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ORIGINAL ARTICLE

Vaticanol C, a novel resveratrol tetramer, reduces lymph node and lung metastases of mouse mammary carcinoma carrying p53 mutation

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Abstract
Purpose The effects of vaticanol C (Vat-C), a novel resveratrol tetramer, were studied in a mouse metastatic mammary cancer model carrying mutations in *p53* that produce a metastatic spectrum similar to that seen in human breast cancers.

Methods Mammary tumors, induced by inoculation of syngeneic BALB/c mice with BJMC3879 cells, were subsequently treated with Vat-C at 0, 100 and 200 ppm in their diet.

Results The in vitro study demonstrated that Vat-C induced apoptosis, as inferred by morphological changes, nucleosomal DNA fragmentation and elevated activities of caspases. Although tumor volumes were not apparently suppressed in mice treated with Vat-C, the multiplicity of lymph node metastasis was significantly decreased in the 200-ppm group. Furthermore, the multiplicity of lung metastasis was also significantly lower in the 200-ppm group. In any category of organ metastasis, the number of organs with metastasis tended to be lower in the 200-ppm group, but these findings were not statistically significant. The levels of apoptosis were significantly higher in the 200-ppm group, but DNA synthesis only a tended to be lower in this group. Microvessel density in tumors also tended to be lower in the Vat-C-treated groups. Moreover, the numbers of lymphatic vessels having intraluminal tumor cells was significantly lower in mammary tumors of mice given 100 and 200-ppm Vat-C, indicating a reduction in migrating tumor cells into the lymphatic vessels of tumor tissue.

Conclusions These results suggest that the observed antimetastatic activity of Vat-C may be of clinical significance as an adjuvant therapy in metastatic human breast cancer having *p53* mutations, and may also be useful as a chemopreventative of breast cancer development.

Keywords Vaticanol C · Polyphenol · Mammary cancer · Apoptosis · Metastasis · Chemoprevention

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Abbreviations

BrdU	5′-Bromo-2′-deoxyuridine
HUVEC	Human umbilical vein endothelial cells
MMTV	Mouse mammary tumor virus
PBS	Phosphate-buffered saline
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end-labeling
Vat-C	Vaticanol C
vWF	von Willebrand factor

Introduction

Breast cancer is one of the most lethal carcinomas. In the United States, breast cancer is the second leading cause of cancer death in women, with approximately 200,000 American women diagnosed in 2002 and about 40,000 (20%) ultimately dying from the disease [1]. In Japan, the incidence of breast cancer is continuously increasing and the disease now ranks fifth as a cause of female mortality, the number of deaths rising 2.6-fold between 1975 and 1998 [2]. The lethality of breast cancer is largely due to metastasis; the most common sites are the lungs, lymph nodes, liver, and bone. Thus, there is a need for the development of effective and less toxic chemopreventive agents that can delay progression of breast cancer, resulting in prolonged life.

Increasing attention has been paid to primitive medicinal plants to discover new substances with potentially useful biological activities. Recently, a large number of dietary factors and natural products have been evaluated as potential chemopreventive or therapeutic agents. Among them, the polyphenols are of great interest because of their antioxidative and possible antitumor activity [3]. Resveratrol (3,5,4′-trihydroxystilbene), a typical chemopreventive and therapeutic polyphenol, is one of the most common phytochemical compounds present in grape skin and various medicinal plants. Many studies have revealed resveratrol's multifunctional biological activities such as cancer chemopreventive activity [4] and cardioprotective effects [5]. Our and others' phytochemical studies have indicated that dipterocarpaceous plants are rich in resveratrol oligomers [6]. One of these plants has been traditionally used in Ayurvedic medicine in India for many centuries. Therefore, dipterocarpaceous plants have attracted interest as useful sources of chemopreventive or therapeutic agents in recent years.

In the course of our phytochemical studies to search for biologically active compounds, the resveratrol tetramer Vaticanol-C (Vat-C), isolated from the stem bark of *Vatica rassak* in *Dipterocarpaceae*, was found to be a potent apoptosis-inducing agent in various cancer cells in humans in vitro [7], suggesting antitumoral activity for Vat-C. Here, we investigated the chemopreventive abilities of Vat-C, in particular any antimetastatic ability, in a mouse metastatic mammary cancer model (carrying p53 mutations) showing a metastatic spectrum similar to that seen in human breast cancers [8–10]. In addition, the apoptotic responses of the metastatic mouse mammary carcinoma cells treated with Vat-C were investigated with respect to cell viability, morphology, DNA fragmentation and caspase activity (executers of apoptosis).

Materials and methods

Experimental regimen

Resveratrol oligomers were isolated from the stem bark of *Vatica rassak*, and their structures were determined in our previous study [6]. Among them, a resveratrol tetramer, vaticanol C (Vat-C), was examined in the present study.

Cell line and animals

The BJMC3879 mammary carcinoma cell line used was derived from a metastatic focus within a lymph node from a female BALB/c mouse that had had the mouse mammary tumor virus (MMTV) injected into the inguinal mammary glands [11]. The mammary tumors resulting from MMTV inoculation showed a high metastatic propensity to lymph nodes and lungs [8–10], a trait retained through culture. BJMC3879 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and streptomycin/penicillin in an incubator at 37°C and under 5% CO_2.

Animal experiment

A total of 70 female 6-week-old BALB/c mice were used in this study (Japan SLC, Hamamatsu, Japan). The animals were housed five per plastic cage on wood chip bedding with free access to water and food under controlled temperature (21 ± 2°C), humidity (50 ± 10%), and lighting (12–12 h light–dark cycle). All animals were held for a 1-week acclimatization period before study commencement. All manipulations of mice were performed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals of Osaka Medical College, Japan.

