

Evaluation of Tumor Angiogenesis with a Second-Generation US Contrast Medium in a Rat Breast Tumor Model

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Abbreviations:

BV = blood volume
ENU = ethyl-N-nitrosourea
IDC = invasive ductal carcinoma
MI = mechanical index
MVD = microvessel density
Q = blood flow
ROI = region of interest
rTA = relative time to arrival
SI = signal intensity
TA = time to arrival
TICs = time-intensity curves

Objective: Tumor angiogenesis is an important factor for tumor growth, treatment response and prognosis. Noninvasive imaging methods for the evaluation of tumor angiogenesis have been studied, but a method for the quantification of tumor angiogenesis has not been established. This study was designed to evaluate tumor angiogenesis in a rat breast tumor model by the use of a contrast-enhanced ultrasound (US) examination with a second-generation US contrast agent.

Materials and Methods: The alkylating agent 19N-ethyl-N-nitrosourea (ENU) was injected into the intraperitoneal cavity of 30-day-old female Sprague-Dawley rats. Three to four months later, breast tumors were detected along the mammary lines of the rats. A total of 17 breast tumors larger than 1 cm in nine rats were evaluated by gray-scale US, color Doppler US and contrast-enhanced US using SonoVue. The results were recorded as digital video images; time-intensity curves and hemodynamic parameters were analyzed. Pathological breast tumor specimens were obtained just after the US examinations. The tumor specimens were stained with hematoxylin and eosin (H & E) and the expression of CD31, an endothelial cell marker, was determined by immunohistochemical staining. We also evaluated the pathological diagnosis of the tumors and the microvessel density (MVD). Spearman's correlation and the Kruskal-Wallis test were used for the analysis.

Results: The pathological diagnoses were 11 invasive ductal carcinomas and six benign intraductal epithelial proliferations. The MVD did not correlate with the pathological diagnosis. However, blood volume (BV) showed a statistically significant correlation with MVD (Spearman's correlation, $p < 0.05$).

Conclusion: Contrast-enhanced US using a second-generation US contrast material was useful for the evaluation of tumor angiogenesis of breast tumors in the rat.

Tumor angiogenesis is crucial for cancer growth and is a significant prognostic indicator in breast cancer. Invasive breast carcinomas with higher microvessel counts show more lymph node metastases, more distance metastases, and a higher rate of recurrence (1, 2). In patients receiving chemotherapy, determination of tumor angiogenesis can be used to monitor the response to treatment (3). Many studies have evaluated tumor angiogenesis through noninvasive imaging methods. MRI with dynamic contrast enhancement, including T1-weighted and T2-weighted angiogenesis MRI, is the most common method for evaluating angiogenesis (4–6). Some studies have suggested that contrast enhancement of MRI, especially early signal enhancement or early signal enhancement with rapid washout of intravenous contrast correlates with intratumoral microvessel density

(MVD) representing tumor angiogenesis (7, 8), and other studies have suggested that the degree and pattern of contrast enhancement is correlated with the density and distribution of microvessels (9). However, other studies have suggested that there was no significant correlation between contrast enhancement of MRI and MVD (10, 11).

Color and power Doppler ultrasound (US) are also used to evaluate tumor vascularity, but these modalities can detect only larger vessels with signals above the noise level or flow velocities above the threshold of the wall filter. Unfortunately, quantification methods for tumor angiogenesis have not yet been established.

Contrast-enhanced US imaging using second-generation contrast medium and a low-mechanical index (MI) can overcome the limitation of detection of only the fast blood flow of large vessels of the above-described methods. The purpose of this study is to evaluate quantification of tumor angiogenesis in a rat breast tumor model by the use of a contrast-enhanced US examination with a second-generation US contrast medium.

MATERIALS AND METHODS

The Institutional Committee for Animal Research approved this study, which was in accordance with the guidelines and the recommendations of the Committee on Animal Research at Asan Medical Center.

Rat Breast Tumor Model

Breast tumors were induced by injection of the alkylating agent 19N-ethyl-N-nitrosourea (ENU) into the peritoneal cavities of 30-day-old specific pathogen-free (SPF) female Sprague-Dawley rats with a 70–80 gram body weight. ENU is a strong carcinogen, inducing various types of tumors at various sites. We administered 180 mg/kg and 45 mg/kg of ENU to 10 and 20 rats for the induction of malignant and benign breast tumors, respectively (12).

