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Research Article

# Treatment with Tenascin C Antibody and/or α-Mangostin Reduces Tumor Growth and Lymph Node Metastasis in a Model of Metastatic Mammary Cancer

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## Abstract

### Background

Antibodies have become a major tool therapeutic in cancer treatment because of their high target specificity; treatment efficacy, however, seems to be higher if the antibodies used are able to affect multiple, rather than single, molecular targets. In this study, we investigated both the anti-tumor effect of tenascin C antibody (TNC Ab) alone and combined with α-mangostin, another anti-tumorigenic compound that uses a different molecular mechanism, in a mouse metastatic mammary cancer model that mimics human disease.

### Methods

Before commencement of main study, localized accumulation of TNC Ab into mouse mammary tumors was confirmed by fair-red luminescence technology. We induced metastatic mammary tumors in BALB/c mice by inoculation of syngeneic BJMC3879Luc2 cells into inguinal mammary fat pads. These mice were subsequently exposed to TNC Ab i.p twice weekly, to continuous dietary α-mangostin, or to a combined TNC Ab + α-mangostin treatment. Control mice received saline twice weekly i.p. The study was terminated after 6 weeks of agent administration and tumor parameters were analyzed.

### Results

We saw no significant differences in group body weights between tumor-bearing animals, but tumor volumes were significantly decreased in all treated groups, as were the bioluminescence imaging for metastatic expansion, the multiplicities of lymph node and lung metastases. Both TNC Ab and α-mangostin also significantly decreased the multiplicity of metastatic foci in any other organ. Lymphatic invasion in tumors was significantly decreased in all treated groups as compared to control. Tumor blood microvessel density was also lower in all treated groups, but not to a statistically significant level in mice receiving TNC Ab alone. While tumor cell proliferation was significantly decreased in all treated groups, α-mangostin, alone and combined with TNC Ab, significantly increased apoptotic levels within mammary tumors.

### Conclusion

Since lymph node involvement is the most important prognostic factor in breast cancer patients, the anti-metastatic activity of both TNC Ab and α-mangostin may be of clinical significance. Unfortunately, the therapeutic effects of TNC Ab and α-mangostin were not enhanced by combination in this model.

## Introduction

According to IARC's GLOBOCAN project - which estimates cancer incidence, mortality and prevalence worldwide - approximately 1,677,000 new breast cancer cases were diagnosed in 2012, accounting for 25% of all newly reported cancers. In that year alone (2012), 522,000 women worldwide died of breast cancer, making the disease the fifth ranked cause of cancer mortality overall [1]. Breast cancer is actually the second most common cancer in women worldwide, and, perhaps more worrisome, is the increasing incidence among younger women under 40 years of age [2-4]. What makes breast cancer so lethal is its propensity to metastasize, preferentially to lymph nodes, lung and bone [5]; less toxic and more effective chemopreventive and anti-metastatic treatments are desperately required if there is to be any advancement in reducing both mortality and morbidity. Because natural antibodies have higher target specificity, lower systemic toxicity, and longer half-

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life [6], there is increasing interest in the therapeutic aspect of antigen-antibody interaction.

Tenascin C (TNC), a glycoprotein of the extracellular matrix, was initially described as a stromal marker of epithelial malignancy and is overexpressed in breast cancers [7,8] and associated with local invasion, distant metastasis and, thus, poor prognosis [9-11]. But TNC is not unique to breast cancers; it is present in the stroma of most solid cancers, and it has further been shown to play a role in proliferation [12], angiogenesis [13,14], invasion, and distal metastasis [10,11] during the process of tumorigenesis [15]. The protein may further be a niche metastatic component; a recent report indicates that breast cancer cells infiltrating the lungs support their own metastasis-initiating ability by expressing TNC [16], and treatment with TNC antibody (TNC Ab) proved effective in inducing regression of U87 glioma cell tumor grafts in mice [17]. These studies implicate TNC as a suitable antigenic target for antibody therapy.

The pericarp of the mangosteen fruit, *Garcinia mangostana* Linn (Figure 1A), has a long history of use as a medicinal plant in Southeast Asia [18]. When damaged, the pericarp secretes a yellow substance that functions to protect the fruit from bacterial infection and also acts as an insect repellant [19]. This yellow exudate contains a class of compounds called xanthones, which includes α-, β-, and γ-mangostin, along with garcinones B and E, mangostinone, tannins, and a flavonoid called epicatechin [18]. The beneficial actions of mangosteen extracts have some scientific support and it has become a popular natural health dietary supplement. In mammary cancer cells, mangosteen extracts, particularly α-mangostin, induce apoptosis using a mitochondrial pathway [20,21], cause cell cycle arrest via induction of $p21^{cip1}$ [21,22] and p27 [20] and promote Akt dephosphorylation [20,21] – all mechanistically associated with suppression of *in vivo* tumor growth and metastasis in mouse mammary cancer models [21,23]. Several other animal models demonstrate the anti-tumorigenic effects of mangosteen extracts, e.g., formation of aberrant crypt foci, a putative preneoplastic lesion in rat colon carcinogenesis, is significantly suppressed by dietary administration of mangosteen extracts [24]. Mangosteen extracts induce a similar anti-tumor effect in human HCT 116 colorectal carcinoma transplanted to nude mice [25]; further, tumor growth associated with cell cycle arrest was significantly suppressed by α-mangostin in a mouse xenograft model of prostate cancer [26].

Since TNC participates in such processes as cellular proliferation and tumor angiogenesis, invasion and metastasis, and since α-mangostin has similar anti-metastatic properties, albeit via different means, we felt both were suitable candidates for investigation as therapeutic agents. We thus looked at the potential anti-tumor efficacy of TNC Ab and at whether or not that efficacy could be enhanced in combination with α-mangostin, using a mouse metastatic mammary cancer model (carrying a *p53* mutation) with a metastatic spectrum similar to that seen in human breast cancers [21,27]. In addition, we analyzed the effects of these single and combined treatments on specific processes such as apoptosis, cell proliferation, angiogenesis and lymphatic invasion in mammary cancers generated in the model.

## Materials and Methods

### TNC Ab and α-mangostin

We produced anti-human tenascin C monoclonal antibody (TNC Ab) from hybridomas induced by the fusion of mouse myeloma NS1 cells with those of rats immunized with human tenascin-C, previously extracted from the supernatant of human myeloma cells cultured in GIT medium. Purified TNC Ab was then isolated by Protein G column separation.