Cell growth and apoptosis in mammary carcinoma cells treated with Vat-C in vitro

BJMC3879 cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma, Tokyo) and 2 mM L-glutamine under an atmosphere of 95% air and 5% CO_2 at 37°C. The cytotoxicity and cell viability were determined by the trypan blue dye-exclusion assay. To evaluate the IC50, 2×10^5 ml^{-1} cells were treated with Vat-C at various concentrations for 72 h. Vat-C concentration at 8 μM used in the in vitro study was determined based on cell growth in the IC50 concentration. For morphological examination of apoptotic changes, cells were stained with Hoechst 33342 (5 μg/ml) at 37°C for 30 min, rinsed twice with phosphate-buffered saline (PBS), pipetted dropwise onto a glass slide, and examined by fluorescence microscopy using an Olympus microscope (Tokyo, Japan) equipped with an epi-illuminator and appropriate filters. The response of BJMC3879 cells to Vat-C over time was determined by examining the effects of 8 μM Vat-C on cell growth for 24, 48 and 72 h.

DNA was extracted from BJMC3879 cells treated with 8 μM Vat-C for 24, 48 and 72 h, and RNase was added to the DNA solution at a final concentration of 20 μg/ml, and the mixture then incubated at 37°C for 30 min. After electrophoresis through a 2.5% agarose gel, DNA was visualized by ethidium bromide staining.

BJMC3879 cells were plated the day before Vat-C treatment at 1×10^4 cells/well in a 96-well plate. Cell viability was measured in cells treated with 8 μM Vat-C or vehicle alone (dimethyl sulfoxide; DMSO) for 48 and 72 h using a fluorescent assay kit (CellTiter-Blue Cell Viability Assay, Promega, Madison, WI, USA) and then the activities of caspase-8, caspase-9 and caspase-3 were measured using a luminescent assay kit (Promega, USA). Caspase activity was recorded in terms of the luminescent signal produced by caspase cleavage of the corresponding substrate using a Luminoskan Acent (ThermoElectron Co., Helsinki, Finland). The caspase activity data was then adjusted to account for the corresponding cell viability.

In vivo analysis of the effect of Vat-C on a metastatic mammary cancer model

Based on the results of a separate study, the dietary dosages of Vat-C were set at 200 ppm for the high dose and 100 ppm for the intermediate dose. For the current study, BJMC3879 cells (5×10^6 cells/0.3 ml in PBS) were inoculated subcutaneously into the right inguinal region of 30 female BALB/c mice. Two weeks later, when tumors had developed to approximately 0.2 cm in diameter, mice were given 0-, 100- and 200-ppm Vat-C in their diet for 8 weeks. Individual body weights were recorded weekly and food consumption was measured over a 2-day period and Vat-C intake (mg/kg/day) was then calculated. Each mammary tumor was also measured weekly using calipers, and tumor volumes were calculated using the following formula: maximum diameter × (minimum diameter)^2 × 0.4 [12].

Histopathological analysis

At necropsy, tumors and lymph nodes—routinely those from the axillary and femoral regions as well as those appearing abnormal—were removed, fixed in 10% formaldehyde solution in phosphate buffer and processed through to paraffin embedding. Lungs were inflated with formaldehyde solution prior to excision and immersion in fixative; the individual lobes were subsequently removed from the bronchial tree and examined for metastatic foci and similarly processed to paraffin embedding. All paraffin-embedded tissues were cut into 4-μm-thick sections and then sequential sections were stained with hematoxylin and eosin for histopathological examination. Unstained sections were used for immunohistochemistry.

p53 immunohistochemistry

The avidin–biotin complex method was used for p53 immunohistochemistry. Unstained sections were immersed in distilled water and heated in a microwave oven for antigen retrieval prior to incubation with a p53 mouse monoclonal antibody (Clone Pab240, Santa Cruz Biotechnology, Santa Cruz, CA, USA) that reacts to the mutant protein in fixed specimens.

DNA synthesis in mammary tumors

All animals received 100 mg/kg 5′-bromo-2′-deoxyuridine (BrdU; Sigma Co., St. Louis, MO, USA) i.p. at 1 h prior to ether euthanization, and the tumors from five animals from each treatment group were subsequently evaluated for DNA synthesis rates as inferred by BrdU incorporation. Using unstained paraffin-embedded tissue sections, DNA was denatured in situ by incubation in 4N HCl solution for 20 min at 37°C. The incorporated BrdU was visualized after exposure to an anti-BrdU mouse monoclonal antibody (Clone Bu20a, Dako, Glostrup, Denmark). The number of BrdU-positive S-phase cells per 5,000 cells was counted in five random high power (×400) fields of viable tissue and the BrdU labeling indices then expressed as a percentage of total cells counted.

Cell death in mammary tumors

For quantitative analysis of cell death, sections from paraffin-embedded tumors were assayed using the terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end-labeling (TUNEL) method in conjunction with an apoptosis in situ detection kit (Wako Pure Chemical Industries) with minor modifications to the manufacturer's protocol. TUNEL-positive cells (mainly regarded as apoptotic cells) were counted in viable regions peripheral to areas of necrosis in tumor sections. The number of TUNEL-positive cells per 5,000 cells was counted in five random high power (×400) fields and expressed as a percentage of the total cells counted.

Lymphatic and blood microvascular densities in mammary tumors

To quantitatively assess blood and lymphatic microvessel density in primary mammary carcinomas we used the avidin–biotin immunohistochemical complex method (LSAB kit; DakoCytomation). A rabbit polyclonal antibody against the von Willebrand factor (vWF) (DakoCytomation), a specific marker for blood vessel endothelium, and a hamster monoclonal antibody anti-podoplanin (AngioBio Co., Del Mar, CA, USA), a lymphatic endothelium marker, were used. The number of immunopositive blood microvessels was counted as described previously [13]. Briefly, the slides were scanned at low-power (×100) magnification to identify those areas having the highest number of vessels; the five areas of highest microvascular density were then selected and counted at higher (×200–400) magnification to obtain mean ± SD values. In addition, the number of podoplanin-positive lymphatic vessels having intralumenal tumor cells present was also counted and expressed as the mean ± SD.