After three to four months, tumors developed along the mammary lines from the axilla to inguinal areas in seven rats in the malignant group and five rats in the benign group. Among the lesions, we selected tumors larger than 1 cm in size for inclusion in an US examination. A total of 17 mammary tumors from nine rats were included in the study.

US Examinations

US images were obtained with a Logic 9 unit (GE Medical Systems, Milwaukee, WI) using a 10-MHz linear array probe. US parameters including acoustic gain, depth, and focus were optimized for each tumor. Conventional

and color Doppler US examinations were performed on all lesions before the contrast-enhanced study. Gray-scale images along the axis of the tumors, where the shape of the entire tumor was well demonstrated, were obtained to evaluate tumor size and shape. On color Doppler US, we evaluated the vascularity within the tumors and the flow of the input arteries around the tumors; these arteries were used as reference arteries in analyzing tumor angiogenesis.

Contrast-enhanced US images were obtained using a second-generation US contrast agent (SonoVue®; Bracco SpA, Milan, Italy), which consists of microbubbles that contain a hydrophobic gas (sulfur hexafluoride) instead of air. We rapidly infused 0.25 ml/kg of the US contrast agent followed by a 0.3 ml saline flush intravenously through the tail vein of the rats. Contrast-enhanced US images were obtained with a pulse-inversion coded harmonic US technique with a low MI of 0.12. Continuous scanning started immediately after injection of the US contrast agent and lasted for 90 seconds. All contrast-enhanced US studies were recorded as digital video images.

Analysis of the US Images

After recording the US examinations, we restored the digital video images on a desktop computer. The US images were analyzed with custom-made software (single compartment angiogenesis analyzing program) using Matlab 7.0 (The MathWorks, Inc., Natick, MA).

Time-intensity curves (TICs) were derived by tracking changes in contrast signals on a pixel-by-pixel basis from the contrast-enhanced US images after setting the region of interest (ROI). The TICs of contrast-enhanced US images differed from pixel to pixel within the same tumor, and the hemodynamic parameters derived from the TICs analysis of the ROI was influenced by the area included in the ROI. In particular, the signal intensity (SI) of contrast increased greatly when a vessel visible on color Doppler US was included in the ROI. Therefore, we set the ROI in the well-enhancing parenchyma of the tumor around 1,000 pixels, avoiding vessels observed on the color Doppler US before contrast-enhanced US imaging (Fig. 1). We used the mean of calculated parameters from the TICs in three different ROIs.

In order to extract quantitative hemodynamic parameters, the SI time curves were fitted to a gamma variate function described by the equation (13–15)

$$C_{tissue}(t) = K(t - T_0)^\alpha e^{-(t - T_0)^\beta} + SI_{base} \quad [1]$$

where t is the time, and $C_{tissue}(t)$ is the measured SI as a function of time which is related to the concentration of the dye. K is a constant scale factor. α , β are parameters that define the shape of the curve. SI_{base} is base signal and

T_0 is time of arrival. From the fitted values for α and β , one can deduce perfusion indices such as the time to reach peak concentration (t_p) and the apparent mean transit time (τ_{app}) as follows (15):

$$t_p = \alpha\beta \quad [2]$$

$$\tau_{app} = \beta(\alpha + 1) \quad [3]$$

Additionally, from measurements of the tissue and arterial concentration curves ($C_{tissue}[t]$ and $C_{arterial}[t]$) the volume of distribution of the agent was calculated directly as follows (14):

$$V = \frac{\int_0^\infty C_{tissue}(t) dt}{\int_0^\infty C_{arterial}(t) dt} \quad [4]$$

The central volume principle relates the terms perfusion (f), blood tissue partition coefficient (p) and the mean transit time (τ) (13, 15):

$$f = p / \tau \quad [5]$$

This principle applies to any agent, whether it is extracted from the blood or not. We assumed that contrast agents behave as intravascular agents during the first pass. Therefore, the mean transit time equals τ_{app} and the partition coefficient can be determined as the distribution volume (V).

$$f = p / \tau = V / \tau_{app} \quad [6]$$

These hemodynamic parameters, including blood volume (BV), blood flow (Q), mean transit time (MTT, τ_{app}), time to arrival (TA), and relative time to arrival (rTA), were obtained from the TICs of the first-pass (5, 13–16). The TICs of the input arteries of tumors were used as the reference of the arterial input function.

