BMC Medical Imaging

STUDY PROTOCOL

Open Access

Ultrasound localization microscopy in the diagnosis of breast tumors and prediction of relevant histologic biomarkers associated with prognosis in humans: the protocol for a prospective, multicenter study

Jia Li[1], Lei Chen[1], Ronghui Wang[2], Jiang Zhu[3,4], Ao Li[5], Jianchun Li[6], Zhaojun Li[7], Wen Luo[8], Wenkun Bai[1], Tao Ying[1], Cong Wei[1*], Di Sun[1*] and Yuanyi Zheng[1*]

Abstract

Background Benign and malignant breast tumors differ in their microvasculature morphology and distribution. Histologic biomarkers of malignant breast tumors are also correlated with the microvasculature. There is a lack of imaging technology for evaluating the microvasculature. Ultrasound localization microscopy (ULM) can provide detailed microvascular architecture at super-resolution. The objective of this trial is to explore the role of ULM in distinguishing benign from malignant breast tumors and to explore the correlations between ULM qualitative and quantitative parameters and histologic biomarkers in malignant breast tumors.

Methods/design This prospective and multicenter study will include 83 patients with breast tumors that will undergo ULM. 55 patients will be assigned to the malignant group, and 28 patients will be assigned to the benign group. The primary outcome is the differences in the qualitative parameters (microvasculature morphology, distribution, and flow direction) between benign and malignant breast tumors on ULM. Secondary outcomes include (1) differences in the quantitative parameters (microvasculature density, tortuosity, diameter, and flow velocity) between benign and malignant breast tumors based on ULM; (2) diagnostic performance of the qualitative parameters in distinguishing benign and malignant breast tumors; (3) diagnostic performance of the quantitative parameters in distinguishing benign and malignant breast tumors; (4) relationships between the qualitative parameters and histologic biomarkers in malignant breast tumors; (5) relationships between the quantitative parameters and histologic biomarkers in malignant breast tumors; and (6) the evaluation of inter-reader and intra-reader reproducibility.

*Correspondence:
Cong Wei
weicong1985@163.com
Di Sun
Sundy316@163.com
Yuanyi Zheng
zhengyuanyi@163.com

Full list of author information is available at the end of the article

Discussion Detecting vascularity in breast tumors is of great significance to differentiate benign from malignant tumors and to predict histologic biomarkers. These histologic biomarkers, such as ER, PR, HER2 and Ki67, are closely related to prognosis evaluation. This trial will provide maximum information about the microvasculature of breast tumors and thereby will help with the formulation of subsequent differential diagnosis and the prediction of histologic biomarkers.

Trial registration number/date Chinese Clinical Trial Registry ChiCTR2100048361/6th/July/2021. This study is a part of that clinical trial.

Keywords Breast tumors, Ultrasound localization microscopy, Microvasculature, Histologic biomarkers

Background

Breast cancer is the most frequent malignant disease and one of the main causes of mortality in women worldwide. Pathological angiogenesis is a hallmark feature of solid tumors [1]. Microvasculature density and distribution are highly correlated with tumor invasion, metastasis, and prognosis [2, 3]. Histologic biomarkers of breast tumors, such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor 2 (HER2) and Ki67, are prognostic factors. ER-negative or Ki67-positive breast cancer have high microvasculature density [4]. In addition, microvascular morphologic features are helpful for the differentiation of breast tumors [5]. The vessels in benign tumors are natural and straight, whereas vessels in malignant tumors are irregular and tortuous [6]. Thus, it is important to evaluate the microvasculature of breast tumors to differentiate benign from malignant tumors and predict histologic biomarkers.

To detect tumor vessels, various noninvasive imaging modalities, such as magnetic resonance imaging (MRI) and ultrasound (US), are employed in clinical practice. Although MRI, with high sensitivity and deep penetration, can evaluate the perfusion and vascularization of tissues, its spatial resolution is limited to the submillimeter or millimeter scale [7]. Therefore, the microvascular architecture of tumors cannot be depicted on MRI. US has been a popular vasculature visualizing tool for many years. US can provide many approaches to detect tumor vessels, especially contrast-enhanced US (CEUS), which generates marked amplification of the flow signals by microbubble (MB) contrast agents [8] and has superior capabilities in the visualization of the microvasculature. Reportedly, the axial resolution of CEUS is approximately 150 μm, and the lateral resolution is approximately 200 μm at a depth of 5 mm [9]. However, its resolution is still confined by the diffraction limit [10], which hinders the application of CEUS to assess microvasculature. It turns out that comprehensive and detailed microvascular architecture is difficult to obtain.