Statistical analysis

Significant differences in the quantitative data containing dose-response effects between groups were analyzed using the Norman–Kleus test. The differences in metastatic incidence were examined by Fisher's exact probability test.

Results

Effects of Vat-C on mouse mammary carcinoma cells in vitro

The cell viability of mammary cancer cells (BJMC3879) was significantly lower after 48 and 72 h of 8 μM Vat-C treatment (Fig. 1a). BJMC3879 cells treated with 8 μM Vat-C for 72 h showed apparent apoptotic features by Hoechst 33342 staining, namely fragmented nuclei with condensed chromatin and apoptotic bodies (Fig. 1c), as compared to the control (Fig. 1b). In addition, a DNA ladder was apparently observed in cells treated with 8 μM Vat-C for 72 h (Fig. 1d). Significantly elevated activities of caspase-8, caspase-9 and caspase-3 were observed in BJMC3879 cells treated with 8 μM Vat-C for 48 and 72 h as compared to the respective controls (Fig. 1e).

Food consumption and Vat-C intake

The average food consumption was 5.4 g/day/mouse in the control group, and 5.3 g/day/mouse in both the 100- and 200-ppm groups (Table 1). The average Vat-C intake was 22.9 mg/kg/day/mouse in the 100-ppm group and 44.7 mg/kg/day/mouse in the 200-ppm group.

Body weights and mammary tumor growth of mice treated with Vat-C

The body weight changes in the control and Vat-C-treated mice bearing mammary tumors are shown in Fig. 2a. The weights of mice given 200 ppm of dietary Vat-C appeared to be slightly higher than the controls in weeks 6–8, but these differences were not statistically significant. In the 100-ppm group, no appreciable differences in body weights between control and treated mice were observed. One of the control mice and two of the 200-ppm Vat-C mice died accidentally. One mouse in the 100-ppm group died of liver metastasis in week 7.

Tumor volumes are presented in Fig. 2b. Tumor growth, as inferred by computed volume, did not differ between the Vat-C-treated and control groups. However, at week 8 (the end of the experiment), the average tumor volume tended to be smaller in the Vat-C treated mice than in the control animals (control, 1678 ± 334 mm^3; 100 ppm, 1513 ± 528 mm^3; 200 ppm, 1377 ± 295 mm^3).

Metastasis of mammary carcinomas in mice treated with Vat-C

Histopathologically, the mammary carcinomas induced by BJMC3879 cell inoculation proved to be moderately differentiated adenocarcinomas (Fig. 3a) which contain p53 mutations, as inferred by immunohistochemistry (Fig. 3b).

Lymph node metastasis: Lymph node metastasis occurred in 89% of control animals (Fig. 3c), in 100% of the 100-ppm group, and in 56% of the 200-ppm