Correlation of the US findings with the Pathological Findings

After contrast-enhanced US imaging, rats were sacrificed by IV injection of a lethal dose of anesthetic agents. Pathological specimens were stained with hematoxylin and eosin (H & E) and with a specific immunohistochemical stain for CD31 for a histological diagnosis of the tumors, and to determine the MVD. The MVD was defined as the total number of vessels counted within six high-power fields ($\times 200$) of the tumor parenchyma that had the highest microvessel counts, except for vessels within the muscular walls.

We evaluated the correlations between hemodynamic parameters from contrast-enhanced US images, MVD and the histological diagnosis using Spearman's correlation and the Kruskal-Wallis test.

RESULTS

In all cases, the input arteries were relatively well visualized on color and power Doppler US examinations before the contrast-enhanced US examinations. When there were multiple input arteries, the largest one was selected as a reference artery. The hemodynamic parameters measured from the TICs of the contrast-enhanced US are shown in Table 1. BV ranged from 3.10 to 12.87, and Q ranged from 42.56 to 137.32.

When analyzing the hemodynamic parameters from the TICs derived from the contrast-enhanced US images, BV showed a statistically significant correlation with tumor MVD ($p < 0.05$); however, the other parameters did not correlate with MVD (Table 2).

The histological diagnoses of the tumors were 11



Fig. 1. US results of intraductal proliferation. Color Doppler US (A) shows input artery in right side of tumor (arrow) and intratumoral vasculature within tumor (arrowhead). Time-intensity curve of tumor (B) was obtained from three areas of enhancing parenchyma without vascular structures within tumor.

invasive ductal carcinomas (IDC) and six benign intraductal epithelial proliferations (IP) (Fig. 2). As these tumors were induced by intraperitoneal administration of a carcinogen, the histological features of the rat breast tumors were not the same as those of human breast tumors that naturally develop; even the benign intraductal proliferation consisted of somewhat atypical cells and there were no areas with normal glandular structures due to exposure to the carcinogen. However, the histological

diagnoses were based on areas with findings relatively typical of breast tumors. MVD counted in six high-power fields ($\times 200$) varied from 27 to 176 (Fig. 3). MVD did not correlate with the histological diagnosis of the tumors. In addition, the hemodynamic parameters driven from TICs of contrast-enhanced US also did not correlate with the histological diagnosis of the tumor. The histological diagnosis and MVD of each tumor are shown in Table 3.

Table 1. Parameters of Tumor Angiogenesis on Contrast-Enhanced US

Number of Tumor	BV	Q	TA/rTA	MTT
1	5.88	61.18	22.27	5.31
2	4.65	66.74	22.04	3.88
3	11.64	137.32	8.39	5.31
4	12.67	72.94	24.86	9.57
5	6.35	46.83	31.99	4.35
6	5.74	58.56	13.20	5.77
7	4.98	109.00	13.51	2.96
8	3.45	61.28	7.79	3.93
9	6.71	55.03	15.41	6.51
10	6.62	62.00	18.66	5.78
11	3.81	86.58	22.36	2.80
12	3.10	96.61	10.38	1.98
13	5.37	62.35	12.67	4.88
14	3.52	42.56	16.08	4.77
15	4.76	56.74	13.86	4.85
16	5.11	63.85	14.36	4.19
17	3.88	69.94	19.47	3.40

Note.— BV = blood volume, Q = blood flow, TA = time to arrival, rTA = relative time to arrival, MTT = mean transit time

DISCUSSION

Color or power Doppler US has been used to evaluate vascularity within tumors to evaluate the characteristics of breast lesions. However, Doppler US is not sensitive to the slow flow and small volume blood flows in capillaries within the tumor parenchyma. In malignant tumors with increased interstitial pressure, slow intratumoral blood flow is particularly difficult to evaluate with color Doppler US (17). Power Doppler US can compensate for slow blood flow to some degree, but flow that is visible on

Table 2. Correlation between Multiple Parameters of Angiogenesis Analysis and MVD of Tumors

		BV	Q	TA /rTA	MTT
Spearman's rho (R)	Correlation coefficient	0.529*	0.070	0.217	0.286
	P values (2-tailed)	0.029	0.790	0.402	0.265

