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Original article

Her-2/*neu* overexpression is associated with thrombospondin-1-related angiogenesis and thrombospondin-1-unrelated lymphangiogenesis in breast cancer

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ABSTRACT

Introduction: Her-2/*neu* overexpression is linked to poor prognosis and is a significant predictor of the response to trastuzumab (i.e., herceptin) therapy in breast cancer patients. Her-2/*neu* promotes angiogenesis with thrombospondin-1 (TSP-1) as a mediator in breast cancer development and progression. However, the clinical quantitative evaluation of Her-2/*neu* and specified localization of TSP-1 are currently lacking.

Materials and methods: In this study, immunohistochemistry was performed to elucidate the *in vivo* relationships between Her-2/*neu* and TSP-1 expression, angiogenesis, and lymphangiogenesis in breast cancer. Based on immunohistochemical staining for anti-Her-2/*neu* and related quantitative scoring of tumor cells, 28 individuals with high Her-2/*Neu* expression (i.e., with a score of $\geq$2.0) were recruited into the study group. Another 28 individuals with low Her-2/*neu* (i.e., with a score of $<$2.0) were recruited for comparison. These tumors were further stained immunohistochemically for the estrogen receptor (ER), the progesterone receptor (PR), Ki-67, TSP-1, CD31 (an vascular endothelial marker), and D2-40 (a lymphatic endothelial marker).

Results: The individuals with Her-2/*neu* overexpression had significantly lower ER and PR expression and a higher Ki-67 index, microvessel count, and lymphatic vessel density in comparison to the individuals with low Her-2/*neu* expression. High Her-2/*neu* expression was associated with lower TSP-1 expression in tumor cells (TSP-1/T) and higher TSP-1 expression in the adjacent stroma (TSP-1/S; 17 of 28 individuals). By contrast, low Her-2/*neu* expression was associated with high TSP-1/T expression and low TSP-1/S (23 of 28 individuals). In one situation, patients with high expression of TSP-1 in tumor cells (a score of $>$2.0, with 2.0 as the cut-off value) had a significantly lower microvessel density, compared to individuals with low-TSP-1 expression ($p = 0.018$). However, the lymphatic vessel density was not significantly different from the TSP-1 levels ($p = 0.333$).

Conclusion: Our *in vivo* results showed that Her-2/*neu* affects the biological manifestations of breast cancer by increasing angiogenesis (which is TSP-1-related) and lymphangiogenesis, which is TSP-1-unrelated).

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Introduction

Overexpression of the human epidermal growth factor receptor-2 (Her-2/*neu*) occurs in 25–30% of breast cancers and is linked to a more aggressive disease course.[1] The Her-2/*neu* (also referred to as c-erbB-2) oncogene is a member of the erbB-like oncogene family; its amplification aggravates tumor proliferation, migration,

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invasion, metastasis, and angiogenesis.[2–4] In clinical practice, the detection of Her-2/*neu* oncogene amplification is necessary for selecting cancer patients for recombinant monoclonal antibody trastuzumab (i.e., herceptin) therapy against Her-2/*neu* (Genetech Inc, South San Francisco, CA, USA), the monoclonal anti-Her-2/*neu* antibody, and for providing prognostic information.[1] Herceptin plays a novel role in antiangiogenic therapy in breast cancer by inducing normalization and regression of the vasculature in an experimental human breast tumor that overexpresses Her-2/*neu* in mice. It works by modulating the effects of different angiogenesis activators and inhibitors, including the downregulation of angiogenic inhibitors, [e.g., thrombospondin-1 (TSP-1)] and reducing angiogenic activators [e.g., vascular endothelial growth factor (VEGF)].[5] The knockdown of Her-2/*neu* expression by RNA interference (RNAi) is associated with the upregulation of TSP-1.[4] However, the quantitative evaluation and clinical correlation of Her-2/*neu*-induced angiogenesis and lymphangiogenesis is lacking.

Among the mediators of Her-2/*neu*, the thrombospondins (TSPs) are a family of extracellular proteins that participate in cell-to-cell and cell-to-matrix communication.[6] Of the five family members, TSP-1 (an endogenous angiogenic inhibitor) plays an important role in the angiogenic switch in carcinomas of the skin,[7] prostate,[8] bladder, and cervix.[9] It has multiple diverse functions such as a proinvasive role in *in vitro* and *in vivo* models of breast cancer.[10,11] However, TSP-1 has been linked with stimulatory[12,13] and with inhibitory[14,15] roles in tumor invasion and progression. The overexpression of TSP-1 is able to block *in vivo* neovascularization and decrease malignant tumor growth.[14,16] However, TSP-1 promotes tumor cell adhesion, migration, and invasion.[12] Thus, TSP-1 has controversially been described as having a dual role as a tumor suppressor and tumor promoter, and a proinvasive and antiangiogenesis role. The presence of TSP-1 expression in stromal cells (i.e., desmoplasia) is correlated with higher microvessel density (MVD)[17]; however, there is a lack of specific localization of TSP-1 in either tumor or stromal cells in breast cancer.