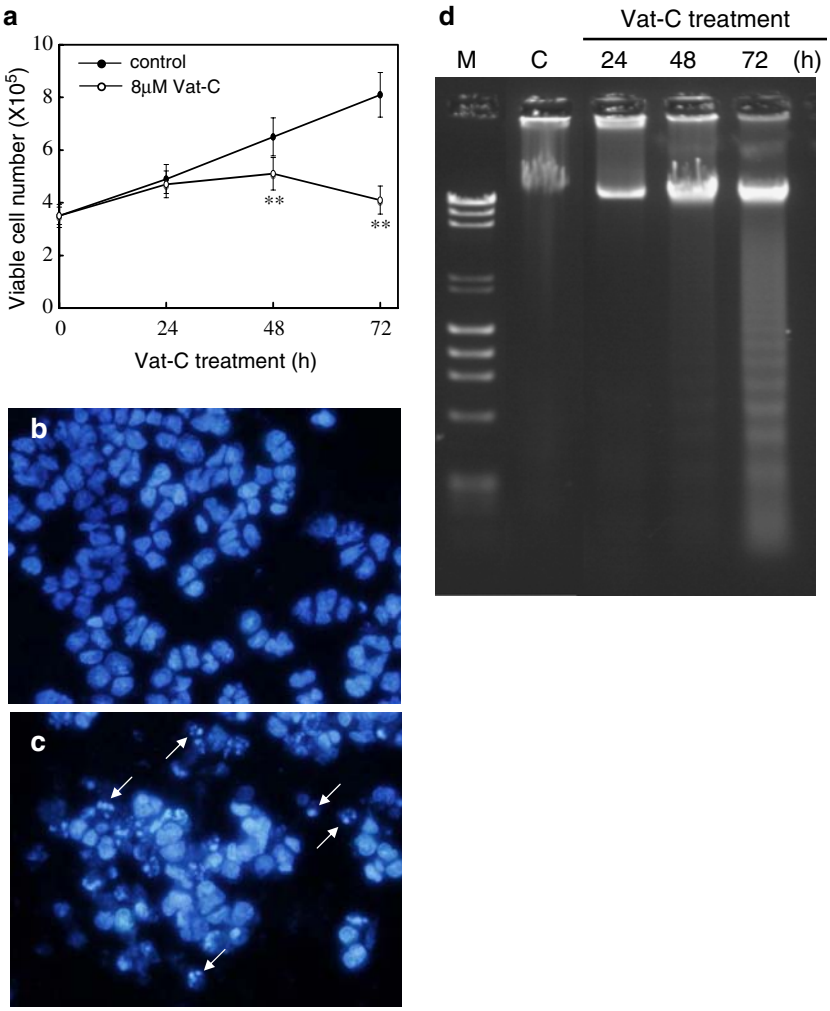

Fig. 1 **a** Cell viability was significantly lower in mouse mammary carcinoma BJMC3879 cells treated with 8 μM Vat-C for 48 and 72 h (**$P < 0.01$). Data were calculated from three repeated experiments of duplicate samples in control and Vat-C-treated cells. Data represent mean ± SD. **b, c** Compared to the morphology of BJMC3879 cells treated with the vehicle control DMSO (**b**), the cells treated with 8 μM Vat-C for 72 h showed fragmented nuclei with condensed chromatin and apoptotic bodies (*arrows*, **C**). **b, c** Hoechst 33342 stain, ×400. **d** Nucleosomal DNA fragmentation of BJMC3879 cells treated with 8 μM Vat-C for up to 72 h. Lane *M* contains a DNA size marker. DNA fragmentation was apparent after exposure to 8 μM Vat-C for 72 h. **e** Caspase activities were evaluated using a luminescent assay. Activities of caspase-8, caspase-9 and caspase-3 were significantly elevated in BJMC3879 cells treated with 8 μM Vat-C for 48 and 72 h (*$P < 0.05$, **$P < 0.01$). Five samples each of control and Vat-C-treated cells were examined. Data represent mean ± SD

group (Fig. 3d); this difference with the 200-ppm group was not statistically significant ($P = 0.06$). However, the number of metastasis-positive lymph nodes per mouse was significantly lower in the 200-ppm group compared to the control group (Fig. 4a).

Lung metastasis: Lung metastasis occurred in 78% of control animals (Fig. 3e), in 60% of the 100-ppm group, and in 63% of the 200-ppm group (Fig. 3f). The number of lung metastatic foci greater than 200 μm in diameter per mouse was significantly lower in the 200-ppm group (Fig. 4b).

Overall metastasis: In metastasis to other organs except for lymph nodes and lungs, metastatic foci were observed in the liver (in four controls, in five 100-ppm mice and in two 200-ppm mice) (Fig. 5a), kidney (in one control and in two 100-ppm mice) (Fig. 5b), ovaries (in one control and one 100 ppm) (Fig. 5c), pancreas (in one 100-ppm mouse) (Fig. 5d), adrenals (in one 100-ppm mouse) and trachea (in one control). In the 200-ppm group, only liver metastasis was seen in addition to lymph node and lung metastasis. The multiplicities of metastasis are presented in Table 2. In metastasis to organs except for the lymph nodes and lungs, both the numbers of organs with metastasis and the average number of organs with metastasis appeared to be lower in the 200-ppm group. In addition, with

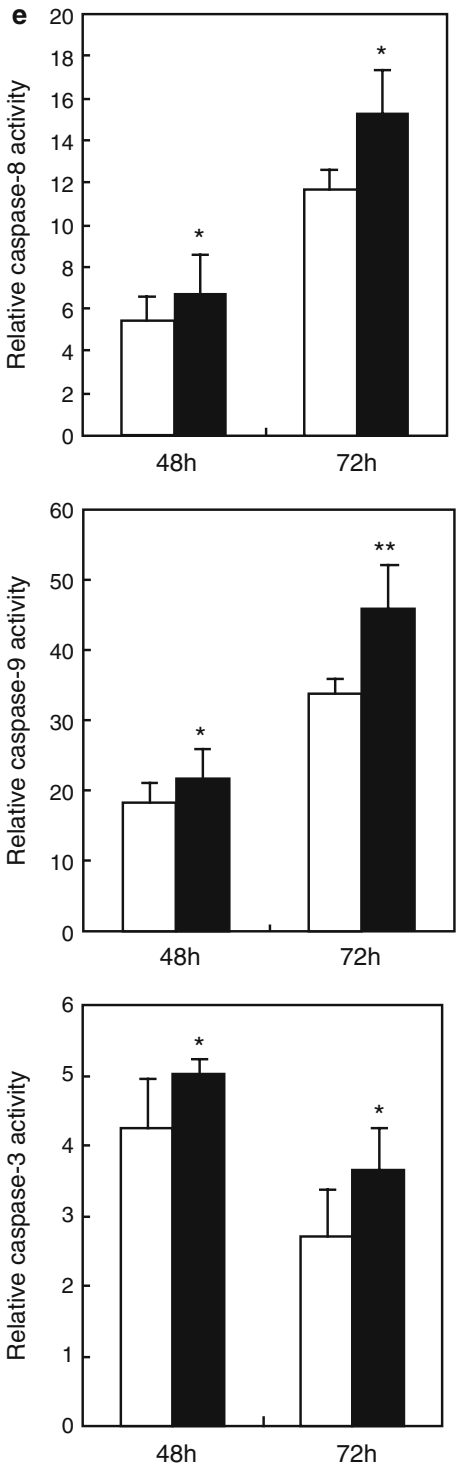

Fig. 1 continued

respect to any category of organ metastasis, both the numbers of organs with metastasis and the average number of organs with metastasis also tended to be lower in the 200-ppm group. However, these differences were not statistically significant.