Mangosteen (*Garcinia mangostana* Linn) pericarps (Figure 1A) collected in Thailand were dried, ground, and successively extracted in water and 50% ethanol. After freeze- drying the ethanol extract, the resultant dried powder was suspended in water partitioned with ethyl acetate and purified by silica gel chromatography using the n-hexane-ethyl acetate system. The end product was recrystallized

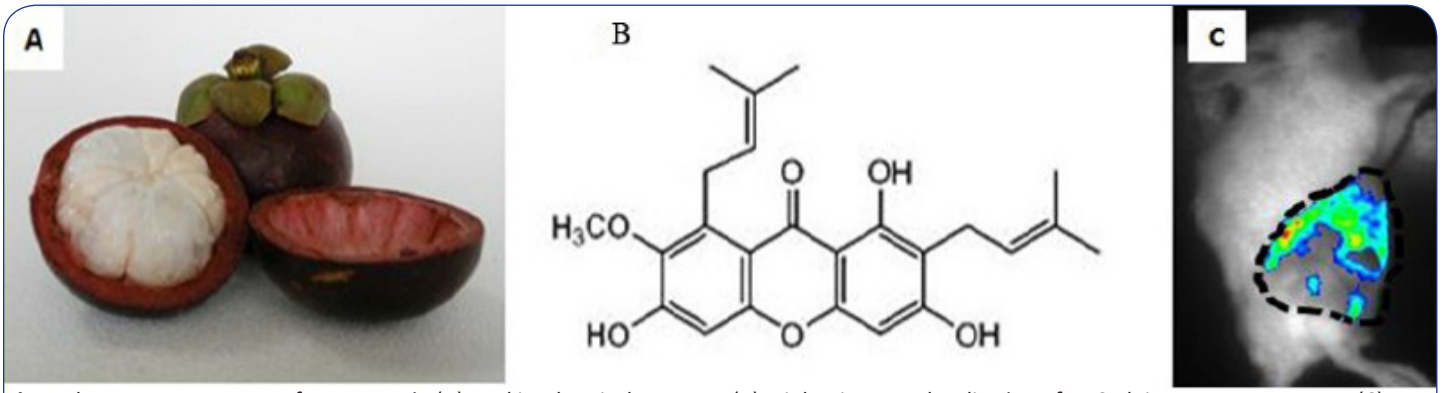


**Fig 1:** The gross appearance of α-mangostin (A), and its chemical structure (B). Bioluminescent localization of TNC Ab in a mammary tumor (C)
A. Mangosteen pericarp is thick but soft and easily cut. The snow white edible endocarp is botanically defined as an aril; the circle of wedge-shaped arils contains 4 to 8 segments. The brown pericarp contains the biologically active compounds including xanthones α-, β- and γ-mangostin.
B. The chemical structure of α-mangostin ($C_{24}H_{26}O_6$, MW 410).
C. In a preliminary pilot study, localized accumulation of anti-tenascin antibody (TNC Ab) in mammary tumors, delineated by the broken line, was confirmed by far-red luminescence technology.

to give α-mangostin at >98% purity, the chemical structure of which is shown in Figure 1B. The extracted α-mangostin was authenticated by Professor Munekazu Iinuma (Pharmacognosy, Department of Bioactive Molecules, Gifu Pharmaceutical University). The voucher specimen of α-mangostin (no.AMG-OMC2011) was deposited at Laboratory of Anatomy and Histopathology, Graduate School of Health Sciences, Osaka Health Science University (1-9-27, Temma, Kita-ku, Osaka 530-0043, Japan).

### Cell line and animals

The parent BJMC3879 murine mammary adenocarcinoma cell line was derived from a metastatic focus isolated from a lymph node of a BALB/c mouse from an earlier study; the cell line continues to show a high metastatic propensity, especially to lymph nodes and lungs [27-29], a trait retained through culture. The BJMC3879Luc2 mammary carcinoma cell line used in our investigations was generated by stable transfection of the *luc2* gene (an improved firefly *luciferase* gene) into the parent BJMC3879 cell line [30]. We maintain this line in RPMI-1640 or DMEM medium containing 10% fetal bovine serum with streptomycin/penicillin at 37°C under 5% $CO_2$.

For the primary study, fifty 6-week-old female BALB/c mice were purchased from Japan SLC, Hamamatsu, Japan. As approved by the Animal Experiment Committee of Osaka Medical College, the animals were housed 5 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) conditions. All animals were held for a 1-week acclimatization period before study commencement. Mice were treated in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals at Osaka Medical College, the Japanese Government Animal Protection and Management Law (No.105) and the Japanese Government Notification on Feeding and Safekeeping of Animals (No.6). The study protocol and ethics were approved by the Animal Experiment Committee of the Laboratory Animal Center in Osaka Medical College.

### Pilot study to test accumulation of TNC Ab in mammary tumors

Prior to initiation of the larger study, we ran a small pilot using far-red luminescence imaging technology [31] in order to confirm attraction and accumulation of TNC Ab into mouse mammary tumors generated by our protocol. Briefly, a far-red probe, made by conjugation of far-red bioluminescent protein (biotinylated) and avidin-bound TNC Ab, was injected either i.p. or into the tail veins of a small number of BALB/c mice bearing tumors initiated by inguinal injection of BJMC3879Luc2 mammary carcinoma cells. Five minutes later, the animals were anesthetized and scanned. As shown in Figure 1C, strong luminescent signals indicated significant accumulation of TNC Ab specifically within tumors. The far-red bioluminescent protein and *Cypridina* luciferin were kindly provided by the National Institute of Advanced Industrial Science and Technology, Sapporo, Japan. Mr. Koki Endo of Wako Pure Chemical Industries, Osaka, Japan, generously provided the avidin-bound TNC Ab.

### Primary *in vivo* study design

At 7 weeks of age, we inoculated $2.5 \times 10^6$ BJMC3879Luc2 cells in 0.3 ml phosphate-buffered saline (PBS) subcutaneously into the right inguinal region of all 50 female BALB/c mice. Two weeks later, 40 mice bearing tumors with an approximate diameter of 0.5-0.7 cm in were selected and remaining 10 mice were killed because of a poor tumor growth. The selected 40 mice were randomly divided into 4 groups of 10 as follows: Group 1 mice (control) received PBS i.p. 2/wk with normal feed; Group 2 mice were treated with 125 μg TNC Ab/mouse in PBS i.p. 2x/wk with normal feed; Group 3 mice received PBS i.p. 2/wk with 4000 ppm α-mangostin in the feed; Group 4 mice were treated with 125 μg TNC Ab/mouse in PBS i.p. 2x/wk also with 4000 ppm α-mangostin continuously in the feed. Mice received treatment for 6 weeks and we recorded individual body weights weekly. Each mammary tumor that developed was also measured weekly using digital calipers, and tumor volumes were calculated using the formula of maximum diameter × (minimum diameter) $^2$ × 0.4 [32]. All surviving mice were euthanized with isoflurane anesthesia after week 6.

### Bioluminescence imaging *in vivo* at study termination

At the end of experimental week 6, we anesthetized 6 mice (for an economical reason) from each treated group using isoflurane inhalation administered via the SBH Scientific anesthesia system (SBH Designs, Inc., Windsor, Ontario, Canada). Only 4 mice from the control group were analyzed by bioluminescence since only 4 mice had survived. Each anesthetized mouse received an i.p. injection of 3 mg of D-luciferin potassium salts (Wako Pure Chemical Industries, Osaka, Japan) prior to imaging with a Photon Imager (Biospace Lab, Paris, France). The bioluminescent signals received during the 6 min acquisition time were quantified using Photovision software (Biospace Lab).

### Histopathological analyses

Following isoflurane euthanasia at the end of week 6, the tumors and lymph nodes from the axillary and femoral regions of all mice were routinely removed, along with any other lymph nodes that appeared abnormal at necropsy. Kidneys, adrenals, ovaries and uterus were also routinely harvested and scanned microscopically as well as macroscopically for metastatic involvement. Other organs that appeared abnormal were also excised. We routinely inflated the lungs with 10% phosphate buffered formaldehyde (PBF) solution prior to excision and immersion in fixative; this allowed us to subsequently remove fixed individual lobes from the bronchial tree and more closely examine them for metastatic foci before paraffin embedding. All tissues were preserved in 10% PBF and processed through to paraffin embedding and sequential sectioning at 4 μm for routine hematoxylin and eosin (H&E) staining and/or for immunohistochemical (IHC) analysis.