The aim of this study was to develop a quantitative measurement and to investigate clinical correlations of Her-2/*neu* and its mediator TSP-1—which acts as an endogenous fence to inhibit angiogenesis that occurs during early carcinogenesis[9] and plays a potential physiologic gatekeeper role in cancer prevention[18]—in regulating angiogenesis and lymphangiogenesis in breast cancer. In addition to angiogenesis, lymphangiogenesis (i.e., the formation of new lymphatic vessels) has recently drawn considerable attention and has become a new frontier of tumor metastasis research.[19] With the advancement in the development of new antibodies, lymphatic microvessels can be detected and measured with appropriate antibodies. A selective monoclonal immunohistochemical marker, D2-40, was used to detect lymphatic invasion in conventionally processed formalin-fixed and paraffin-embedded tissue specimens.[20] To provide a more objective and sophisticated method for immunohistochemistry stains, we used the Automated Cellular Imaging System (ACIS) digital microscope (ChromaVision Medical Systems, San Juan Capistrano, CA, USA), which combines automated digital microscopy and computerized image processing; ACIS is less time consuming and less dependent on the experience of analysts in characterizing breast cancers, compared to traditional, manual light microscopy.[21] In this study, we used the ACIS to evaluate Her-2/*neu* and TSP-1 in a more objective and quantitative way. We also specifically localized TSP-1 expression separately in tumor and stromal cells.

Table 1
The clinicopathologic parameters of the high Her-2/*neu* and low Her-2/*neu* groups.

	Group	High Her-2/*neu*	Low Her-2/*neu*	*p*
		≥2	<2	
No.		28	28	
Her-2/*neu*		2.76 ± 0.74	0.89 ± 0.46	
Age		52.8 ± 11.7	56.5 ± 13.4	0.266
Grade	I	2	16	<0.001
	II	8	5	
	III	18	7	
Tumor size	T1	20	24	0.329
	T2	7	4	
	T3	1	0	
Lymph node[a]	+	7 (25.0)	8 (28.6)	0.763

Data are expressed as mean ± SD or *n* (%).
[a] Fisher's exact test was used to analyze tumor size.

Materials and methods

Specimen samples

From January 2004 to September 2004, specimens of stage I or stage II invasive ductal or lobular invasive breast cancer were collected at WCP Pathology Laboratories, Inc. (St. Louis, MO, USA) by using the standard procedure described later. Her-2/*neu* protein expression of each tumor was quantitatively measured by using an ACIS, and ranged from 0.5 to 3.0. Twenty-eight patients with high Her-2/*neu* expression (i.e., a score of ≥2.0) were recruited into the study group; another 28 patients with low Her-2/*neu* expression (i.e., a score of <2.0) were recruited as the comparison group. Table 1 provides other clinicopathological features of these 56 patients (e.g., age, histological grading, tumor size, and lymph node metastasis) that were obtained through reviewing their medical records. Representative paraffin-embedded tissue blocks from these patients were selected for additional immunohistochemical staining, which included staining for TSP-1, CD31, and D2-40. A detailed description follows.

Immunohistochemical staining

Specimens of invasive ductal or lobular breast carcinoma were stained immunohistochemically for Her-2/*neu* protein expression by using the anti-Her-2/*neu* antibody CBE-356 (dilution of 1:150; Novocastra Laboratories, Newcastle upon Tyne, UK) by using the Bond-max autostainer (Vision Biosystem, Norwell, MA, USA). Other antibodies and dilution concentrations were as follows: estrogen receptor (ER, 1:100), progesterone receptor (PR, 1:100), Ki-67 (1:30), p53 (1:400), and TSP-1 (1:150), which were all obtained from Novocatra Laboratories; CD31 (1:100), obtained from Ventana Medical System, Inc, (Tucson, AZ, USA); and D2-40 (1:40), obtained from Signet Laboratories (Dedham, MA, USA). The protocol for Her2 immunohistochemical assays has been described elsewhere.[22] In brief, paraffin-embedded tissue blocks were cut to 5-μm sections, deparaffinized, and heat treated for antigen retrieval. The primary polyclonal anti-Her-2/*neu* antibody, CBE356, chosen as previously described, was used at the optimal dilution of 1:150 determined in our laboratory. Biotinylated secondary antibody with streptavidinbiotin labeling and related reagents were provided in the level 2 USA UltraStreptavidin Multi-Species Detection System (Signet Laboratories). All immunohistochemical assays were performed on an automated immunostainer. A positive immunohistochemical reaction was defined as a dark brown reaction product on the cell membrane. Positive controls, (e.g., spleen, thymus, placenta) were used in accordance with the manufacturer's user manual.