Table 1 Average food consumption and Vat-C intake

Vat-C (ppm in diet)	Average food consumption (g/day/mouse)	Average Vat-C intake (mg/kg/day/mouse)
0	5.4	0
100	5.3	22.9
200	5.3	44.7

Groups of 10 mice were given 0-, 100- or 200-ppm Vat-C in the diet for 8 weeks. Vat-C intakes were calculated from data on food consumption and body weight

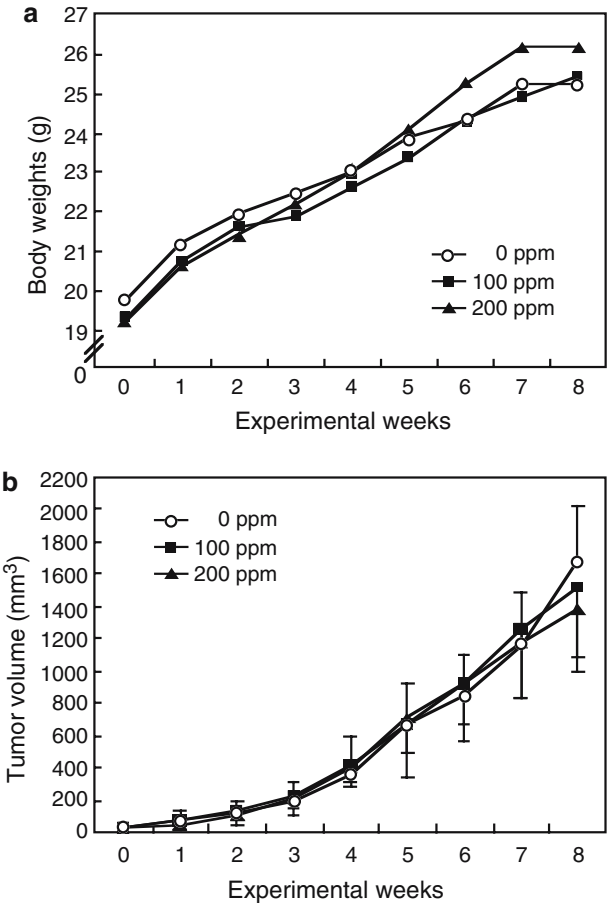

Fig. 2 Body weights (**a**) and tumor volumes (**b**) in mammary carcinomas from female BALB/c control mice and mice treated with 100- and 200-ppm dietary Vat-C. Each group consisted of 10 mice. **a** There were no differences in body weight between the Vat-C-treated and control groups. **b** Tumor volume was also similar between the Vat-C-treated and control groups. Data represent the mean ± SD

DNA synthesis and apoptosis in mammary cancers of mice treated with Vat-C

The DNA synthesis levels in mammary carcinomas of Vat-C-treated mice, as inferred by the BrdU labeling indices, are shown in Fig. 6a. DNA synthesis in

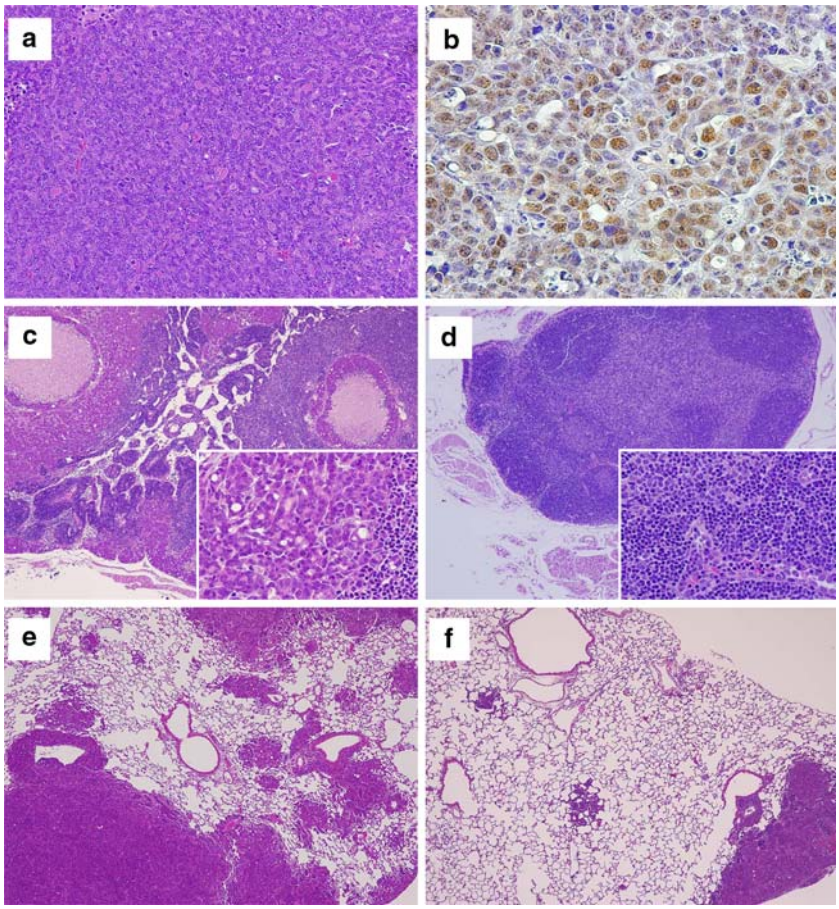

Fig. 3 Histopathological analysis of mammary tumors in mice treated with Vat-C. **a** The implanted mammary carcinomas proved to be moderately differentiated adenocarcinoma, ×200. **b** p53 immunohistochemistry of mammary carcinoma induced by BJMC3879 cell inoculation. Note nuclear staining for abnormal p53 protein, indicating that these cells carry mutant p53, ×400. **c** Metastasis to a lymph node, a frequent occurrence in control mice, ×40. Metastatic carcinoma cells were filled with sinusoidal space (×400, *inset*). **d** A lymph node from a mouse given 200-ppm Vat-C. Fewer lymph nodes with metastasis were found. No tumor cells were observed (×400, *inset*). **e** Metastatic foci in the lung of a control mouse. Many metastatic foci and nodules were seen, ×40. **f** Metastatic foci in the lung of mouse given 200-ppm Vat-C. Metastatic lung foci were much smaller in the 200-ppm group than in the control group. X40. **a, c–f** H&E stain, **b** p53 immunohistochemistry

tumors tended to be lower in the 200-ppm group but this was not statistically significant. The results of the quantitative analysis of apoptosis in lesions, as assessed by the TUNEL assay, are shown in Fig. 6b; the number of TUNEL-positive cells was significantly greater in tumors from the 200-ppm group (Fig. 5f) compared to the levels seen in tumors from control mice (Fig. 5e).