### Apoptosis in mammary tumors

For quantitative analysis of apoptotic cell death, sections from unstained paraffin-embedded tumors were assayed using terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end-labeling (TUNEL) in conjunction with an apoptosis *in situ* detection

kit (Wako Pure Chemical Industries), performed with minor modifications to the manufacturer's protocol. TUNEL-positive cells indicated apoptosis and were counted in viable tissues peripheral to areas of necrosis in tumor sections. We initially scanned the slides at low-power magnification (i.e., ×100) to identify those areas having the highest density of TUNEL-positive cells. We then chose four 250 $mm^2$ fields neighboring an area of high TUNEL positivity and counted those at higher magnification (×200-400); the numbers of TUNEL-positive cells were thus expressed as number per $cm^2$.

### Cell proliferation in mammary tumors

We chose the labeled streptavidin-biotin (LSAB) method (Dako Co., Glostrup, Denmark) for IHC analyses of cell proliferation. Unstained sections were immersed in citrate buffer at pH6.0 and heated at 110°C for 10 min for antigen retrieval; we were than able to perform IHC to quantitatively assess cell proliferation in primary mammary carcinomas using a rabbit polyclonal antibody against proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Tumors from 5 animals from each treatment group were evaluated for cell proliferation as inferred by PCNA-positive nuclear staining. We counted the numbers of PCNA-positive cells in each of four randomly selected 250 $mm^2$ microscopic fields of viable tissue at high power (×400); cell proliferation was then quantitatively expressed as number per $cm^2$.

### Blood microvascular density in mammary tumors

We used IHC to quantitatively assess blood microvessel density in primary mammary carcinomas, employing the LSAB method (Dako), which utilizes a rabbit polyclonal antibody against CD31 (Lab Vision Co., Fremont, CA, USA), a marker specific for blood microvessel endothelium. Unstained paraffin-embedded mammary tumor sections were treated with antigen retrieval and then exposed to CD31 Ab. The numbers of blood microvessels positive for CD31 were counted as previously described [33]; briefly, slides are scanned at low-power (×100) magnification to identify those areas having the highest number of vessels and the five areas of highest microvascular density are selected and counted at higher (×200-400) magnification.

### Dilated lymphatic vessels with invading cancer cells

Mammary tumor sections from paraffin-embedded tissues were treated with antigen retrieval and immunohistochemically stained using the LSAB method (Dako) just as for evaluation of microvessel density, this time using a hamster anti-podoplanin monoclonal antibody against a lymphatic endothelium marker (AngioBio Co., Del Mar, CA, USA). As an indication of potential metastases, we wanted to quantitatively assess the number of lymphatic vessels having intraluminal tumor cells in the areas peripheral to primary mammary carcinomas, which indicates lymphatic invasion. As with IHC studies for previously described characteristics, slide-mounted tumor tissues exposed to podoplanin Ab were scanned at low-power (x100) magnification to first identify podoplanin-positive lymphatic vessels, which were then examined at higher (x200-400) magnification for the presence of intraluminal mammary cancer cells [27].

### Statistical analysis

Survival rates were analyzed using the Holm-Sidak method. Significant differences in the quantitative data between groups were analyzed using the Welch's Student's *t*-test, which provides for insufficient homogeneity of variance. The differences in metastatic incidence were subjected to Fisher's exact probability test, with either $P<0.05$ or $P<0.01$ considered to represent a statistical significance.

## Results

### Survival rates, body weights and mammary tumor growth

Accidental deaths (dosing and husbandry mishaps), as well as removal due to health problems, accounted for reductions within all groups in the number of mice reaching the 6 week study termination; these mice were excluded from the end analyses. Therefore, effectively 50% (4/8) of the controls, 78% (7/9) of TNC Ab only, 100% (8/8) of α-mangostin alone, and 100% (7/7) TNC Ab + α-mangostin mice survived in 6 weeks of the commencement of the study.

Changes in average body weights are shown in Figure 2A; as can be seen, weights of both control and treated mice bearing mammary tumors did not differ statistically throughout the experiment. However, tumor growth as inferred by computed volumes (Figure 2B) was significantly inhibited in all treated groups from week 1 to 6 as compared to the control group. By the end of the experiment, the average tumor volume in control animals was 1722 ± 508 $mm^3$, 1175 ± 292$mm^3$ in mice receiving TNC Ab alone, 1042 ± 168 $mm^3$ in mice receiving α-mangostin alone, and 872 ± 142 $mm^3$ in the combination group.

### Metastasis with TNC Ab, α-mangostin or combination treatment

**Histopathology of primary mammary tumors:** All mammary tumors induced by BJMC3879Luc2 cell inoculation proved histopathologically to be moderately differentiated adenocarcinomas containing *p53* mutation as previously determined by IHC [27].

**Lymph node metastasis by bioluminescence imaging and histopathology:** Bioluminescence imaging showed signal indicative of metastatic growth in the mandibular, axillary and inguinal lymphatic regions of all groups; however, all mice receiving TNC Ab, α-mangostin or the combination of both showed a tendency for decreased metastatic expansion signals (Figure 2C). Since cause of the death was tumor metastasis in case of the death in 5 weeks of the study and thereafter with exception of the accidental death was effectively examined histopathologically (effective number of mice was 8 in control, 9 in TNC Ab, 8 in α-mangostin and 7 in the combination groups). Representative examples of lymph node metastases in H&E-stained tissues are shown in Figures 3A-D. Lymph node metastasis occurred in almost all mice independent of treatment, but the number of metastasis-positive lymph nodes per mouse was significantly lower in all groups receiving treatment