Recently, ultrasound localization microscopy (ULM), also known as ultrasound super-resolution microcirculation imaging (USRmi) and super-resolution ultrasound (SRUS) imaging, is an advanced tracking technique that reconstructs microvasculature images in tissues by precisely locating isolated MB contrast agents and tracking their displacements from a series of CEUS images [11]. As an emerging technology, ULM breaks the diffraction limit of imaging systems [12, 13] and has a resolution of blood vessels up to 10 μm [13]. Therefore, it can improve the ability to obtain highly detailed images of the microvasculature. Furthermore, ULM can offer qualitative and quantitative parameters [14], especially some parameters (e.g., blood flow direction and velocity) that are inaccessible by CEUS. Therefore, ULM demonstrates great advantages in visualizing the microvasculature.

To date, the microvasculature imaging of ULM has been tested successfully in various tissues of animal models, including tumor, skeletal muscle, lymph node, kidney, brain, ear, and atherosclerotic plaque [10, 14–26]. For human studies, ULM has been preliminarily applied in imaging the microvasculature of breast tumor, lower limb, prostate cancer, healthy liver, healthy kidney, and pancreatic tumor [12, 14, 27–29]. For breast tumors, Dencks et al. [12] mapped the microvasculature in 1 case of breast cancer and extracted relevant parameters, including relative blood volume, flow velocity, and flow direction. However, the vessel trees were imaged incompletely, which may affect relevant parameters. Opacic et al. [14] imaged the microvasculature in 3 cases of breast cancers and acquired relevant parameters, including relative blood volume, flow distribution, flow velocity, and flow direction. Similar to the previous study, the problem is that vessel trees may also be imaged incompletely. Huang et al. imaged [27] the microvasculature in 1 case of breast cancer and obtained relevant parameters, such as flow velocity and flow direction. However, the patient had been treated with chemotherapy before ULM, so the results could not provide a reference for preoperative diagnosis and differential diagnosis.

The above breast tumor studies only demonstrate the proof of concept, but few studies have explored their application to benign breast tumors. Thus, it is still unclear whether ULM can differentiate benign and malignant breast tumors. In addition, the relationships between ULM parameters and histologic markers have not been evaluated.

We hypothesize that ULM would be helpful to differentiate breast tumors and predict relevant histologic biomarkers associated with prognosis. The objectives of this multicenter study are to explore the role of ULM in distinguishing benign from malignant breast tumors and to explore the correlations between ULM qualitative and quantitative parameters and histologic biomarkers in malignant breast tumors.

Methods/design

Study design

The protocol for analyzing breast tumors with ULM is a prospective, multicenter study conducted by Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, and the study is led in 15 municipal tertiary hospitals nationally. This trial follows the STARD-2015 [30], and Additional file 1 is the STARD checklist.

Patient and public involvement

Patients were not invited to participate in the design, recruitment, conduct of the protocol, or to write or edit the manuscript. The ultimate outcomes will be communicated to the public by mass media. At the end of the published papers and conference presentations, all of patients as a whole will be acknowledged.

Patient recruitment

Figure 1 presents the patient flow diagram of this study. Patients undergoing CEUS of breast tumors in each center will be recruited. Pathological examination is considered the gold standard in distinguishing benign and malignant tumors.

Grayscale US will be performed initially to scan the breast tumors. Then, color Doppler flow imaging (CDFI) will be conducted in different planes to evaluate intra- and extra-tumoral vascularity. The section with the richest vessels is selected for CEUS according to CDFI.

Medical evaluation and enrollment procedure

Inclusion criteria

► Age ≥ 18 years old and ≤ 75 years old, female;
► Isolated microbubbles in tumors;
► CEUS and histological results of breast tumors;
► Recently diagnosed (within 3 months) with breast tumors based on clinical or radiological findings.