ACIS

Digital microscopy was performed with the ACIS digital microscope (ChromaVision Medical Systems). The ACIS system consists of an automated robotic bright-field microscope module, a

computer, and a Windows NT-based software interface.[23] The robotic microscope module scanned the immunohistochemically stained slides, and a computer monitor displayed the digitized tissue images. The images were captured with an analog camera and digitizer, transformed into pixels, and then quantified by hue (i.e., color), saturation (i.e., color purity), and luminosity (i.e., intensity). The ACIS was implemented for routine operation in the WCP Laboratories. Membrane staining was not highlighted on the image; however, the ACIS system was able to distinguish cell membrane staining from cytoplasmic staining by using color space transformation (CST) proprietary technology. The ACIS then specifically quantitated Her-2/*neu* protein staining on cell membrane. In this study, immunohistochemical staining was quantitated [without knowledge of the manual immunohistochemical or fluorescence *in situ* hybridization (FISH) scores] by using the manufacturer's preset circular marks to select the areas of invasive tumor cells. The ACIS recognized 256 levels of immunohistochemical staining intensity and converted these to fractional scores for the selected individual cells. An average score for all selected areas also was calculated.

Scoring and quantitation

The manufacturer recommended quantitation of five areas with the highest staining intensity. In this study, six areas of invasive component with highest staining intensity were quantitated to reduce potential sampling variations. The ACIS measured the average positive percentage of ER, PR, Ki67, and p53. A positive result for ER or PR was defined as >5% of the tumor cells staining positive for ER or PR; for Ki-67, 20% of cells staining positive; and for p53, 10% of cells staining positive.[24,25] The average staining intensity of Her-2/*neu* was measured by ACIS. Patients with an average score of 2.0 or higher were considered to have Her-2/*neu* protein overexpression and classified in the high Her-2/*neu* group. The patients with an average score <2.0 were classified in the low Her-2/*neu* group. Similar techniques and principles were applied to determine the TSP-1 level by using the same cut-off score of 2.0. Patients with an average score of ≥2.0 were classified in the high TSP-1 group, whereas patients with an average score lower than 2.0 were classified in the low TSP-1 group. The observers (MPW) were blinded with regard to the staging information and demographic characteristics of the patients.

Vascular microvessel density and lymphatic vessel density

Vascular microvessel density, although it has some inherent limitations, is a very commonly used quantitative assessment of tumor vasculature. The hotspots were measured within isolated regions of high vessel concentration by using different vascular endothelial markers.[26] We used anti-CD31 to stain blood and D2-40 to stain lymphatic vessels. The monoclonal antibody D2-40, which reacts with the oncofetal membrane antigen M2A, is a new selective marker for the lymphatic endothelium, and is useful in identifying the presence of lymphatic invasion in various malignant neoplasms.[27] To identify areas of capillaries, sections were scanned at low magnification (10× objective lens). The vessels that were defined as any positively stained single cell or cluster of cells were counted for MVD to measure the number of vessels per high-power microscopic field (40× objective lens). The MVD was scored in three fields of each lesion by the microvessel counting protocols and criteria developed by Weidner et al[28] with minor modifications. Microvessels or lymphatic vessels were defined as CD31-positive, D2-40-positive, or circular structures. Other isolated or clusters of positive cells were not included. Two investigators performed simultaneous quantitation by using a multi-headed microscope. They had to concur on the areas to be analyzed and on vessel identification numbers and counts.

Statistical analysis

The Chi-square test was used for data analysis, which included age, histologic grade, lymph node metastasis, ER, PR, and Ki-67 status. The Fisher's exact test was used for the analysis of tumor size and p53. All values of Her-2/*neu*, TSP-1, MVD, and lymphatic vessel density (LVD) were reported as the mean ± the standard deviation (M ± SD). The Student *t* test was used to analyze Her-2/*neu*, TSP-1, MVD, and LVD. A p value < 0.05 was considered significant. The Pearson correlation coefficient (r) was used to evaluate the correlation of Her-2/*neu* with MVD and LVD and the correlation of TSP-1 with MVD and LVD. A p value < 0.05 was considered statistically significant. In this study, a retrospective power analysis was performed to determine the association of MVD and LVD with the high and low Her-2/*neu* groups. In accordance with the observed data, this analysis showed that the study had approximately 99% power (high Her-2/*neu* group) and 91% power (low Her-2/*neu* group) to detect a significant difference (effect sizes were 5.9 and 1.4, respectively), assuming a significance level of $\alpha = 0.05$.

Results

Clinicopathologic parameters and ER, PR, Ki-67, and p53 status in the different Her-2/neu groups

By using the ACIS, we evaluated Her-2/*neu* in a more objective and quantitative manner with scores ranging from 0.5 to 3.0 (Fig. 1). Compared to the low Her-2/*neu* group, the high Her-2/*neu* group had a higher histologic grading ($p < 0.001$), but no significant difference in tumor size ($p < 0.329$) or lymph node metastasis ($p < 0.763$; Table 1). However, the high-Her-2/*neu* group had less ER expression, less PR expression, and more Ki-67 expression ($p < 0.001$, $p < 0.001$, $p < 0.001$, respectively; Table 2). More p53-positive results (photos not shown) was found in the high Her-2/*neu* group, although this was not statistically significant ($p = 0.051$; Table 2).