Blood microvascular density and lymphatic vessels in mammary cancers of mice treated with Vat-C

Microvessel density, determined by immunohistochemical analysis with the blood vessel endothelial cell marker vWF, tended to be lower in the Vat-C-treated groups in a dose-dependent manner but these findings were not statistically significant (Fig. 6c).

The lymphatic vessels in mammary tumors were stained with anti-podoplanin antibody, as demonstrated in Fig. 5g, h. Independent of treatment, these lymphatic vessels were well developed in the outer, superficial layers of the mammary tumors in a somewhat hexagonal network pattern. There were tumor cells within the lumina of the dilated lymphatic vessels of tumors in both control (Fig. 5g) and Vat-C-treated animals (Fig. 5h). However, the number of lymphatic vessels having intraluminal tumor cells was significantly lower in mammary tumors of mice given 100- and 200-ppm dietary Vat-C (Fig. 6d), indicating a reduction in migration of tumor cells into the lymphatic vessels of tumor tissues.

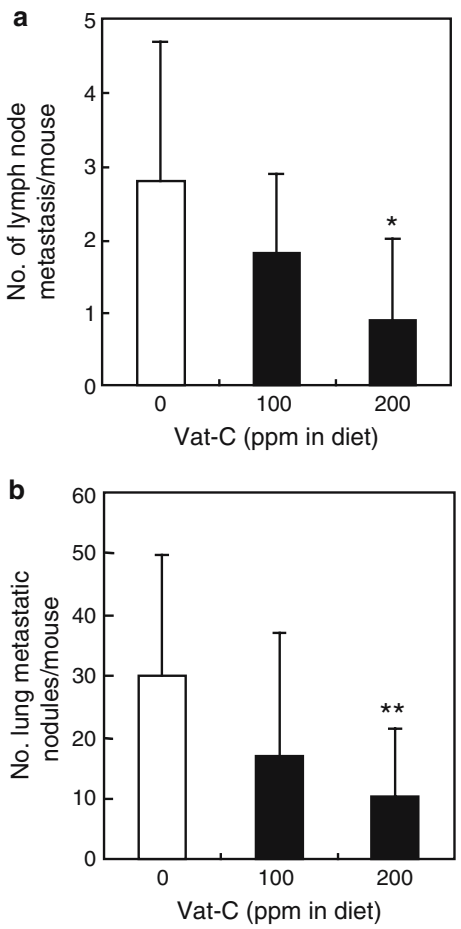

Fig. 4 **a** The multiplicity of lymph node metastasis was significantly lower in the 200-ppm Vat-C (*$P < 0.05$). **b** The multiplicity of lung metastasis was also significantly lower in the 200-ppm Vat-C group (**$P < 0.01$). Data represent mean ± SD

Discussion

Resveratrol is a natural polyphenol occurring in grape skin, red wine, soybeans and various other plants with medicinal properties associated with reduced cardiovascular disease [14]. Furthermore, resveratrol has been shown to be an antitumor agent and chemopreventive agent, and affects cell proliferation through its action on tumor initiation, promotion and progression [4–16]. Two representative resveratrol tetramer, vaticanols B and C (Vat-C) have been isolated from three dipterocarpaceous plants, as the major products [6]. Recently, we demonstrated that Vat-C suppresses cell growth and induction of apoptosis via a mitochondrial pathway in various cancer cell lines [7], suggesting that Vat-C is possibly a chemopreventive agent for cancer.

The present study indicates that Vat-C does not inhibit tumor growth, but significantly suppresses the multiplicity of metastasis to the lymph nodes and lungs, in a mouse metastatic mammary carcinoma model. Since DNA synthesis in tumor cells only tended to slightly decrease in the Vat-C-treated groups, it follows that there would be no suppression in tumor growth. Human breast cancers metastasize mainly to the lymph nodes, lungs, liver, and bone, and intractable metastasis leads to death. Carter et al. [17] have reported that tumor size and nodal status are practical parameters for estimating disease prognosis. Therefore, a treatment involving Vat-C that offered suppression of metastasis would have significant clinical implications.

The present study demonstrated that Vat-C significantly induces apoptosis in murine mammary carcinoma cells both in vitro and in vivo. There are two pathways currently proposed to play major roles in regulating apoptosis in mammalian cells: one mediated by death receptors (extrinsic pathway; execution by caspase-8) and one mediated by the mitochondria (intrinsic pathway; execution by caspase-9)[18]. Caspase-3, involved in terminal execution of apoptosis, is subsequently activated in both pathways. We previously demonstrated that Vat-C induced mitochondria-mediated apoptosis as indicated by loss of mitochondrial membrane potential and concurrent cytochrome *c* release and activation of caspase-9 and caspase-3 [7]. In addition, Vat-C also prevents Bcl-2 expression (an apoptosis inhibiting protein) [7]. We also demonstrated in the present study that Vat-C induces increases in the activities of caspase-8, caspase-9 and caspase-3 in mammary carcinoma cells BJMC3879, suggesting at the very least that Vat-C induces mitochondria-mediated apoptosis. Since caspase-8 has been shown to be autoactivated by ligand- and DISC-independent pathways [19], Vat-C may directly or indirectly activate caspase-8. The death receptor and mitochondrial pathways are linked by the cleavage of Bid by caspase-8. It is known that cleaved Bid translocates to the mitochondria and induces mitochondria-mediated apoptosis [20]. The elevated activities of caspase-8, -9 and -3 in our Vat-C-treated cells suggest that both the mitochondrial and death receptor pathways are involved in Vat-C-induced apoptosis.