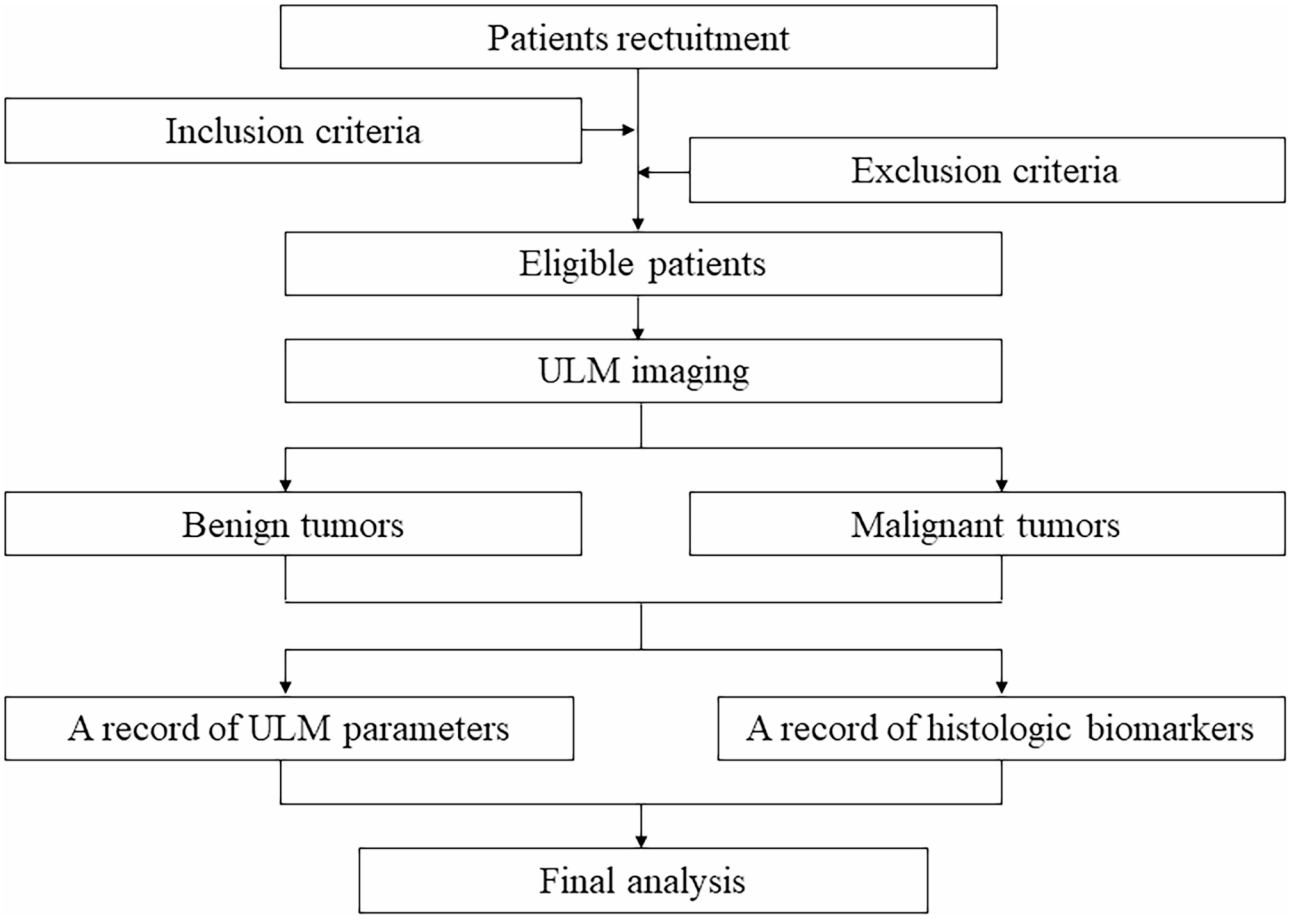

Fig. 1 Patient flow diagram

Table 1 ULM qualitative and quantitative parameters

Parameters	Variables
Qualitative parameters	microvasculature morphology (dot-like, line-like or branch-like patterns)
	microvasculature distribution (central or peripheral)
	microvasculature flow direction (toward the transducer or away from the transducer)
Quantitative parameters	microvasculature density
	microvasculature tortuosity (max tortuosity and mean tortuosity)
	microvasculature diameter (max diameter and mean diameter)
	microvasculature flow velocity (max flow velocity and mean flow velocity)

Table 2 Study evaluation procedures and timeline

Study procedure	Medical evaluation	Enrolment visit
Determine eligibility	√	
Obtain signed consent		√
Baseline characteristic		√
Machine parameters		√
Outcome measures of ULM		
Qualitative parameters		√
Quantitative parameters		√

Exclusion criteria

► Poor quality of dynamic CEUS images;

► Large tissue motions;

► Low frame rate in contrast mode;

► The time interval between CEUS and histological results is more than 3 months.