Her-2/neu overexpression is associated with high MVD and LVD

We evaluated angiogenesis by the immunohistochemical staining of CD31 for MVD (Fig. 2A and B) and D2-40 for lymphangiogenesis assessment (i.e., LVD; Fig. 2C and D). Angiogenesis was significantly higher in the high Her-2/*neu* group than in the low Her-2/*neu* group ($p < 0.001$). The specificity of D2-40 for lymphatic vessels was demonstrated in which some arterioles with lumen-like characteristics showed no staining for D2-40. This can provide further evidence of the specificity of D2-40 for lymphatic vessels instead of blood vessels. The lymphangiogenic response was significantly higher in the high Her-2/*neu* group than in the low Her-2/*neu* group ($p = 0.002$; Table 2).

Her-2/neu overexpression is correlated with low TSP-1 expression in tumor cells and high TSP-1 expression in the stromal area

The ACIS was used to quantitate and specifically localize TSP-1 expression in the tumor cells and in the surrounding stromal cells, respectively. Individual scores and statistical analysis showed that the high Her-2/*neu* group had a significantly low expression of TSP-1 in tumor cells (TSP-1/T; $p < 0.001$) and high expression of TSP-1 in stromal cells (TSP-1/S; $p < 0.001$), compared to the low Her-2/*neu* group (Table 2). We found two distinct expression

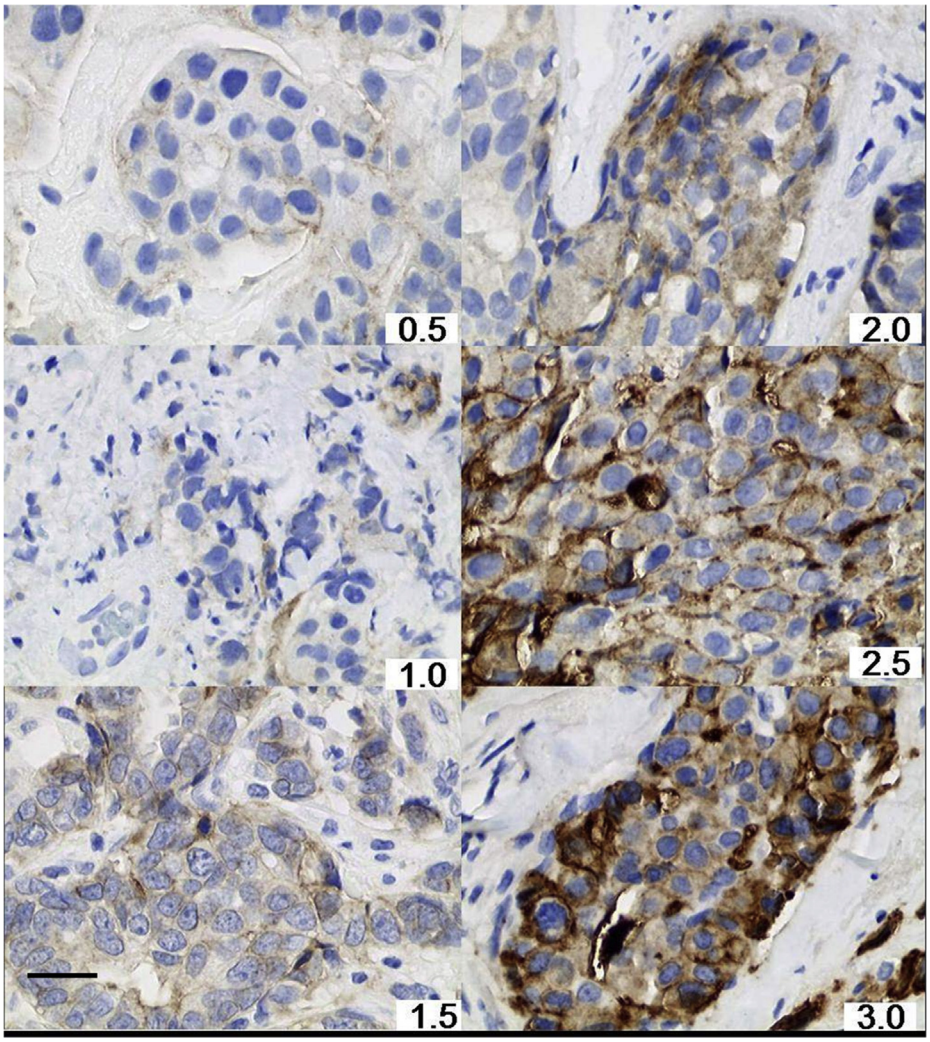

Fig. 1. Representative photographs of immunohistochemical stains for different levels of Her-2/*neu* expression, ranging from low expression (0.5) to high expression (3.0). Characteristic scores (i.e., 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0) from the ACIS scoring system are shown. The scale bar represents 2.5 μm.

patterns of TSP-1 in the breast cancer specimens: pattern I showed that low TSP-1/T was associated with high TSP-1/S (Fig. 3A and B) and pattern II showed that high TSP-1/T was associated with low TSP-1/S (Fig. 3C and D). In both patterns, TSP-1/T and TSP-1/S often manifestly compensated each other. Pattern I manifested more commonly in the high Her-2/*neu* group, whereas pattern II manifested more commonly in the low Her-2/*neu* group. In the high Her-2/*neu* group, there were 17 patients with pattern I; four patients with pattern II; and seven patients with concordant expression (four patients had high TSP-1 expression in the tumor and stromal cells and three patients had low TPS-1 expression in both cell types). In the low Her-2/*neu* group, there were four patients with pattern I, 23 patients with pattern II, and one patient with concordant expression (i.e, high TPS-1 expression in both cell types).