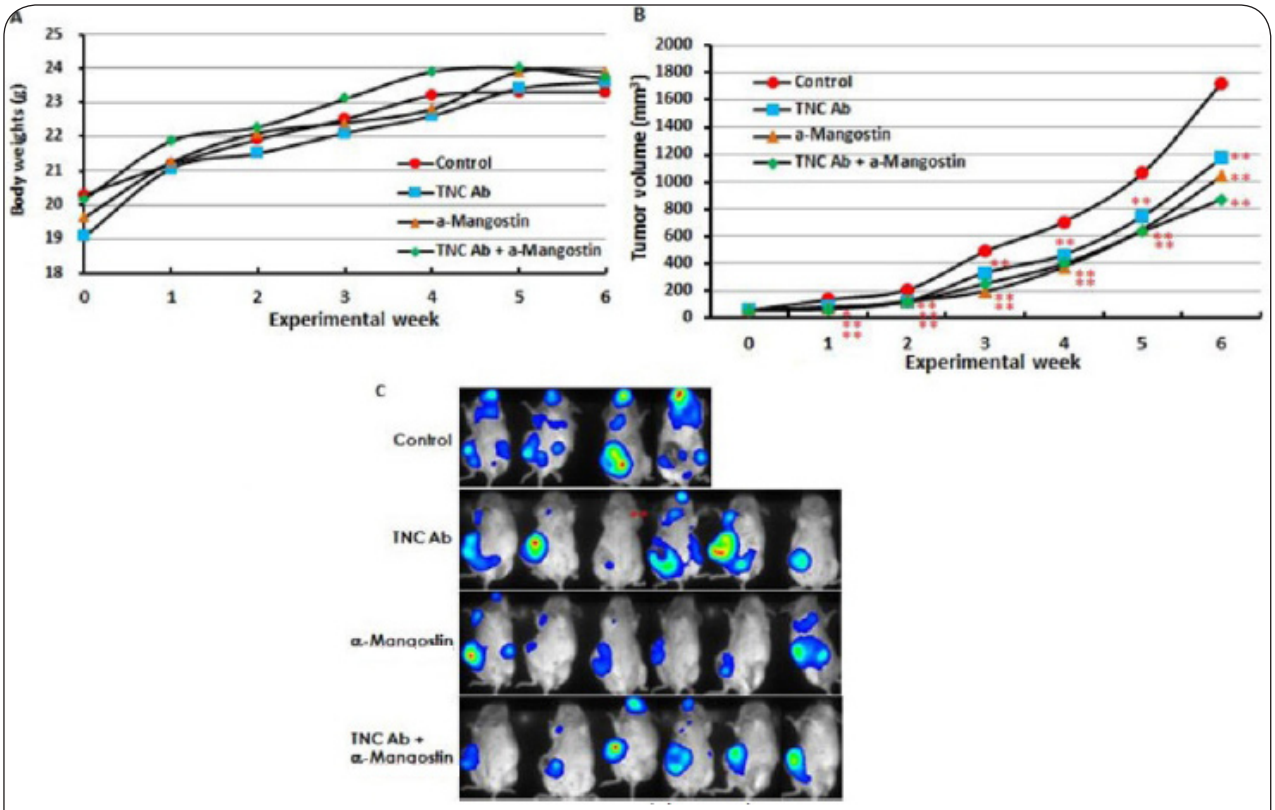


**Fig 2: Comparison of mouse body weights (A), tumor volumes (B) and bioluminescent imaging for metastasis extension (C)**

A. No significant differences in the average body weights were observed among treatment groups. B. Tumor volumes were significantly decreased in mice receiving TNC Ab, α-mangostin, or both agents (*P<0.05; **P<0.01; data are presented as mean ± SD) C. Bioluminescent imaging in 6 representative mice from each of the 3 treated groups and in all 4 surviving control mice. When compared to controls, imaging showed a tendency toward reduction in the extent of metastasis in treated mice.

when compared those in the control group (Figure 4A).

**Lung and overall metastasis**: Lung metastasis also occurred in almost all mice regardless of treatment (Figures 3E-H). When considering the size of lung lesions, however, the numbers of metastatic lung nodules >1mm in diameter per mouse were significantly decreased in all treated groups when compared to those in control mice (Figure 4B).

Metastatic foci were also seen in ovary, kidneys, adrenals and uterus. For ease in evaluation of bilateral organs, each organ was considered separately such that metastasis to one of the paired organs was counted as one and metastases to both as two. The multiplicity of overall metastasis is presented in Figures 4C and D. As can be seen, the total number of organs with metastasis was much smaller in all treated groups than in the control group (Figure 4C), while the average total number of organs with metastasis per mouse was significantly decreased in animals receiving TNC Ab, α-mangostin or both agents as compared to the control group (Figure 4D).

### Apoptosis and cell proliferation in mammary cancers

Representative examples of TUNEL-positive cells within mammary tumor tissues are presented in Figures 5A -D. The results of the quantitative analysis for apoptosis in mammary tumors, as assessed by the TUNEL assay, are shown in Figure 6A. The number of TUNEL-positive cells was significantly higher in tumors from the α-mangostin and the α-mangostin + TNC Ab groups as compared to the numbers in tumors from the control mice.

Figures 5E-H illustrate *in vivo* PCNA-positive tumor cells in tissue sections. Cell proliferation levels, as inferred by the percentage of cells immunopositive for PCNA within the carcinomas, were significantly decreased in tumors from all treated groups as compared to those of control animals (Figure 6B).

### Blood microvessel density and lymphatic invasion in mammary cancers

Immunohistochemical analysis using the blood vessel endothelial cell marker CD31 (Figures 7A-D) showed significantly lower blood microvessel density in tumors exposed to α-mangostin alone and in combination with TNC Ab, but although TNC Ab treatment alone similarly reduced microvessel density, the difference was not statistically significant ($P$=0.07) when compared control values (Figure 6C).

Anti-podoplanin-stained lymphatic vessels, many of which are dilated, are demonstrated in mammary tumors in Figures 7E-H. Tumor cells were frequently found within the lumina of lymphatic vessels in tumors from all groups; however, the number of lymphatic vessels containing intraluminal tumor cells was significantly decreased in mammary tumors from all treated groups (Figures