Blind reviews

The investigators will be blinded to the results of the pathology, clinical histology, and other examinations before ULM. As such, the pathologist will be blinded to the results of clinical histology and other examinations.

ULM imaging and observations

MATLAB R2021a will be used to postprocess data from the CEUS video clips of breast tumors in a personal computer. A series of ULM images, super-resolution microbubble density map, density map with directions, flow velocity magnitude map, and flow angle map, will be generated, and the qualitative and quantitative parameters will be extracted based on the ULM images. Qualitative parameters such as microvasculature morphology, distribution, and flow direction will be assessed. The microvasculature morphology will be divided into dot-like, line-like or branch-like patterns and chaotic patterns. The microvasculature distribution will be divided into central or peripheral or both. The microvasculature flow direction will be divided into toward the transducer or away from the transducer. Quantitative parameters, such as microvasculature density, tortuosity, diameter, and flow velocity, will be assessed. The microvasculature tortuosity will be divided into max tortuosity and mean tortuosity. The microvasculature diameter will be divided into max diameter and mean diameter. The microvasculature flow velocity will be divided into max flow velocity and mean flow velocity. Table 1 shows ULM qualitative and quantitative parameters.

To evaluate inter-reader reproducibility, two readers will individually read the ULM images and record the results. As such, to evaluate intra-reader reproducibility, the principal reader will read the ULM images again 1 month after the initial read.

Histologic analysis

Malignant breast tumors will be subjected to immunohistochemical tests. The biomarkers comprise ER, PR, HER2, and Ki67. The conditions of positivity are as follows: a score more than 2 points for ER and PR; membrane 3+immunohistochemistry, or membrane 2+HER2 gene amplification visualized on sliver in-situ hybridization for HER2 staining; 14% for Ki67 expression [4, 31].

Data management

The associated data will be collected at the time of inclusion, including baseline characteristics of the patients, machine parameters of CEUS, and ULM qualitative and quantitative parameters. Table 2 shows study evaluation procedures and timeline.

Outcome measurement

Primary outcome

Differences in the qualitative parameters between benign and malignant breast tumors based on ULM.

Secondary outcome

► Differences in the quantitative parameters between benign and malignant breast tumors based on ULM.

► Diagnostic performance of the qualitative parameters in distinguishing benign and malignant breast tumors, evaluated using the area under the curve (AUC), sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV).

► Diagnostic performance of the quantitative parameters in distinguishing benign and malignant breast tumors, evaluated using the AUC, sensitivity, specificity, accuracy, PPV, and NPV. The optimal cut-off values of the quantitative parameters will be analyzed by receiver operating characteristic (ROC) curves.

► Relationships between the qualitative parameters and histologic biomarkers in malignant breast tumors.

► Relationships between the quantitative parameters and histologic biomarkers in malignant breast tumors.

► The evaluation of inter-reader and intra-reader reproducibility.

Premature ending of patient participation

Patients will be excluded from the research if they have one of the following conditions:

► Withdrawal of informed consent before the end of the research;

► Discovery of any exclusion criteria after registration.

These patients will be promptly excluded from the research and replaced by new patients. Meanwhile, the reason why the patient is excluded and the corresponding date will be recorded. Withdrawal of informed consent will not influence the patient's medical treatment.

Follow-up

No specific follow-up will be carried out for the patients during and after the research.

Sample size calculation

Because this is the first study to distinguish benign and malignant breast tumors by using ULM, there is no available literature for sample size consideration. Hence, the sensitivity (0.9) and specificity (0.83) to differentiate benign from malignant breast tumors using CEUS in a meta-analysis were used for sample size determination [32]. Sample size was calculated by PASS software. The estimated sample size assumes a two-sided α of 5% and a power of 90%. 75 patients will be included in this study. Considering a 10% drop-out rate, 83 patients will be finally enrolled in the study. Since the number of patients with malignant tumors is larger than that of patients with benign tumors, the patients will be divided into a malignant group and a benign group at a ratio of 2:1. In the end, 55 patients and 28 patients will be included in the malignant group and the benign group, respectively.

Quality assurance/monitoring/management

To clarify the responsibilities of all parties, the principal investigator shall play the role of management and monitoring, and each center will strictly follow the research plan to ensure the quality control of the clinical research and the implementation of the quality assurance system. In this clinical research, quality control will be carried out at each stage of data processing to ensure data integrity, accuracy, authenticity and reliability.