Note.— * $p < 0.05$

MVD = microvessel density, BV = blood volume, Q = blood flow, TA = time to arrival, rTA = relative time to arrival, MTT = mean transit time

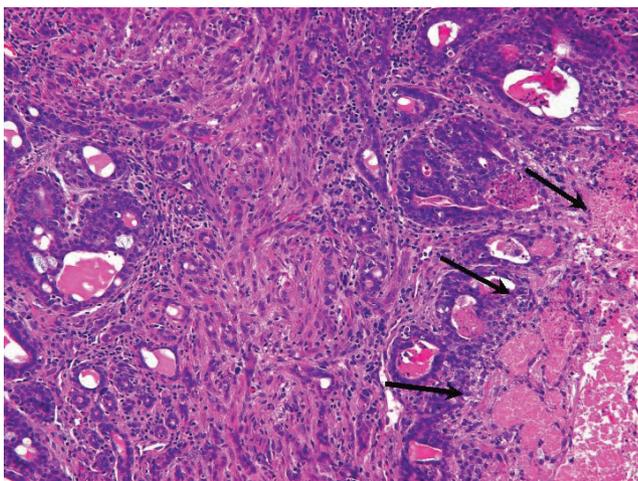


Fig. 2. Invasive ductal carcinoma induced by intraperitoneal injection of ethyl-N-nitrosourea. Tumor consists of infiltrating cords and tubules in background of atypical ductal hyperplasia. Arrows on right side indicate tumor necrosis (Hematoxylin & Eosin staining, $\times 100$).

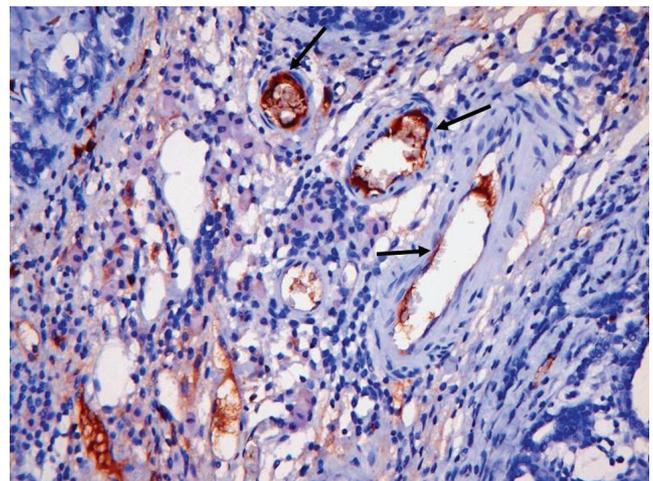


Fig. 3. Immunostaining for CD31. Endothelial cells (arrows) lining lymphovascular spaces are immunoreactive for CD31 ($\times 200$).

Table 3. Pathological Diagnosis and MVD of Tumors

Number of Tumor	Diagnosis	MVD (microvessels in 6 HPF)
1	IDC	176
2	IDC	122
3	IDC	137
4	IP	105
5	IP	119
6	IDC	64
7	IDC	105
8	IDC	64
9	IDC	58
10	IDC	92
11	IP	28
12	IP	36
13	IP	27
14	IP	44
15	IDC	50
16	IDC	100
17	IDC	28

Note.—IP = intraductal proliferation, IDC = invasive ductal carcinoma
MVD = microvessel density, HPF = high power fields

power Doppler US differs substantially from the histological MVDs (18). Contrast-enhanced US has been widely studied for the evaluation of hepatic tumors, for the diagnosis of testicular torsion or in the evaluation of myocardial angiogenesis in patients following a myocardial infarction (19–21). First-generation US contrast agents were small, stabilized air microbubbles with a mean diameter of 2–3 μm and contrast enhancement using first-generation US contrast agents under a high MI relied more on microbubble destruction than on the concentration of the contrast agent (19). Second-generation US contrast agents consist of 1 to 10 μm (mean 2.5 μm) microbubbles that contain a hydrophobic gas in place of air (22). These agents can circulate within the blood pool for a long time without being destroyed, as they are much less soluble and much more stable in the blood pool (23). Contrast-enhanced US using a second-generation contrast agent with a low MI can display the simple backscattering sound wave of microbubbles with minimized bubble destruction and permit prolonged evaluation of the hemodynamic distribution of the contrast agent in real time without serious complications (24–26).