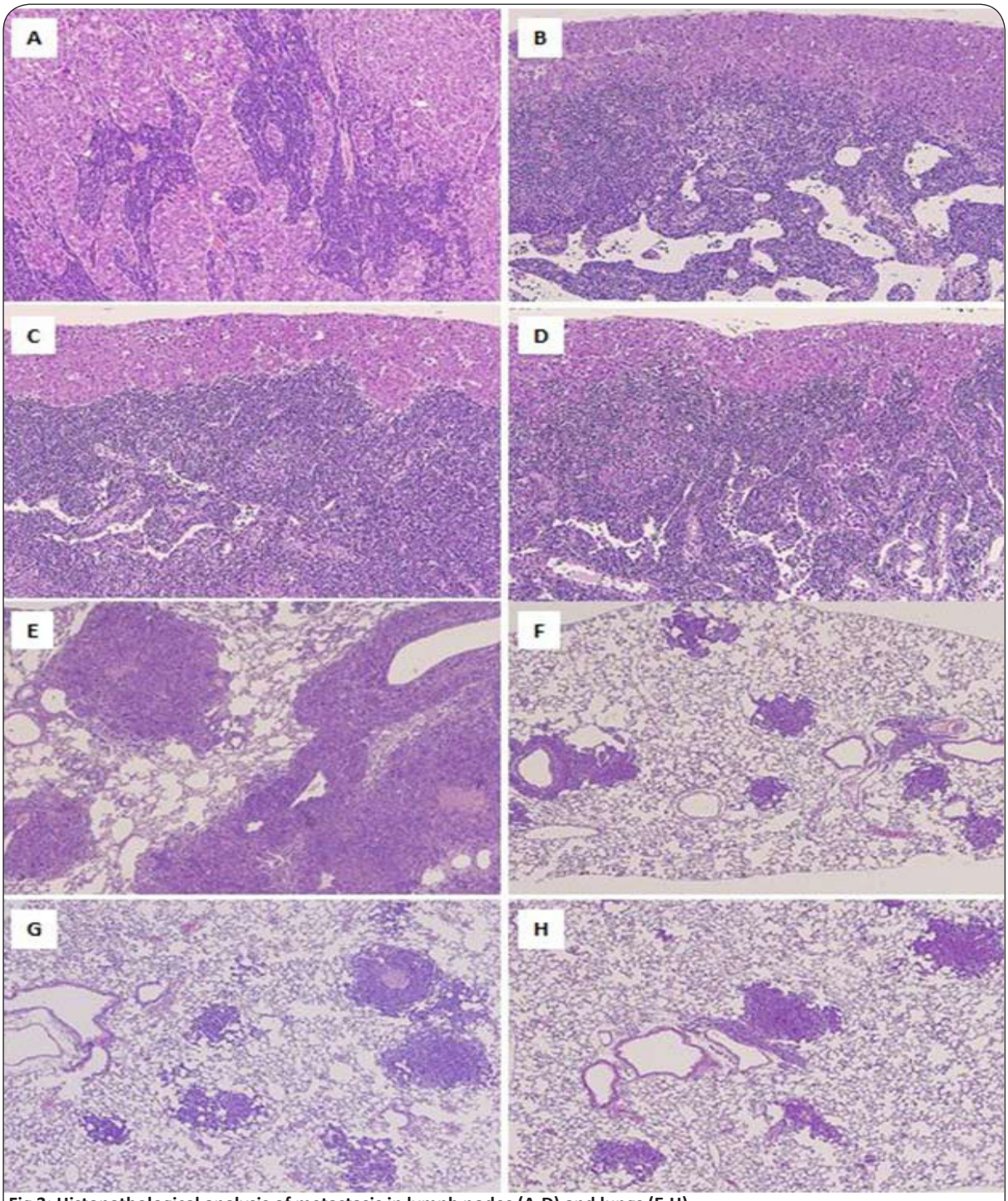


**Fig 3: Histopathological analysis of metastasis in lymph nodes (A-D) and lungs (E-H)**

Metastasis to a lymph node in a control mouse (A), from a mouse treated with TNC Ab only (B), with α-mangostin treatment only (C), and with TNC Ab + α-mangostin (D). Carcinoma cells have infiltrated and filled the sinusoidal space in the control node (A), but are more confined to the subcapsular sinus in nodes from treated mice.

Metastatic foci in the lung of a control mouse (E), in a TNC Ab-treated mouse (F), with α-mangostin treatment (G), and with TNC Ab + α-mangostin exposure (H). Metastatic foci appeared dramatically smaller in the treated groups as compared to those in control animals. (A-D, H&E ×100; E-H, H&E ×40)

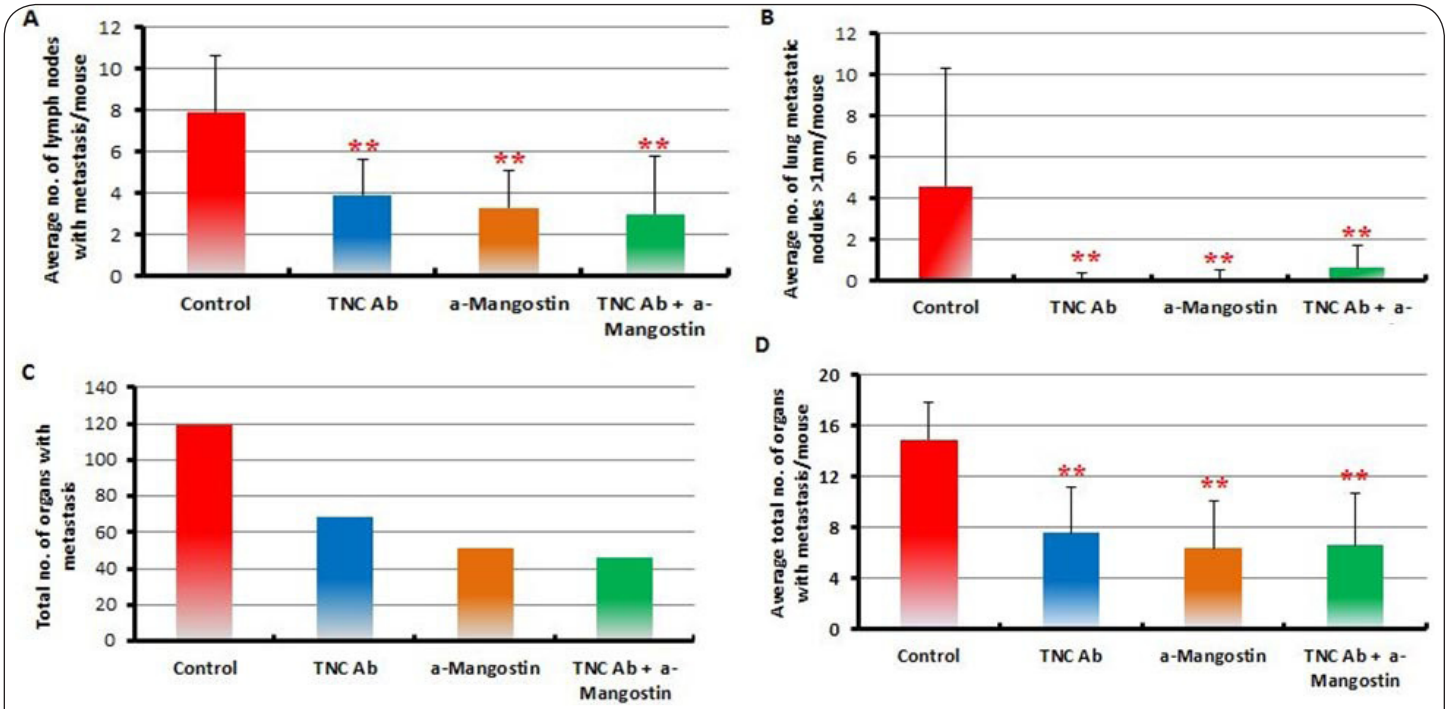


**Fig 4: Quantitative analyses of mammary carcinomas metastasis.**

A. The multiplicity of lymph node metastasis per mouse was significantly reduced in treated groups (**P<0.01). B. The number of larger (> 1 mm) metastatic lung nodules was significantly reduced in all treated groups (**P<0.01). C. TNC Ab and α-mangostin, alone and in combination, tended to reduce overall tumor metastasis to other organs. D. The average number of organs per mouse with metastases was significantly decreased in by TNC Ab and α-mangostin, alone and in combination (**P<0.01). (Data are presented as mean ± SD.) Since cause of the death was tumor metastasis in case of the death in 5 weeks of the study and thereafter with exception of the accidental death was effectively examined histopathologically (effective number of mice was 8 in control, 9 in TNC Ab, 8 in α-mangostin and 7 in the combination groups).

7F-H) when compared to tumors from the control animals (Figure 7E; Figure 6D), indicating a reduction in lymphatic tumor cell dissemination with administration of TNC Ab and α-mangostin alone and in combination.

## Discussion

We were able to demonstrate growth suppressive and anti-metastatic properties for both TNC Ab and α-mangostin, alone and in combination, in a murine mammary tumor model having a *p53* mutation that induces a metastatic spectrum similar to that seen in human breast cancers [21,27]. Suppression of tumor growth and metastasis was further associated with decreases in intratumoral cell proliferation and angiogenesis and with lymphatic invasion by mammary tumors induced in the study mice. In terms of relevance, *p53* plays a critical role in regulating cell cycle progression, DNA repair, and apoptotic cell death and fully half of all molecularly analyzed human cancers include mutations in *p53* [34]; indeed, in sporadic human breast cancers, *p53* mutations are associated with stroma-specific loss of heterozygosity and/or allelic imbalance *and* metastasis to regional lymph nodes [35]. The apparent ability of TNC and α-mangostin to act independent of *p53* status is thus highly significant to clinical therapeutics.