The collected data will be kept anonymous and stored in an electronic database. The information that can identify participants will not be disclosed to members outside the research team unless permission is obtained from the participants. All study members are required to keep the identities of the participants confidential. The identities of the research participants will be kept in locked- filling cabinets for researcher access only. To ensure that the research is carried out in accordance with the regulations, if necessary, the members of the government management department or the ethics review committee can access the participant's personal data according to regulations. When our results are published, any information about the participants will not be disclosed.

Statistical analysis

Normal distributions of the data will be verified using the Kolmogorov-Smirnov analysis. Continuous variables will be reported as the means ± standard deviations or median (P25, P75) according to the type of data. Categorical variables will be reported as frequencies and percentages. Student's *t* test or Mann-Whitney *U* test will be performed to compare the quantitative parameters according to the type of data and to evaluate the correlations between quantitative parameters and histologic biomarkers. The Pearson $\chi 2$ test or Fisher's exact test will be performed to compare the qualitative parameters and to compare the correlations between the qualitative parameters and histologic biomarkers. A ROC curve will be applied to assess the diagnostic performance of the qualitative and quantitative parameters. Sensitivity, specificity, accuracy, PPV, and NPV will be computed. We will determine the optimal cut-off values of the quantitative parameters by ROC curves. Inter-reader and intra-reader reproducibility will be assessed by the intraclass correlation coefficient accompanied by 95% confidence intervals (CI). A *P* value lower than 0.05 will be considered statistically significant. Statistical analysis will be processed using SPSS software and MedCalc software.

Discussion

Detecting vascularity in breast tumors is of great significance to differentiate benign from malignant tumors and to predict histologic biomarkers. However, the capacity of various imaging modalities in evaluating the microvasculature is limited. That is why other techniques are needed. Recently, ULM has been proposed because of unprecedented resolution in imaging microvasculature.

In this prospective and multicenter study, ULM will be used to provide multiple qualitative and quantitative parameters, such as microvasculature morphology, distribution, flow direction, density, tortuosity (max tortuosity and mean tortuosity), diameter (max diameter and mean diameter), and flow velocity (max flow velocity and mean flow velocity). The primary aim of this study is to explore the differences in the qualitative parameters between benign and malignant breast tumors based on ULM. Quantitative parameters will be recognized as auxiliary indicators. Compared to other imaging techniques, ULM can image the microvasculature at super-resolution [19, 33]. Moreover, ULM can directly generate quantitative parameters instead of measuring them indirectly [11]. It is worth mentioning that microvasculature flow direction (toward the transducer or away from the transducer) is a new indicator for assessing breast tumors in our trial. Until now, its value in breast tumors has remained unclear. To the best of our knowledge, this is the first study to investigate the role of flow direction in differentiating benign from malignant tumors. In the field of US, CDFI is a routine method to evaluate flow direction. ULM has some advantages over CDFI. First, ULM not only has high sensitivity in the microvasculature but also intuitively observes the flow directions of all microvasculatures on a velocity map. Second, this technology is angle-independent. Therefore, it will help to obtain more information about flow direction with ULM.

This trial will also assess the relationships between qualitative and quantitative parameters based on ULM and histologic biomarkers in malignant breast tumors. These histologic biomarkers, such as ER, PR, HER2 and Ki67, are closely related to prognosis evaluation. Previous studies have assessed the associations between histologic biomarkers and US characteristics. However, the results have some overlaps. For example, perfusion defects on CEUS are often correlated with ER-negative, HER2-positive and Ki67-positive breast tumors [34, 35]. The retraction pattern in the coronal plane of three-dimensional US is correlated with ER-positive and PR-positive breast tumors [36]. As a new technology, ULM is able to offer detailed information on microvasculature compared to conventional US imaging techniques [13, 37], thus, it has the potential to be helpful for predicting the prognosis of malignant breast tumors.

This trial will have one limitation: ULM has high requirements for CEUS video clips, which relies on the operators of CEUS. This may lead to bias. Nevertheless, unified training will be given to the operators to reduce bias. The principal investigator will standardize the types of contrast agents, dosages, injection speeds, and ultrasound parameters, and provide detailed video tutorials to ensure consistency and high quality of operations across all centers. They will establish image evaluation standards, regularly assess the image quality collected by each center, and provide detailed improvement suggestions based on the evaluation results.