Table 2
Comparison of ER, PR, Ki-67, p53, MVD, LVD between the high Her-2/*neu* and low Her-2/*neu* groups.

Cutoff (%)		High Her-2/*neu*	Low Her-2/*neu*	*p*
		≥2	<2	
ER	≥5	9 (32.1)	23 (82.1)	<0.001
PR	≥5	4 (14.3)	16 (57.1)	0.001
Ki-67	≥20	26 (92.9)	13 (46.4)	<0.001
p53	≥10	7 (25.0)	1 (3.6)	0.051
MVD		13.8 ± 2.4	7.9 ± 3.6	<0.001
LVD[a]		4.4 ± 1.6	3.0 ± 1.5	0.002
TSP-1/T		1.69 ± 0.62	2.54 ± 0.58	<0.001
TSP-1/S		2.44 ± 0.64	1.51 ± 0.48	<0.001

Data are expressed as mean ± standard deviation or *n* (%).
ER = estrogen receptor; LVD = lymphatic vessel density; MVD = microvessel density; PR = progesterone receptor; TSP-1 = thrombospondin-1; TSP-1/S = TSP-1 expression in the stroma; TSP-1/T = TSP-1 expression in tumor cells.
[a] Fisher's exact test was used for the analysis of tumor p53 status.

TSP-1 expression is correlated with different MVD levels, but not with LVD

We further compared angiogenesis in the different TSP-1 expression groups. For statistical analysis, the patients with different TSP-1 scores were categorized into the high TSP-1 group or low TSP-1 group with a cut-off value of 2.0 by using the same principles in measuring Her-2/*neu* with ACIS. The MVD was significantly lower in the high TSP-1/T group ($p = 0.018$) and significantly higher in the high TSP-1/S group ($p = 0.015$), compared to the low TSP-1/T group. The LVD was not significantly different in the TSP-1/T group ($p = 0.333$) or in the TSP-1/S group ($p = 0.372$; Table 3). In summary, the expression of Her-2/*neu* was positively correlated with angiogenesis and lymphangiogenesis

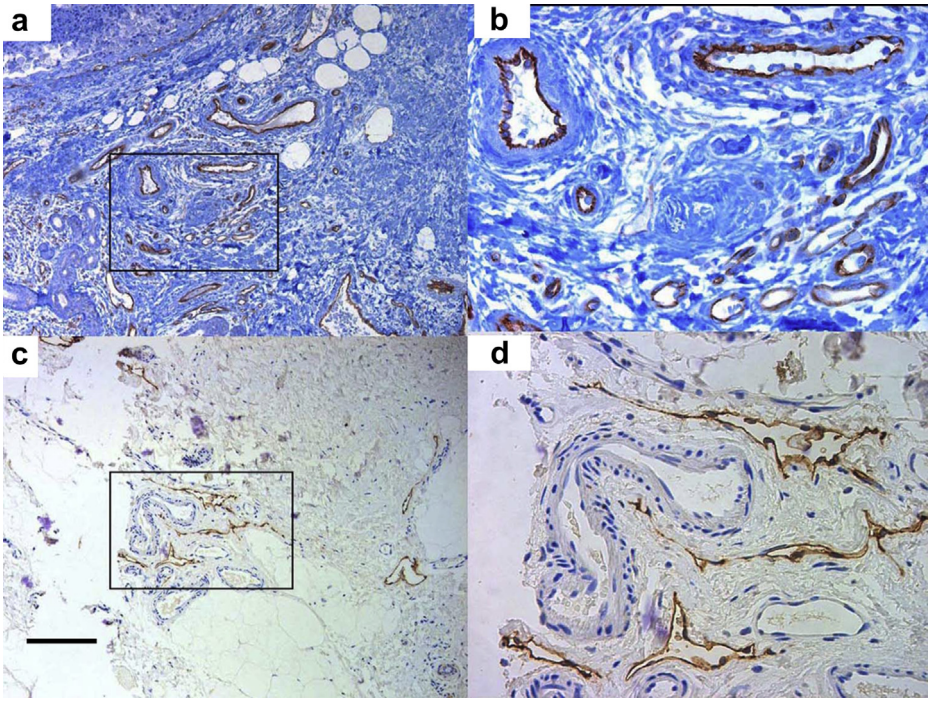

Fig. 2. Representative photographs of immunohistochemical stains for two blood vessels (i.e., CD31-stain) and lymphatic vessels (i.e., D2-40 stain). The blood vessels are defined as a positive CD31-stained tube or circular structure, and viewed through the objective lens of the microscope at (A) 10× magnification and at (B) 40× magnification. Lymphatic vessels are defined as D2-40-stained tube or circular structures, and are shown at (C) 100× magnification and at (D) 400× magnification. Isolated or clusters of positively stained cells are not included. Some arterioles with characteristic lumen-like structures show no D2-40 staining, which provides evidence of the specificity of D2-40 for lymphatic vessels, instead of blood vessels. The scale bar represents 40 μm (A, C), 10 μm (B, D).