As was previously stated, metastasis is one of the most negative prognostic factors in human breast cancer [36] and metastasis often occurs through dissemination of tumor cells via the lymphatic system. In the mammary tumors generated in this study, we found a significant decrease in the number of dilated lymphatic vessels containing intraluminal cancer cells, a phenomenon associated with active lymphatic invasion, using both routine H&E and specific IHC staining. The significant decrease in overall distant site metastases and in the multiplicity of metastatic foci in any affected organ is corroborating evidence of the efficacy of TNC Ab and/or α-mangostin treatment.

Neovascularization, or angiogenesis, is a key process in the growth of solid tumors, and tumors will not grow beyond a few cubic millimeters unless a vascular network is established to feed further expansion [37]. Hematogenous spread of tumor cells is also a major pathway of metastasis. TNC has been associated with angiogenesis in various physiological and pathological conditions involving cancer [38]. The switch to a highly angiogenic phenotype is clinically characteristic of malignant glioma, and increased perivascular TNC localization in gliomas is considered to be a marker for tumor aggressiveness [13]. In a mouse melanoma transplant model, TNC-deficient mice show reduced blood VEGF levels correlating with impaired tumor angiogenesis, suggesting VEGF regulation by TNC affects tumor vascularization [14]. *In vitro* studies have demonstrated TNC expression localized to sprouting bovine aortic endothelial cells, but not to nonsprouting cells, further implying a

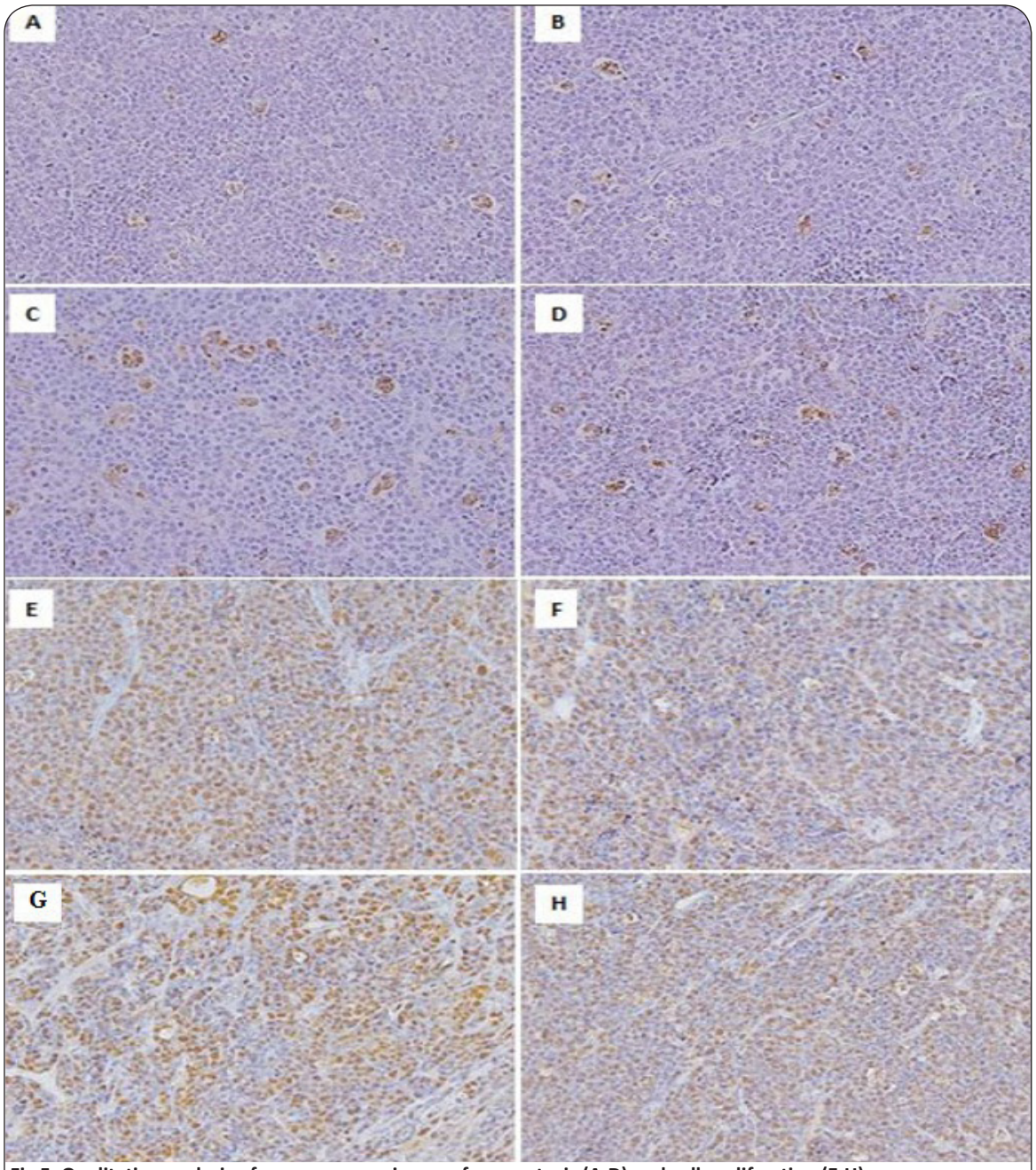


**Fig 5: Qualitative analysis of mammary carcinomas for apoptosis (A-D) and cell proliferation (E-H).**

A. TUNEL-positive apoptotic cells (brownish stain) in a mammary tumor of a control mouse treated with saline only. B. The relative number of apoptotic cells with TNC Ab treatment appears similar to control, indicating little effect. C. The number of apoptotic cells increased, however, α with exposure to α-mangostin and to α-mangostin + TNC Ab (D). E. Cells undergoing proliferation are immunopositive to PCNA (brown nuclear staining) in a mammary tumor of a control mouse treated with saline alone. F. Reduction of PCNA-positive cells in a mammary tumor treated with TNC Ab only, with α-mangostin only (G), and with TNC Ab + α-mangostin (H). (A-D, TUNEL staining, ×200; E-H, PCNA immunohistochemistry, ×200).