For our primary and secondary outcomes, we anticipate that our study results will provide maximum information about the microvasculature of breast tumors and thereby will help with the formulation of subsequent differential diagnosis and the prediction of histologic biomarkers.

Abbreviations

ULM	Ultrasound localization microscopy
USRmi	Ultrasound super-resolution microcirculation imaging
ER	Estrogen receptor
PR	Progesterone receptor
HER2	Human epidermal growth factor 2
CT	Computed tomography
MRI	Magnetic resonance imaging
US	Ultrasound
CEUS	Contrast-enhanced ultrasound
MB	Microbubble
AUC	Area under the curve
PPV	Positive predictive value
NPV	Negative predictive value
ROC	Receiver operating characteristic
CI	Confidence intervals

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12880-024-01535-7.

Supplementary Material 1: **Additional file 1**: STARD checklist.

Acknowledgements

The authors thank all the patients who will participate in this trial.

Author contributions

Jia Li is the primary investigator. Jia Li, Cong Wei, Di Sun, Yuanyi Zheng Participated in the development of the study design. Jia Li, Lei Chen, Ronghui Wang, Jiang Zhu, Ao Li, Jianchun Li, Zhaojun Li, Wen Luo, Wenkun Bai, Tao Ying, Cong Wei, Di Sun, Yuanyi Zheng participated in the study conduct. Jia Li drafted the manuscript under Cong Wei, Di Sun, Yuanyi Zheng supervision. Yuanyi Zheng contributed to applying for and gaining funding. All authors contributed to the content and critical revision and approved the final draft of the manuscript.

Funding

Key Project of the National Natural Science Foundation of China (No. 82030050, T2394534). The funding bodies played no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Data availability
The datasets generated and/or analyzed during the current study and the full protocol will be available from the corresponding author on reasonable request after study completion, except for the patients' personal information.

Declarations

Ethics approval and consent to participate
This trial was validated by the Ethics Committee of Shanghai Sixth People's Hospital (lead Clinical Centre No. 2021 − 138). Written informed consent will be signed by every patient. The patients have the right to withdraw from the trial at any time. All clinical investigations will be conducted in accordance with the guidelines of the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Trial status
The trial began in July 2021 and was completed in June 2024. Recruitment began in August 2021 and ended in May 2024.

Author details
[1]Department of Ultrasound in Medicine, Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200233, China
[2]Department of Ultrasound, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China
[3]Department of Ultrasound, Women's Hospital, Zhejiang University School of Medicine, Hangzhou 310006, China
[4]Zhejiang Provincial Key Laboratory of Precision Diagnosis and Therapy for Major Gynecological Diseases, Women's Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310006, China
[5]Department of Ultrasound, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China
[6]Cancer Center, Department of Ultrasound Medicine, Zhejiang Provincial People's Hospital, Affiliated People's Hospital, Hangzhou Medical College, Hangzhou, Zhejiang 310014, China
[7]Department of Ultrasound, Shanghai General Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200080, China
[8]Department of Ultrasound, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China

Received: 17 December 2022 / Accepted: 16 December 2024
Published online: 08 January 2025