Microvessel density is commonly used as a surrogate for angiogenesis (1). Assessment of tumor angiogenesis is important in predicting the prognosis of breast cancer before treatment, and monitoring the response to treatment (1–3). Assessment of tumor angiogenesis is also important in selecting an optimal treatment for individual

patients and deciding an optimal drug dosage of an antiangiogenic agent (27, 28). Imaging tumor angiogenesis is more advantageous than measuring MVD directly as it is noninvasive and can be used to assess much larger volumes than biopsy samples (16, 29).

There are a few studies that have evaluated the quantification of tumor angiogenesis, and most of the studies used TICs derived from dynamic contrast-enhanced MRI (4–6, 16). Changes of hemodynamics in breast cancer after treatment (16), and tumor perfusion in benign and malignant breast tumors (31), as well as prognostic factors of breast cancer have been evaluated with the use of dynamic contrast-enhanced MRI (6, 32–34). A high degree of contrast enhancement, rapid peak enhancement, and peripheral enhancement pattern on MRI were correlated with a higher tumor grade, advanced nodal stage, and with active cellular proliferation (32–34). However, MRI with dynamic contrast enhancement represents the permeability of the vessels more than the angiogenesis of the tumor (12, 30, 31). Moreover, some studies showed no significant correlation between contrast enhancement on MRI and tumor angiogenesis (10, 11), and suggested that contrast enhancement on MRI could not be explained solely by MVD, but from other contributing factors (6, 10, 11).

In this study, we evaluated the quantification of tumor angiogenesis using contrast-enhanced US, which is an intravascular agent without diffusion (23). One hemodynamic parameter, BV, from the TICs of contrast-enhanced US showed a moderate degree of correlation (correlation coefficient, 0.529) to MVD. In previous studies on the hemodynamics of breast tumor using dynamic contrast-enhanced MRI, similar results as compared to the present study were seen, where the BV or ratio of BV between the tumor and surrounding normal parenchyma were the most meaningful parameters among all of the hemodynamic parameters (4, 5). Although the degree of correlation between BV and MVD was not high, this result suggests that MVD can be imaged non-invasively and that the quantification of tumor angiogenesis using contrast-enhanced US is possible to achieve.

The degree of US contrast enhancement correlated with MVD but did not correlate with the histological diagnosis of the breast tumors. Previous studies using dynamic contrast-enhanced MRI (31, 35), showed more rapid and stronger contrast enhancement in malignant breast tumors. However, the MVD did not differ between benign and malignant breast tumors, as in previous studies (6, 31) and the present study. The exact reason is still unknown, but Kuhl et al. (31) suggested that the BV effect may be attributable to increased blood flow or an increased ratio of

perfused versus non-perfused capillaries.

Based on our findings, we suggest that imaging of MVD using contrast-enhanced US should not be used as a method of differential diagnosis between benign and malignant breast tumors, but should be considered as representative of tumor angiogenesis in both benign and malignant breast tumors, and is useful for the detection of vascular changes in tumors.

This study has some limitations. The SonoVue contrast agent produces the best images with the combination of a low MI around 0.1 and a low MHz transducer around 4 MHz (36); but we used a 10-MHz transducer with a low MI of 0.12 due to the lesion characteristics of the rat breast tumors that were small lesion with superficial locations in our study.

Another limitation was the method of MVD assessment. The histological diagnosis of the tumor was based on the areas representative of invasive tumor components in malignant lesions. However, when we counted the number of microvessels within the tumor in six high power fields, including both the periphery and center of the tumor, the more invasive foci as well as the less invasive areas were included among the counted high power fields. Therefore, tumors with a small malignant portion within a large area of benign lesion were histologically diagnosed as IDCs, but the MVD of the tumors represented the entire tumor, not just the small malignant portion of the tumor. This may influence the result of correlation between the MVD and pathological diagnosis of the tumor. Finally, the degree of contrast enhancement in contrast-enhanced US is largely influenced by technical conditions, including acoustic shadow, acoustic pressure, depth of the lesion, and echogenicity of the surrounding tissue (37, 38). Poor images caused by technical difficulties result in the poor detection of the contrast signal that represents angiogenesis. All of these factors influence the quantitative analysis of contrast-enhanced US. Therefore, to use contrast-enhanced US for the quantification of tumor angiogenesis, more studies are needed to establish proper technical imaging conditions and analysis methods.