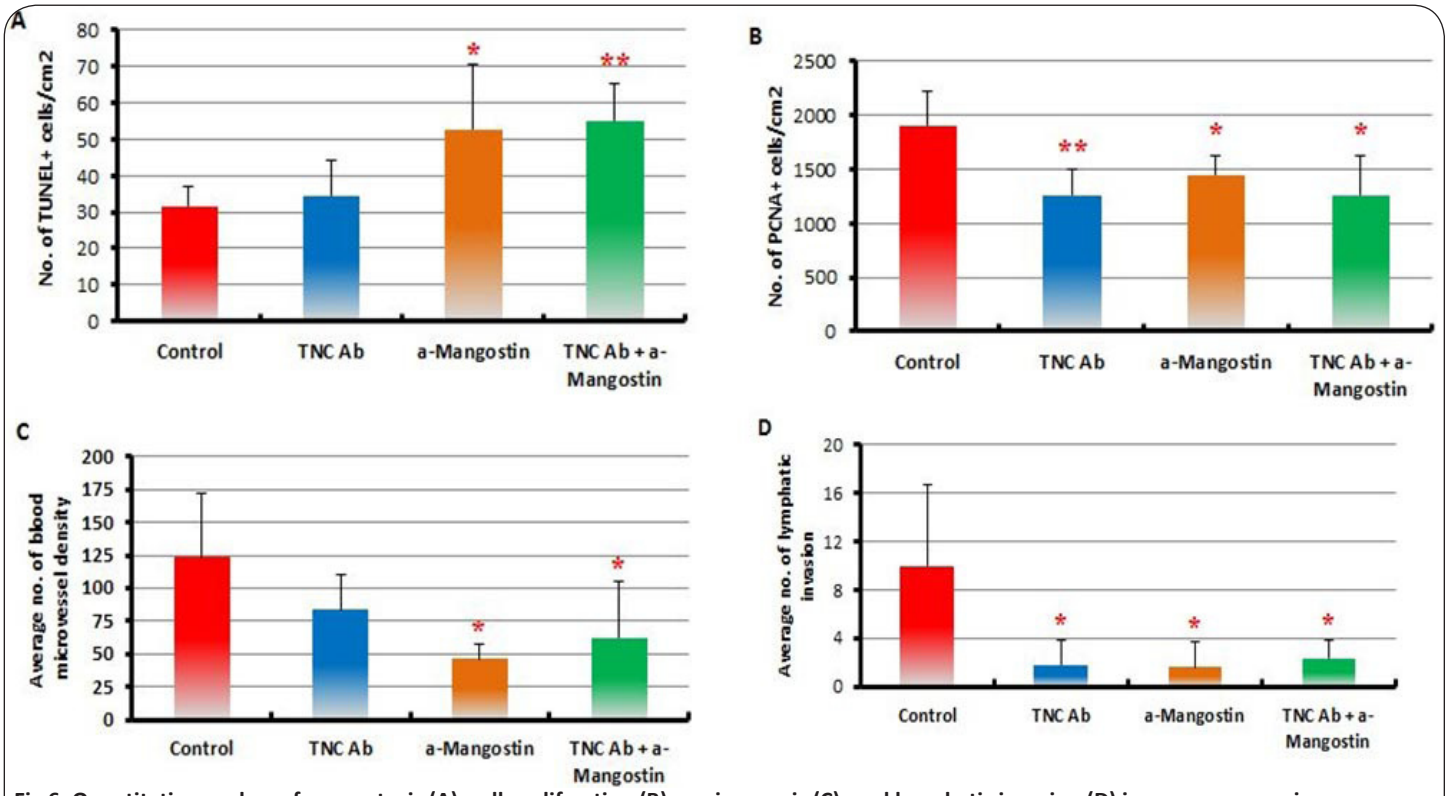


**Fig 6: Quantitative analyses for apoptosis (A), cell proliferation (B), angiogenesis (C), and lymphatic invasion (D) in mammary carcinomas.**

A. Apoptosis, as assessed by TUNEL staining, was significantly higher only in α-mangostin alone (*P<0.05) and α-mangostin + TNC Ab (**P<0.01) groups than in the control group. B. Cell proliferation, as assessed by PCNA-positivity, was significantly decreased in all treated groups. C. Blood microvessel density, inferred by the frequency of blood vessels showing CD31-positive endothelium, was significantly lower in in tumors exposed to α-mangostin alone and α-mangostin + TNC Ab; TNC Ab alone also reduced microvessel density, but not to a statistically significant level (P=0.07). D. The number of dilated lymphatic vessels containing intraluminal tumor cells, a measure of metastatic potential, was significantly lower in all treated groups as compared to control. (Data are presented as mean ± SD.)

role for TNC in neovascularization. Angiogenesis, as inferred by blood microvessel density measurable with specific endothelial markers, was markedly reduced in primary tumors exposed to TNC Ab and α-mangostin; however, TNC Ab alone was not as effective as α-mangostin alone or in combination with α-mangostin (*P*=0.07, marginal for established statistical significance at *P*<0.05 or *P*<0.01). It is biologically intriguing, therefore, that lymph nodes, lungs and overall metastases were significantly reduced with TNC Ab treatment alone even though the decrease in tumor blood microvessel density seen was not, as mentioned, statistically significant.

As we previously discussed, the growth of primary tumors is angiogenesis-dependent and endothelial cells recruited by a tumor in the process of neovascularization have become an important target of cancer therapy [39]. Mangosteen extracts, including α-mangostin, apparently have somewhat more effective anti-angiogenic properties *in vivo* in the mammary cancer model used in this study [21,23]; however, the mechanism of angiogenic inhibition by α-mangostin may be related to a reduction in Akt phosphorylation [19-21], rather than to antigen-antibody interactions; phosphatidylinositol 3-kinase (PI3K) exerts regulatory functions on tumor growth and angiogenesis, as well as on cell proliferation, cell transformation and apoptosis, through downstream targeting of Akt [40]. α-Mangostin inhibited angiogenesis in human umbilical vein endothelial cells and was associated with inhibited phosphorylation of VEGFR2 *in vitro* [41].

Correlated with reductions in tumor growth (quantitatively measured as tumor volume) and overall metastasis, cell proliferation was significantly decreased in mammary tumors from mice receiving either TNC Ab or α-mangostin alone or in combination, as assessed by PCNA immunohistochemistry. Apoptosis was significantly elevated over control in mammary tumors in mice given α-mangostin alone or α-mangostin + TNC Ab, but not with TNC Ab treatment alone, as indicated by TUNEL staining. α-Mangostin has been shown to inhibit cell proliferation by cell cycle arrest at G1 and is correlated with induction of either of the cyclin-dependent kinase inhibitors $p21^{Cip1}$ [21,22] or p27 [20]. Mangosteen extracts have further been reported to induce apoptosis through a mitochondrial pathway in a variety of cancer cells *in vitro* [20-22] and in mouse mammary cancers *in vivo* [21,23]. TNC's role in apoptosis is still equivocal at present; it apparently induces resistance to apoptosis in pancreatic cancer cells via activation of the ERK/NF-kB pathway [42], whereas upregulation of TNC by platelet-derived growth factor receptor activation promotes neural