(for both, $p < 0.001$; Fig. 4). The TSP-1/T was negatively correlated with angiogenesis ($p = 0.002$), whereas the TSP-1/S was positively correlated with angiogenesis ($p < 0.001$). Neither TSP-1/T nor TSP-1/S was correlated with lymphangiogenesis ($p = 0.138$ and $p = 0.185$, respectively; Fig. 4).

Discussion

Our study is the first to provide a quantitative analysis with clinical correlation that Her-2/*neu* induces angiogenesis and lymphangiogenesis and plays a role in the biological and behavioral

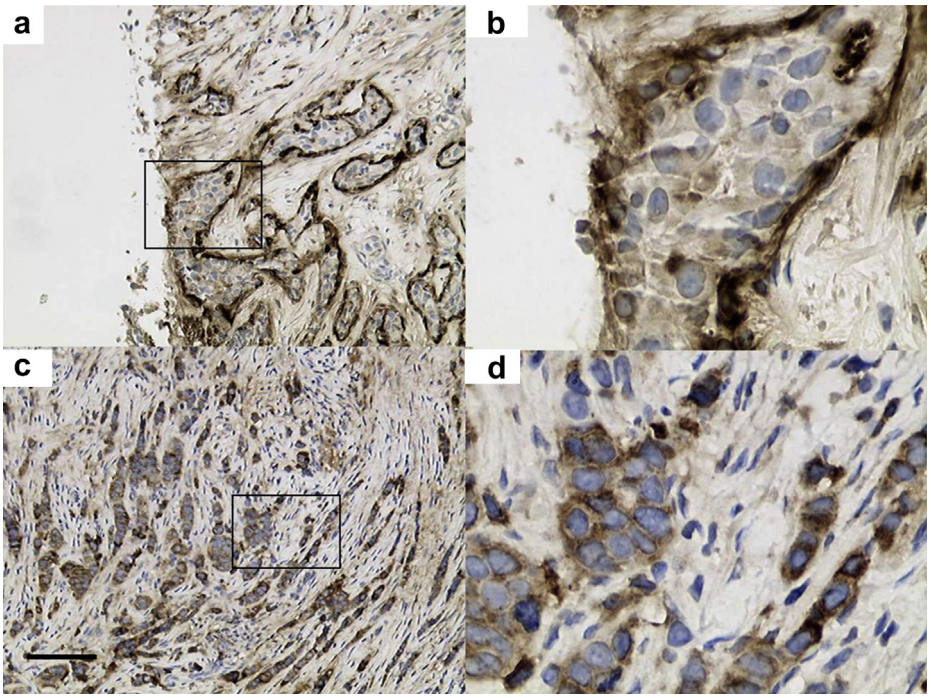

Fig. 3. Two TSP-1 expression patterns are demonstrated: pattern 1 is low thrombospondin-1 (TSP-1) expression in the tumor area (TSP-1/T) accompanied by high TSP-1 expression in the stroma area (TSP-1/S), shown at (A) 100× magnification and at (B) 400× magnification. Pattern 2 is high TSP-1/T accompanied by low TSP-1/S, shown at (C) 100× magnification and at (D) 400× magnification. The scale bar represents 10 μm (A, C) and 2.5 μm (B, D).

Table 3
The correlation of angiogenesis (MVD) and lymphangiogenesis (LVD) with TSP-1 expression in the tumor or stromal area.

	TSP-1/T		p	TSP-1/S		p
	<2	≥2		<2	≥2	
No.	23	33		27	29	
MVD	12.4 ± 4.5	9.8 ± 3.7	0.018	9.4 ± 3.6	12.2 ± 4.4	0.015
LVD	4.0 ± 1.9	3.6 ± 1.5	0.333	3.5 ± 1.5	3.9 ± 1.9	0.372

Data are expressed as mean ± standard deviation.
LVD = lymphatic vessel density; MVD = microvessel density; TSP-1/S = thrombospondin-1 expression in the stromal area; TSP-1/T = thrombospondin-1 expression in the tumor area.

changes and the pathogenesis of human breast cancer. Her-2/*neu* amplification has a greater prognostic value than most currently used prognostic factors, including hormonal receptor status, in early breast cancer or lymph node-positive disease.[29] Its significance remained even after adjusting for other known prognostic factors.[1] In our data, Her-2/*neu* expression was correlated with less ER and PR expression and more Ki-67 expression. It also supports the concept that ER-negative tumors are a morphologically and phenotypically distinct entity and provides a rationale for the study and for use of newer agents in breast cancer treatment.[30]

