of the *I. obliquus* extract could suppress tumorigenesis by supporting normal metabolic reactions in the organism, including thermogenesis. Continuous intake of the *I. obliquus* extract at the dose similar to that received by Japanese people through daily Chaga tea drinking (6 mg/kg/day) significantly reduced body weight in middle-aged mice (Fig. 1A), but not in mature adult mice (Fig. 1C), suggesting that *I. obliquus* intake promotes lipolysis in age-accumulated adipose tissue. A previous study showed that water-soluble components extracted from *I. obliquus* improved insulin sensitivity and reduced adiposity in obese mice fed a high-fat diet [17], suggesting beneficial anti-hyperglycemic effects and lipid metabolism enhancement. Consistent with our result, that study detected no difference in body weight between control and *I. obliquus* extract-treated mice fed a normal diet. In view of the link revealed between obesity and multiple types of cancer [3], continuous intake of the *I. obliquus* aqueous extract could have a potential for tumor suppression by promoting lipid metabolism.

As the next step, we examined the anticancer activity in mice taking the *I. obliquus* extracts daily for 3 weeks prior to tumor implantation. As shown in Fig. 2 and Fig. 3, a significant suppression of cancer development was observed both in the tumor growth model and spontaneous metastasis model after the ingestion of the *I. obliquus* extract at the dose corresponding to human daily uptake. Though the mechanisms underlying anti-tumor effects of *I. obliquus* are still unclear, previous studies suggest that apoptosis-inducing activity, changes in tumor microenvironemt such as suppression of angiogenesis, and the improvement of chronic inflammation in adipose tissue could be involved in *I. obliquus*-mediated cancer suppression. Thus, oral administration of the *I. obliquus* aqueous extract ameliorated acute inflammation in dextran sodium sulfate-induced colitis in mice [16]. Here, we observed a tendency for tumor cell agglomeration (Fig. 3A and Fig. 5) and reduced vascularization (Fig. 3B) in mice drinking the *I. obliquus* extract, which can be explained by anti-inflammatory effects exerted by *I. obliquus* . Metabolic regulation may also be an important aspect of the anti-cancer effects of *I. obliquus*. Obesity and hypernutrition may affect different tissues simultaneously resulting in a systemic increase in non-esterified fatty acids, insulin, glucose, leptin, and inflammatory cytokines, and decrease in adiponectin [26], which can directly promote cancer cell survival, proliferation, and malignant progression. We measured serum levels of glucose, leptin, and adiponectin in mice drinking the *I. obliquus* extract; however, no difference was found (data not shown). Further studies are needed to understand the molecular mechanism underlying *I. obliquus* effects.

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As recent findings revealed a correlation between tumor development and body temperature [8, 27], we measured the body temperature in mice receiving the *I. obliquus* extract continuously before and after tumor implantation. The results revealed a significantly lower body temperature in the control group at the light-to-dark switching (Fig. 6B), while the body temperature in the *I. obliquus* group remained constant. Although there is no direct evidence of causal relationship between the maintenance of body temperature and tumor suppression, mice drinking the *I. obliquus* extract showed higher body temperature before tumor implantation (Fig. 6A), indicating that the effects occurred prior to tumor suppression and may be involved in the anti-tumor activity. It has been suggested that brown adipose tissue (BAT) plays a key role in energy homeostasis and thermogenesis not only in infants but also in adults [28]. BAT activity is known to positively correlate with energy expenditure during cold exposure and negatively with age, body mass index, and fasting glycemia, suggesting the association between BAT, cold-induced thermogenesis, and energy metabolism [29]. The increase in the body temperature by *I. obliquus* may be mediated by BAT activity and stimulation of lipid metabolism. As hypothermia is known to activate adipocytes to stimulate tumor growth [30], the effect of *I. obliquus* on maintaining the body temperature could play a critical role in tumor suppression. Future studies addressing metabolic and inflammatory mechanisms underlying the responses of the tumor microenvironment to *I. obliquus* are required to substantiate our conclusions.

5. Conclusion

We showed that the continuous intake of the *I. obliquus* extract can potentially suppress cancer development through the maintenance of the body temperature. In addition, middle-aged mice drinking the *I. obliquus* extract exhibited body weight loss. Our findings suggest that the aqueous extract of *I. obliquus* could be used as a natural product for cancer suppression and general health care.

Declarations

Author contribution statement

Satoru Arata: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jun Watanabe: Performed the experiments; Wrote the paper.

Masako Maeda, Masato Yamamoto: Contributed reagents, materials, analysis tools or data.

Hideto Matsuhashi, Mamiko Mochizuki, Nobuyuki Kagami: Performed the experiments.

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Kazuho Honda: Conceived and designed the experiments.

Masahiro Inagaki: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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