In conclusion, BV, one hemodynamic parameter derived from the TICs of contrast-enhanced US, correlates with MVD of the rat breast tumors. This finding suggests that the quantification of tumor angiogenesis is possible with the use of contrast-enhanced US using second-generation contrast agents.

References

- Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, et al. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992;84:1875-1887
- Bosari S, Lee AK, DeLellis RA, Wiley BD, Heatley GJ, Silverman ML. Microvessel quantitation and prognosis in invasive breast carcinoma. *Hum Pathol* 1992;23:755-761
- Toi M, Bando H, Kuroi K. The predictive value of angiogenesis for adjuvant therapy in breast cancer. *Breast Cancer* 2007;7:311-314
- Delille JP, Slanetz PJ, Yeh ED, Kopans DB, Garrido L. Breast cancer: regional blood flow and blood volume measured with magnetic susceptibility-based MR imaging—initial results. *Radiology* 2002;223:558-565
- Brix G, Kiessling F, Lucht R, Darai S, Wasser K, Delorme S, et al. Microcirculation and microvasculature in breast tumors: pharmacokinetic analysis of dynamic MR image series. *Magn Reson Med* 2004;52:420-429
- Teifke A, Behr O, Schmidt M, Victor A, Vomweg TW, Thelen M, et al. Dynamic MR imaging of breast lesions: correlation with microvessel distribution pattern and histologic characteristics of prognosis. *Radiology* 2006;239:351-360
- Frouge C, Guinebretiere JM, Contesso G, Di Paola R, Blery M. Correlation between contrast enhancement in dynamic magnetic resonance imaging of the breast and tumor angiogenesis. *Invest Radiol* 1994;29:1043-1049
- Esserman L, Hylton N, George T, Weidner N. Contrast-enhanced magnetic resonance imaging to assess tumor histopathology and angiogenesis in breast carcinoma. *Breast J* 1999;5:13-21
- Buadu LD, Murakami J, Murayama S, Hashiguchi N, Sakai S, Masuda K, et al. Breast lesions: correlation of contrast medium enhancement patterns on MR images with histopathologic findings and tumor angiogenesis. *Radiology* 1996;200:639-649
- Buckley DL, Drew PJ, Mussurakis S, Monson JR, Horsman A. Microvessel density of invasive breast cancer assessed by dynamic Gd-DTPA enhanced MRI. *J Magn Reson Imaging* 1997;7:461-464
- Hulka CA, Edmister WB, Smith BL, Tan L, Sgroi DC, Campbell T, et al. Dynamic echo-planar imaging of the breast: experience in diagnosing breast carcinoma and correlation with tumor angiogenesis. *Radiology* 1997;205:837-842
- Su MY, Wang Z, Carpenter PM, Lao X, Muhler A, Nalcioğlu O. Characterization of N-ethyl-N-nitrosourea-induced malignant and benign breast tumors in rats by using three MR contrast agents. *J Magn Reson Imaging* 1999;9:177-186
- Thompson HK Jr, Starmer CF, Whalen RE, McIntosh HD. Indicator transit time considered as a gamma variate. *Circ Res* 1964;14:502-515
- Wilke N, Simm C, Zhang J, Ellermann J, Ya X, Merkle H, et al. Contrast-enhanced first pass myocardial perfusion imaging: correlation between myocardial blood flow in dogs at rest and during hyperemia. *Magn Reson Med* 1993;29:485-497
- Wilke N, Jerosch-Herold M, Stillman AE, Kroll K, Tsekos N, Merkle H, et al. Concepts of myocardial perfusion imaging in magnetic resonance imaging. *Magn Reson Q* 1994;10:249-286
- Miller JC, Pien HH, Sahani D, Sorensen AG, Thrall JH. Imaging angiogenesis: applications and potential for drug development. *J Natl Cancer Inst* 2005;97:172-187
- Less JR, Skalak TC, Sevick EM, Jain RK. Microvascular architecture in a mammary carcinoma: branching patterns and vessel dimensions. *Cancer Res* 1991;51:265-273
- Peters-Engl C, Medl M, Mirau M, Wanner C, Bilgi S, Sevelde P, et al. Color-coded and spectral Doppler flow in breast carcinomas—relationship with the tumor microvasculature. *Breast*