Her-2/*neu* stimulates tumor growth, at least in part, by upregulating angiogenesis.[4] Some angiogenic activators and/or inhibitors have been proposed as the mediators that were reported by Izumi et al[5] in herceptin-induced normalization and regression of the vasculature in an experimental Her-2/*neu* overexpressing breast tumor model. The knockdown of Her-2/*neu* expression by small interfering RNA (siRNA) further elucidates the specific association of increased expression of the antiangiogenic factor TSP-1 with decreased expression of the proiangiogenic VEGF.[4] Our data indicated that Her-2/*neu*-induced angiogenesis is related to TSP-1 expression. In addition, Her-2/*neu*-related angiogenesis is also explained by the constitutive activation of oncogenic proteins (e.g., EGRF, Raf, MEK, and PI3K) acting at various levels in the Ras signaling pathway, which simultaneously induces TSP-1 downregulation and VEGF upregulation.[31]

Lymphangiogenesis (in addition to angiogenesis) is a commonly reported histopathological finding and is essential for breast cancer progression and patient survival.[32] Her-2/*neu*-related lymphangiogenesis in breast cancer has attracted attention in recent years.[33,34] In the absence of effective immunohistochemical markers of lymphatic endothelium in paraffin sections, lymphatic invasion can only be identified on the basis of conventional hematoxylin and eosin (H&E) staining because the presence of tumor emboli within vascular channels is distinctly lined by a single layer of endothelial cells. Therefore, some contradictory results have been reported when applying immunohistochemistry because of a lack of standards for antibodies, preparation, fixation, staining procedure, and analysis for different choices of cut-off points.[35] With the availability of a selective marker for the lymphatic endothelium (e.g., the D2-40 monoclonal antibody), the increased accuracy of detecting lymphatic invasion can contribute to the evaluation of the utility of a qualitative and quantitative assessment as a prognostic indicator in invasive cancer.[20] In our study, overexpression of Her-2/*neu* in breast cancer is associated with a high LVD. The specificity of D2-40-stained lymphatic vessels was evidenced by some arterioles showing characteristic lumen-like structures but no staining for D2-40 (Fig. 2B). Bhattacharjee et al[33] recently indicated that Her-2/*neu* interacts with cyclooxygenase-2 in the upregulation of VEGF-C and in lymphangiogenesis.

Our data showed that Her-2/*neu*-induced lymphangiogenesis is unrelated to TSP-1 expression. The role of TSP-1 in lymphagiogenesis was unclear prior to the availability of specific lymphatic markers that distinguish lymphatic from vascular endothelium. Our finding is concordant with the report by Hawighorst et al[36] that showed that TSP-1 overexpression suppresses tumor angiogenesis and distant organ metastasis, but it failed to inhibit tumor-associated lymphangiogenesis or lymphatic tumors from spreading to regional lymph nodes in a transgenic mouse skin cancer model. Concomitant with these results, the endothelial TSP-1 receptor CD36 is mostly absent from cutaneous lymphatic vessels.[36] The

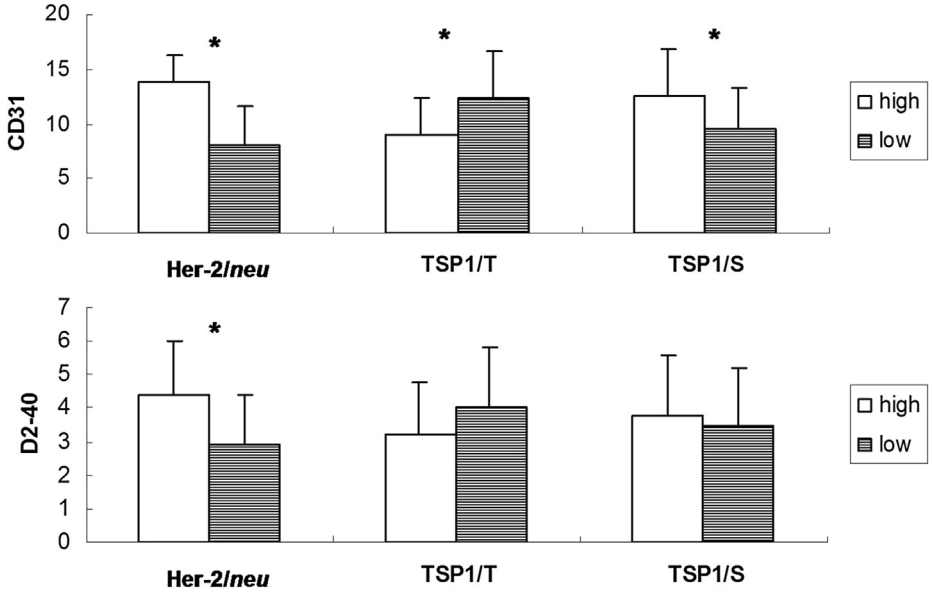

Fig. 4. The correlation between MVD, LVD and the different Her-2/*neu*, TSP-1/T, and TSP-1/S expression groups. The expression of Her-2/*neu* is positively correlated with MVD ($r = 0.662$, $p < 0.001$) and LVD ($r = 0.574$, $p < 0.001$). MVD is negatively correlated with TSP-1/T ($r = -0.414$, $p = 0.002$) and positively correlated with TSP-1/S ($r = 0.471$, $p < 0.001$). LVD is correlated with neither TSP-1/T ($r = -0.201$, $p = 0.138$) nor TSP-1/S ($r = 0.180$, $p = 0/185$). LVD = lymphatic vessel density; MVD = microvessel density; TSP-1/S = thrombospondin-1 expression in adjacent stroma; TSP-1/T = thrombospondin-1 expression in tumor cells.