References

1. Folkman J, Angiogenesis. Annu Rev Med. 2006;57:1–18. https://doi.org/10.1146/annurev.med.57.121304.131306
2. Ruoslahti E. Specialization of tumour vasculature. Nat Rev Cancer. 2002;2:83–90. https://doi.org/10.1038/nrc724
3. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature. 2011;473:298–307. https://doi.org/10.1038/nature10144
4. Park AY, Kwon M, Woo OH, et al. A Prospective Study on the Value of Ultrasound Microflow Assessment to Distinguish Malignant from Benign Solid Breast Masses: Association between Ultrasound Parameters and Histologic Microvessel Densities. Korean J Radiol. 2019;20:759–72. https://doi.org/10.3348/kjr.2018.0515
5. Gong P, Song P, Huang C, et al. Ultrasensitive Ultrasound Microvessel Imaging for Characterizing Benign and Malignant Breast Tumors. Ultrasound Med Biol. 2019;45:3128–36. https://doi.org/10.1016/j.ultrasmedbio.2019.08.009
6. Xiao XY, Chen X, Guan XF, Wu H, Qin W, Luo BM. Superb microvascular imaging in diagnosis of breast lesions: a comparative study with contrast-enhanced ultrasonographic microvascular imaging. Br J Radiol. 2016;89:20160546. https://doi.org/10.1259/bjr.20160546
7. Lin E, Alessio A. What are the basic concepts of temporal, contrast, and spatial resolution in cardiac CT? J Cardiovasc Comput Tomogr. 2009;3:403–8. https://doi.org/10.1016/j.jcct.2009.07.003
8. Chung YE, Kim KW. Contrast-enhanced ultrasonography: advance and current status in abdominal imaging. Ultrasonography. 2015;34:3–18. https://doi.org/10.14366/usg.14034
9. Gessner RC, Frederick CB, Foster FS, Dayton PA. Acoustic angiography: a new imaging modality for assessing microvasculature architecture. Int J Biomed Imaging. 2013;2013:936593. https://doi.org/10.1155/2013/936593
10. van Sloun RJG, Solomon O, Bruce M, et al. Super-Resolution Ultrasound Localization Microscopy Through Deep Learning. IEEE Trans Med Imaging. 2021;40:829–39. https://doi.org/10.1109/TMI.2020.3037790
11. Christensen-Jeffries K, Couture O, Dayton PA, et al. Super-resolution Ultrasound Imaging. Ultrasound Med Biol. 2020;46:865–91. https://doi.org/10.1016/j.ultrasmedbio.2019.11.013
12. Dencks S, Piepenbrock M, Opacic T, et al. Clinical Pilot Application of Super-Resolution US Imaging in Breast Cancer. IEEE Trans Ultrason Ferroelectr Freq Control. 2019;66:517–26. https://doi.org/10.1109/TUFFC.2018.2872067
13. Couture O, Hingot V, Heiles B, Muleki-Seya P, Tanter M. Ultrasound Localization Microscopy and Super-Resolution: A State of the Art. IEEE Trans Ultrason Ferroelectr Freq Control. 2018;65:1304–20. https://doi.org/10.1109/TUFFC.2018.2850811
14. Opacic T, Dencks S, Theek B, et al. Motion model ultrasound localization microscopy for preclinical and clinical multiparametric tumor characterization. Nat Commun. 2018;9:1527. https://doi.org/10.1038/s41467-018-03973-8
15. Lin F, Shelton SE, Espíndola D, Rojas JD, Pinton G, Dayton PA. 3-D Ultrasound Localization Microscopy for Identifying Microvascular Morphology Features of Tumor Angiogenesis at a Resolution Beyond the Diffraction Limit of Conventional Ultrasound. Theranostics. 2017;7:196–204. https://doi.org/10.7150/thno.16899
16. Ghosh D, Peng J, Brown K, et al. Super-Resolution Ultrasound Imaging of Skeletal Muscle Microvascular Dysfunction in an Animal Model of Type 2 Diabetes. J Ultrasound Med. 2019;38:2589–99. https://doi.org/10.1002/jum.14956
17. Zhu J, Rowland EM, Harput S, et al. 3D Super-Resolution US Imaging of Rabbit Lymph Node Vasculature in Vivo by Using Microbubbles. Radiology. 2019;291:642–50. https://doi.org/10.1148/radiol.2019182593
18. Foiret J, Zhang H, Ilovitsh T, Mahakian L, Tam S, Ferrara KW. Ultrasound localization microscopy to image and assess microvasculature in a rat kidney. Sci Rep. 2017;7:13662. https://doi.org/10.1038/s41598-017-13676-7
19. Errico C, Pierre J, Pezet S, et al. Ultrafast ultrasound localization microscopy for deep super-resolution vascular imaging. Nature. 2015;527:499–502. https://doi.org/10.1038/nature16066
20. Christensen-Jeffries K, Browning RJ, Tang MX, Dunsby C, Eckersley RJ. In vivo acoustic super-resolution and super-resolved velocity mapping using microbubbles. IEEE Trans Med Imaging. 2015;34:433–40. https://doi.org/10.1109/TMI.2014.2359650
21. Yu J, Lavery L, Kim K. Super-resolution ultrasound imaging method for microvasculature in vivo with a high temporal accuracy. Sci Rep. 2018;8:13918. https://doi.org/10.1038/s41598-018-32235-2
22. Kierski TM, Espindola D, Newsome IG, et al. Superharmonic Ultrasound for Motion-Independent Localization Microscopy: Applications to Microvascular Imaging From Low to High Flow Rates. IEEE Trans Ultrason Ferroelectr Freq Control. 2020;67:957–67. https://doi.org/10.1109/TUFFC.2020.2965767
23. Liu X, Zhou T, Lu M, Yang Y, He Q, Luo J. Deep Learning for Ultrasound Localization Microscopy. IEEE Trans Med Imaging. 2020;39:3064–78. https://doi.org/10.1109/TMI.2020.2986781
24. Song P, Trzasko JD, Manduca A, et al. Improved Super-Resolution Ultrasound Microvessel Imaging With Spatiotemporal Nonlocal Means Filtering and Bipartite Graph-Based Microbubble Tracking. IEEE Trans Ultrason Ferroelectr Freq Control. 2018;65:149–67. https://doi.org/10.1109/TUFFC.2017.2778941
25. Huang C, Lowerison MR, Trzasko JD, et al. Short Acquisition Time Super-Resolution Ultrasound Microvessel Imaging via Microbubble Separation. Sci Rep. 2020;10:6007. https://doi.org/10.1038/s41598-020-62898-9
26. Lowerison MR, Huang CW, Lucien F, Chen SG, Song PF. Ultrasound localization microscopy of renal tumor xenografts in chicken embryo is correlated to hypoxia. Sci Rep. 2020;10:2478. https://doi.org/10.1038/s41598-020-59338-z
27. Huang C, Zhang W, Gong P, et al. Super-resolution ultrasound localization microscopy based on a high frame-rate clinical ultrasound scanner: an in-human feasibility study. Phys Med Biol. 2021;66. https://doi.org/10.1088/1361-6560/abef45