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- Cancer Res Treat* 1998;47:83-89
19. Kim EA, Yoon KH, Lee YH, Kim HW, Juhng SK, Won JJ. Focal hepatic lesions: contrast-enhancement patterns at pulse-inversion harmonic US using a microbubble contrast agent. *Korean J Radiol* 2003;4:224-233
 20. Yamaguchi A, Hayashi Y, Kojima Y, Miyagawa H, Ito M, Kohri K. Testicular torsion: usefulness of contrast-enhanced power Doppler sonography. *Int J Urol* 2005;12:849-851
 21. Tani T, Tanabe K, Tani M, Ono F, Katayama M, Tamita K, et al. Quantitative assessment of harmonic power doppler myocardial perfusion imaging with intravenous Levovist in patients with myocardial infarction: comparison with myocardial viability evaluated by coronary flow reserve and coronary flow pattern of infarct-related artery. *Cardiovasc Ultrasound* 2005;3:22
 22. Greis C. Technology overview: SonoVue (Bracco, Milan). *Eur Radiol* 2004;14 Suppl 8:P11-15
 23. Schneider M, Arditi M, Barrau MB, Brochot J, Broillet A, Ventrone R, et al. BR1: a new ultrasonographic contrast agent based on sulfur hexafluoride-filled microbubbles. *Invest Radiol* 1995;30:451-457
 24. Lim A, Cosgrove D. Functional studies. *Eur Radiol* 2004;14 Suppl 8:P110-115
 25. Bokor D. Diagnostic efficacy of SonoVue. *Am J Cardiol* 2000;86:19G-24G
 26. Ding H, Wang WP, Huang BJ, Wei RX, He NA, Qi Q, et al. Imaging of focal liver lesions: low-mechanical-index real-time ultrasonography with SonoVue. *J Ultrasound Med* 2005;24:285-297
 27. Rosen L. Antiangiogenic strategies and agents in clinical trials. *Oncologist* 2000;5:20-27
 28. Davis DW, McConkey DJ, Abbruzzese JL, Herbst RS. Surrogate markers in antiangiogenesis clinical trials. *Br J Cancer* 2003;89:8-14
 29. Anderson H, Price P, Blomley M, Leach MO, Workman P. Measuring changes in human tumour vasculature in response to therapy using functional imaging techniques. *Br J Cancer* 2001;85:1085-1093
 30. Turetschek K, Preda A, Novikov V, Brasch RC, Weinmann HJ, Wunderbaldinger P, et al. Tumor microvascular changes in antiangiogenic treatment: assessment by magnetic resonance contrast media of different molecular weights. *J Magn Reson Imaging* 2004;20:138-144
 31. Kuhl CK, Bieling H, Gieseke J, Ebel T, Mielcarek P, Far F, et al. Breast neoplasms: T2* susceptibility-contrast, first-pass perfusion MR imaging. *Radiology* 1997;202:87-95
 32. Mussurakis S, Buckley DL, Horsman A. Prediction of axillary lymph node status in invasive breast cancer with dynamic contrast-enhanced MR imaging. *Radiology* 1997;203:317-321
 33. Szabo BK, Aspelin P, Kristoffersen Wiberg M, Tot T, Bone B. Invasive breast cancer: correlation of dynamic MR features with prognostic factors. *Eur Radiol* 2003;13:2425-2435
 34. Stomper PC, Herman S, Klippenstein DL, Winston JS, Budnick RM, Stewart CC. Invasive breast carcinoma: analysis of dynamic magnetic resonance imaging enhancement features and cell proliferative activity determined by DNA S-phase percentage. *Cancer* 1996;77:1844-1849
 35. Kvistad KA, Lundgren S, Fjosne HE, Smenes E, Smethurst HB, Haraldseth O. Differentiating benign and malignant breast lesions with T2*-weighted first pass perfusion imaging. *Acta Radiol* 1999;40:45-51
 36. Cosgrove D. Future prospects for SonoVue and CPS. *Eur Radiol* 2004;14 Suppl 8:P116-124
 37. Tang MX, Eckersley RJ, Noble JA. Pressure-dependent attenuation with microbubbles at low mechanical index. *Ultrasound Med Biol* 2005;31:377-384
 38. Chen Q, Zagzebski J, Wilson T, Stiles T. Pressure-dependent attenuation in ultrasound contrast agents. *Ultrasound Med Biol* 2002;28:1041-1051