In the present study, we demonstrated that the multiplicities of lymphatic and pulmonary metastasis were significantly lower in the 200-ppm Vat-C group. Furthermore, overall metastasis to any organ tended to be lower in the 200-ppm group. Neovascularization is a key process in the growth of solid tumors, and the growth of both primary tumors and metastases is thus

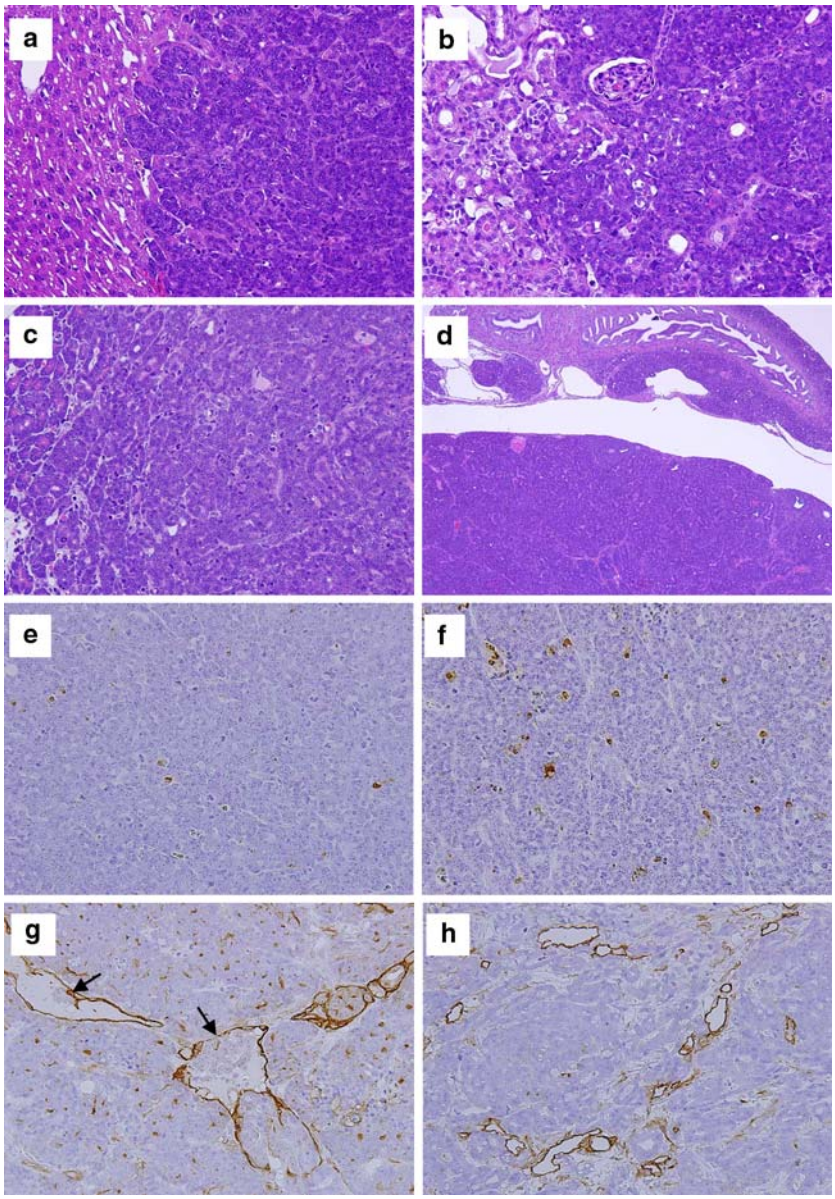

Fig. 5 Representative metastasis to other organs except for lymph nodes and lungs (**a**–**d**), apoptotic cell death (**e**, **f**) and lymphatic vessels of tumors (**g**, **h**). **a** Liver metastasis of a mammary carcinoma of a control mouse, ×200. **b** Kidney metastasis of a mammary carcinoma of a control mouse, ×200. **c** Pancreas metastasis of a mammary carcinoma of a mouse receiving 100-ppm Vat-C, ×200. **d** Ovary metastasis of a mammary carcinoma of a mouse receiving 100-ppm Vat-C. ×40 **e**, **f** TUNEL staining in mammary tumors. Whereas some TUNEL-positive cells are seen in the tumor of a control mouse (**e**), many more TUNEL-positive cells are observed in the tumor of a mouse given 200-ppm Vat-C (**f**), ×200. **g**, **h** Podoplanin-positive lymphatic vessels of a tumor in a control mouse were often dilated and filled with tumor cells (*arrows*, **g**). The Vat-C-treated group had significantly fewer dilated lymphatic vessels containing intralumenal tumor cells. **h** ×200, **a**–**d** H&E stain, **e**, **f** TUNEL stain; **g**, **h** podoplanin immunohistochemistry

Table 2 Multiplicity of metastasis of mammary carcinomas

Vat-C (ppm in diet)	Number of mice examined	Metastasis to organs except for lymph nodes and lungs		Any category of metastasis	
		Number of organs with metastasis	Average no. of organs with metastasis	Total no. of organs with metastasis/mouse	Average no. of organs with metastasis/mouse
0	9	7	0.8 ± 1.1	22	2.4 ± 1.5
100	10	10	1.0 ± 1.1	28	2.8 ± 1.5
200	9	2	0.3 ± 0.5	12	1.5 ± 1.1

Values are means ± SD

angiogenesis-dependent [21]. The microvessel density in our tumors was lower in the Vat-C-treated groups but not significantly. However, since resveratrol has been shown to inhibit tubular formation of human umbilical vein endothelial cells (HUVEC) and deprive angiogenesis in mouse lung cancer [22], a resveratrol analogue of Vat-C may exert antiangiogenetic action. In addition, a reduction in lung metastasis via a hematogenous pathway may be due to antiangiogenic action by Vat-C. Further investigation is required to determine whether Vat-C exerts an antiangiogenic action.