phenomenon can be explained by the findings of other mediators (e.g., the upregulation of VEGF-C and VEGF-D in a Her-2/*neu*-dependent pattern) that facilitate lymphangiogenesis and the spreading of tumors cells to the lymph nodes in breast carcinoma and in lung carcinoma.[19,37,38]

Stromal reaction is a host reaction of connective tissue that, when induced in cancer, produces a progressive and permissive mesenchymal microenvironment, thereby supporting tumor progression.[39] The locally activated host microenvironment, which includes cellular and extracellular elements, then modifies the proliferative and invasive behavior of the tumor cells.[40] Our previous study demonstrated a novel role of TSP-1 in inhibiting the tumor-stroma reaction that could be attributed to the blockage of activated fibroblasts from invading cancer cells, in addition to its antiangiogenesis effect. The increased migration ability and the invasive ability into the tumor cluster of activated fibroblasts are inhibited by TSP-1.[41] The TSP-1 expression in tumors and in the stroma modulates the pericellular environment and can potentially change the tumor-extracellular matrix (ECM) microenvironment to avoid further progression. The remodeling connective tissue interacts with epithelial cells and controls tumor invasion and angiogenesis.[42] Changes in the tumor-ECM microenvironment further support the concept of stromal reaction happening in parallel to tumorigenesis.[41]

In this study, two unique expression patterns of TSP-1 were found, and they were correlated with different Her-2/*neu* expression and angiogenesis. Low TSP-1 expression in tumor cells coexisting with high TSP-1 expression in surrounding stromal areas (i.e., desmoplasia) induced higher angiogenesis. The spatial correlation of high Her-2/*neu* expression and low TSP-1 expression in the tumor area implies that Her-2/*neu* may downregulate TSP-1 in tumor areas, and have a subsequent compensatory response from the surrounding stroma and a final angiogenic balance falling on the side of the tumor area instead of the stroma area. In a study by Bertin et al[17] the presence of TSP-1 in desmoplasia alone—but not the total TSP-1 mRNA expression—was correlated with the angiogenic status. This is conceivable because TSP-1 mRNA was expressed in the tumor and in the surrounding stroma. By using the ACIS, we could further specifically localize and quantitate both areas separately. Another explanation is that there are more factors (e.g., VEGFs) involved in the angiogenesis balance in addition to TSP-1. A single factor cannot explain the whole proangiogenic or antiangiogenic microenvironment.[26] From our observation, a hypothetical model could be proposed in which Her-2/*neu* augments tumor angiogenesis, at least in part, by lowering TSP-1 expression in tumor areas, thereby resulting in high stromal reaction as compensation. Because the resultant angiogenesis status coincided with TSP-1 expression in the tumor area, TSP-1 in stroma areas plays a compensatory role in the change of TSP-1 expression in the stroma area. The hypothetical model is supported by the compensatory response mode of matrix metallopreotease providing an alternative paracrine support for tumor angiogenesis.[43] Thus, TSP-1/T may play an active role and TSP-1/S may play a passive and compensatory role for microenvironmental changes in breast cancer.

The limitations of this study are as follows: (1) only a correlative phenomenon was demonstrated; therefore a cause–effect relationship was not available and (2) some selection bias of LVD may exist because of an insufficient number of lymphatic vessels visible in the high-power-fields on acccount of the inherent paucity of lymphatic vessels. The biological significance of the presence of D2–40 in the normal myoepithelia cells remains unknown. Dumoff et al[27] reported positive D2-40 immunoreactivity in cervical cancer tissues that was correlated with lymphatic invasion and nodal metastasis in early stage cervical squamous cell carcinoma.

In conclusion, our data support the observation that Her-2/*neu* can affect biological manifestations of breast cancer by increasing angiogenesis which is TSP-1-related and increasing lymphangiogenesis which is TSP-1-unrelated. Two distinct expression patterns of TSP-1 occur in accordance with the Her-2/*neu* expression levels, thereby resulting in different angiogenic status. Our findings further provide correlative evidence for the hypothetical concept that Her-2/*neu* augments tumor angiogenesis, at least in part, by lowering TSP-1 expression in tumor areas, thereby resulting in a high stromal reaction as compensation. From our results and observations, combined herceptin and antiangiogenesis treatment may have synergistic antitumor effects in Her-2/*neu* overexpression in breast cancer patients.

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