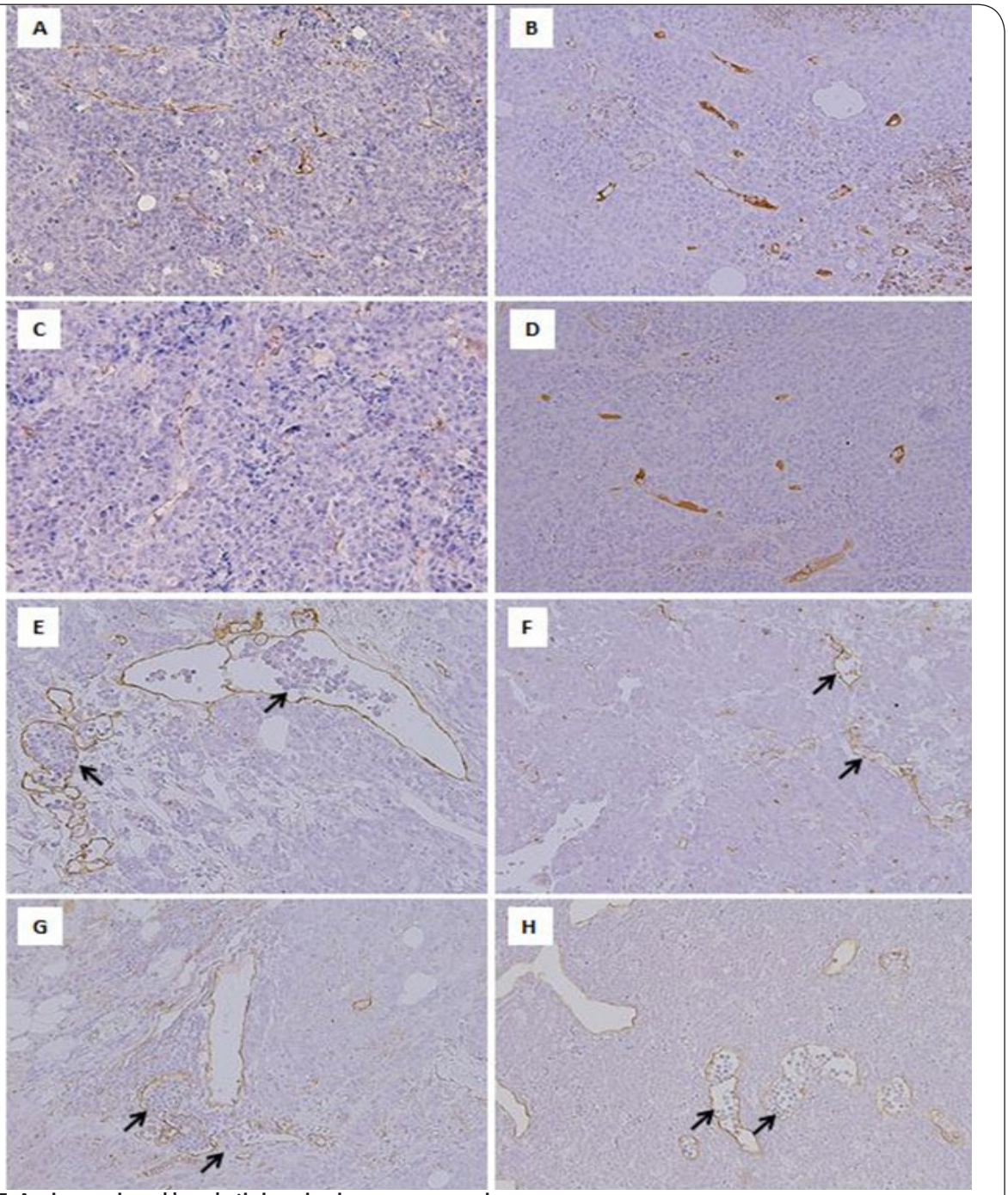


**Fig 7: Angiogenesis and lymphatic invasion in mammary carcinomas.**

Representative section of a mammary tumor from a control mouse showing blood capillary endothelium positive for CD31, a marker for neovascularization (A). CD31-positive capillaries in a TNC Ab-treated tumor (B), in a tumor exposed to α-mangostin only (C), and in a tumor treated with both TNC Ab + α-mangostin (D). Overall, fewer CD31-positive capillaries were detected in tumors receiving TNC Ab and α-mangostin, alone and in combination as compared to control.

Podoplanin, a marker for lymphatic endothelium was used to delineate intratumoral lymph vessels. E. This representative section of a tumor from a control mouse treated with saline illustrates not only the appearance of podoplanin-positive lymphatic endothelium, but also the degree of dilation and the presence of intraluminal tumor cells (arrows) which is taken to indicate lymphatic invasion. Mice treatment with TNC Ab (F), with α-mangostin (G), and with TNC Ab + α-mangostin (H) also have lymphatic invasion as indicated by cell-filled lymphatic lumenae (arrows). (A-D, CD31 immunohistochemistry, ×200; E-H, podoplanin immunohistochemistry, ×200.)

apoptosis via MAPK activation [43].

TNC is expressed in the stroma of most solid carcinoma and it plays critical roles in the tumorigenic processes of cell proliferation, angiogenesis, invasion and metastasis. It is reported to enhance cell migration and proliferation by activation of cell-surface growth factor receptors [38] and participates in carcinogenesis via gene destabilization, providing a niche environment ripe for metastasizing and the survival of cancer stem cells [38]. In a transplant model of the human breast cancer cells, downregulation of TNC by siRNA is correlated to decreased cell proliferation and metastasis in the lungs [44], and, in a transplant model of human breast cancer cells, miR-335 suppressed lung metastasis and invasion by targeting TNC and SOX4, a factor in progenitor cell transcription [45].

In some respects, it may not be surprising that α-mangostin appears to be somewhat more effective than TNC Ab curbing tumor growth and metastasis. We recently reported that even mixed mangostin extracts consisting of 80-90% α-mangostin and 5-10% α-mangostin suppressed tumor growth and metastasis in the same mouse mammary model used in this study as well as purified α-mangostin [21,23]. Crude α-mangostin in the diet also significantly suppressed colon tumor growth in a nude mouse xenograft model using HCT 116 human colorectal carcinoma cells [25], and *in vitro* studies using crude α-mangostin in skin cancer cell lines demonstrated decreased cell motility, adhesion and clonogenicity as well as mitochondrial induction of apoptosis [46]. Other investigators reporting α-mangostin's inhibition of cell invasion and migration in mammary cancer cells have suggested these effects are due to downregulation of MMP-2 and MMP-9 [47].

The development and use of therapeutic agents targeted to specific molecular entities is the rising tide in cancer treatment. In modern medicine, therapies targeting multiple molecular endpoints are considered potentially more effective than a treatment intended to address a single facet of a disease. α-Mangostin is a drug affecting a wide variety of cascades as described above, and TNC also exerts multiple functions dependent on its presence as a structural component of the cellular matrix and on effects resulting from specific binding to cell surface receptors [38]. Hence our interest in TNC Ab and α-mangostin as anti-tumorigenic and/or anti-metastatic interventions in our mammary model, with the possibility of enhanced therapeutic efficacy when used in combination. Unfortunately, no synergistic effects were seen, although we observed marked inhibitory effects on tumor growth and metastasis from both compounds. The reason(s) why enhanced anti-tumorigenic effects were not observed by combination treatment with TNC Ab and α-mangostin is unclear, but the positive therapeutic value of both compounds, even used individually, cannot be dismissed and deserves further study.

## Conclusions

We demonstrated the ability of TNC Ab and α-mangostin to induce significant suppression of tumor growth and metastasis to other organs in a mouse mammary cancer model carrying a *p53* mutation frequent in the human disease. However, we saw no enhancement of these effects when TNC Ab and α-mangostin were combined. Given that the presence or absence of metastasis is the most important prognostic factor in breast cancer patients, we feel the anti-metastatic activities of both TNC Ab and α-mangostin warrant further investigation and clinical consideration in future cancer treatment.

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Authors' contributions as follows: MAS carried out all animal experiments as well as the histopathological analysis. Anti-tenascin antibody was made and purified by MK. All immunohistochemical procedures were conducted by ES and SF. Extraction of α-mangostin from mangosteen periapts was performed by MI. Maintenance of mammary carcinoma cells, transplantation and treatments to mice were performed by JM. MAS and MH-S participated in the design of the study. MAS wrote the first version of the manuscript. All authors have read and approved the final submitted manuscript.

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