28. Harput S, Christensen-Jeffries K, Brown J, et al. Two-Stage Motion Correction for Super-Resolution Ultrasound Imaging in Human Lower Limb. IEEE Trans Ultrason Ferroelectr Freq Control. 2018;65:803–14. https://doi.org/10.1109/TUFFC.2018.2824846
29. Kanoulas E, Butler M, Rowley C, et al. Super-Resolution Contrast-Enhanced Ultrasound Methodology for the Identification of In Vivo Vascular Dynamics in 2D. Invest Radiol. 2019;54:500–16. https://doi.org/10.1097/RLI.0000000000000565
30. Bossuyt PM, Reitsma JB, Bruns DE, et al. STARD 2015: An Updated List of Essential Items for Reporting Diagnostic Accuracy Studies. Clin Chem. 2015;61:1446–52. https://doi.org/10.1373/clinchem.2015.246280
31. Hammond ME, Hayes DF, Dowsett M, et al. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Immunohistochemical Testing of Estrogen and Progesterone Receptors in Breast Cancer. Arch Pathol Lab Med. 2010;134:907–22. https://doi.org/10.1200/JOP.777003
32. Lu J, Zhou P, Jin C, et al. Diagnostic Value of Contrast-Enhanced Ultrasonography With SonoVue in the Differentiation of Benign and Malignant Breast Lesions: A Meta-Analysis. Technol Cancer Res Treat. 2020;19:1533033820971583. https://doi.org/10.1177/1533033820971583
33. Chen Q, Yu J, Rush BM, Stocker SD, Tan RJ, Kim K. Ultrasound super-resolution imaging provides a noninvasive assessment of renal microvasculature changes during mouse acute kidney injury. Kidney Int. 2020;98:355–65. https://doi.org/10.1016/j.kint.2020.02.011
34. Li X, Li Y, Zhu Y, Fu L, Liu P. Association between enhancement patterns and parameters of contrast-enhanced ultrasound and microvessel distribution in breast cancer. Oncol Lett. 2018;15:5643–9. https://doi.org/10.3892/ol.2018.8078
35. Wan CF, Du J, Fang H, Li FH, Zhu JS, Liu Q. Enhancement Patterns and Parameters of Breast Cancers at Contrast-enhanced US: Correlation with Prognostic Factors. Radiology. 2012;262:450–9. https://doi.org/10.1148/radiol.11110789
36. Jiang J, Chen YQ, Xu YZ, et al. Correlation between three-dimensional ultrasound features and pathological prognostic factors in breast cancer. Eur Radiol. 2014;24:1186–96. https://doi.org/10.1007/s00330-014-3135-8
37. Andersen SB, Taghavi I, Hoyos CAV, et al. Super-Resolution Imaging with Ultrasound for Visualization of the Renal Microvasculature in Rats Before and After Renal Ischemia: A Pilot Study. Diagnostics (Basel). 2020;10:862. https://doi.org/10.3390/diagnostics10110862

Publisher's note