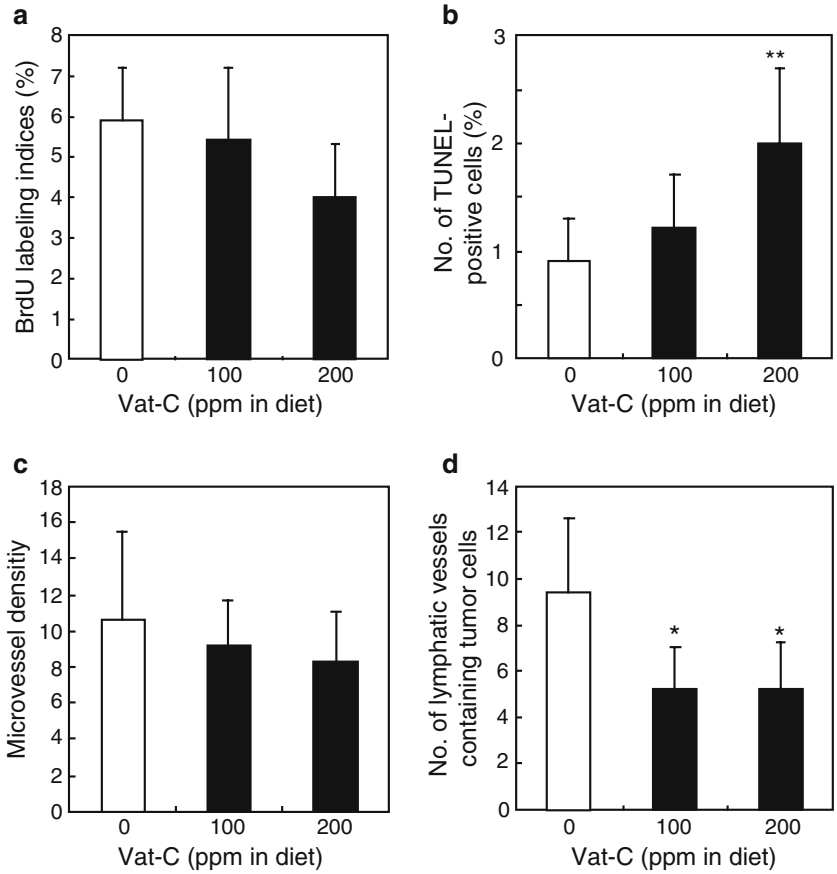

Fig. 6 **a** DNA synthesis, inferred by BrdU labeling indices, tended to be lower in the 200-ppm Vat-C group, but not significantly. **b** Apoptotic cell death, assessed by TUNEL assay, was significantly greater in the 200-ppm Vat-C group (**$P < 0.01$). **c** Microvessel density in tumors, inferred by vWF-positive endothelium, tended to be lower in the 200-ppm Vat-C group, but not significantly. **d** The number of lymphatic vessels containing tumor cells intralumenally was significantly lower in groups receiving 100- and 200-ppm Vat-C than the control group (*$P < 0.05$). Data represent mean ± SD

Cancer cells metastasize to distal sites via the lymphatic system as well as through the vascular system. The lymphatic capillaries present in tissues and tumors provide entrance into the lymphatics, allowing cancer cell migration to the lymph nodes. In the present study, a significant decrease in the number of lymphatic vessels with tumor cells in their lumina was observed in the 100- and 200-ppm Vat-C groups compared to the control group, indicating that Vat-C may have an inhibitory effect on migration. VEGF-C and its receptor VEGFR-3 have been shown to correlate with lymph node metastasis in a variety of human cancers, including breast neoplasms [23–25]. Vat-C may affect the capacity for cancer cell migration by interfering with VEGF-C/VEGFR-3 binding or expression within lymphatic vessels, but further investigation is needed in clarify these issues. Nonetheless, since Vat-C directly acts on cancer cells, i.e., decreases survival by apoptosis of mammary cancer cells, this situation may lead to inhibition of cancer migration to blood and lymphatic vessels. Therefore, it is possible that Vat-C can suppress both lymphatic and hematogeneous metastasis, but further investigation is required.

The present experiments indicate that Vat-C-induced apoptosis in BJMC3879 cells having p53 mutations. In fact, Vat-C has been shown to induce apoptosis in a wide variety of cancer cells, irrespective of p53 status [7]. However, resveratrol is known to induce p53-dependent apoptosis [26–30]. In addition, although Vat-C induces apoptosis in colon cancer SW480 cells having *p53* mutations, Vat-C reduces the amount of the mutant *p53* transcripts, suggesting that it may also modulate the transcription level of mutant *p53* [7]. Further investigation using BJMC3879 cells with deletion of the *p53* allele is required. Since 50% of human cancers have *p53* mutations [31], the fact that Vat-C induces an apoptotic response in cancer cells having *p53* mutations may be highly relevant to inhibiting many human cancers. In cases of non-functional p53 status, p73, the p53 homologue, may play a role for apoptosis induction.

In conclusion, we have demonstrated that treatment with Vat-C significantly suppresses metastasis to both lymph nodes and lungs in a mouse mammary cancer model. The total number of all distant-site metastases was also much lower in the Vat-C-treated mice than the control mice. We believe the antimetastatic activity of Vat-C to be of clinical significance in terms of adjuvant therapy and chemoprevention in metastatic human breast cancer.

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