

# Breast Cancer Detection by B7-H3-Targeted Ultrasound Molecular Imaging

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## Abstract

Ultrasound complements mammography as an imaging modality for breast cancer detection, especially in patients with dense breast tissue, but its utility is limited by low diagnostic accuracy. One emerging molecular tool to address this limitation involves contrast-enhanced ultrasound using microbubbles targeted to molecular signatures on tumor neovasculature. In this study, we illustrate how tumor vascular expression of B7-H3 (CD276), a member of the B7 family of ligands for T-cell coregulatory receptors, can be incorporated into an ultrasound method that can distinguish normal, benign, precursor, and malignant breast pathologies for diagnostic purposes. Through an IHC analysis of 248 human breast specimens, we found that

vascular expression of B7-H3 was selectively and significantly higher in breast cancer tissues. B7-H3 immunostaining on blood vessels distinguished benign/precursors from malignant lesions with high diagnostic accuracy in human specimens. In a transgenic mouse model of cancer, the B7-H3-targeted ultrasound imaging signal was increased significantly in breast cancer tissues and highly correlated with *ex vivo* expression levels of B7-H3 on quantitative immunofluorescence. Our findings offer a preclinical proof of concept for the use of B7-H3-targeted ultrasound molecular imaging as a tool to improve the diagnostic accuracy of breast cancer detection in patients. *Cancer Res*; 75(12); 2501–9. ©2015 AACR.

## Introduction

Breast cancer is the second leading cause of cancer-related deaths in women in the United States, with an estimated 232,670 new diagnoses and 40,000 deaths from this cancer in 2014 (1). If detected early, survival of women with breast cancer can be substantially increased compared with detection at later stages. The 5-year survival rate in patients diagnosed with stage I and II disease is 100% and 98.5% compared with 84.6% and 25.0%, respectively, when detected at stage III and IV disease (1). Next to breast self-exam and clinical breast exam, the American Cancer Society recommends mammography as a screening exam in women ages 40 years and older (2). For high-risk women, mammography is recommended at age 30 years (2).

However, presence of dense or heterogeneously dense breast tissue, which is particularly prevalent in younger patients (3), may decrease diagnostic accuracy of mammography in detecting breast cancer, with sensitivities ranging between 30% and 55%

(4–6). Adding ultrasound to screening mammography is currently being explored as a complementary screening approach for earlier breast cancer detection in women with dense breast tissue (7). Several studies have addressed the value of adding breast ultrasound imaging to screening mammography and demonstrated an increase in cancer detection rates ranging from 0.3 to 7.7 cancers per 1,000 women screened (6–11). Berg and colleagues showed that breast cancer was diagnosed on ultrasound alone in 12 of 40 patients (30%; ref. 11). However, the diagnostic accuracy of current ultrasound screening techniques in breast cancer detection is low with a positive predictive value as low as 8.6% (11) or even lower 5.6% in another study (12), resulting in a large number of unnecessary callbacks and biopsies. In addition, the sensitivity of ultrasound performed alone in detecting invasive breast cancer was only 50% (11) and 27% in another prospective multimodality screening study (13). Therefore, further improvement of the diagnostic accuracy of ultrasound imaging is critically needed for women enrolled in breast cancer screening.

Molecularly targeted contrast-enhanced ultrasound imaging is an emerging imaging strategy with large potential for improving diagnostic accuracy of conventional ultrasound imaging in earlier cancer detection (14–16). Ultrasound contrast agents are gas-filled echogenic microbubbles that can be further modified by adding binding ligands to the microbubble shell, which makes them firmly attach to molecular markers (17, 18). Because microbubbles are several micrometers in size, they remain exclusively within the vascular compartment (18). This property of a purely intravascular contrast agent makes them particularly well suited for visualizing molecular markers expressed on the tumor neovasculature in various cancers, including breast cancer (16, 19). To achieve both high sensitivity and specificity in detecting breast cancer with ultrasound,

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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**doi:** 10.1158/0008-5472.CAN-14-3361

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it is of paramount importance to identify molecular markers as potential molecular imaging targets that are differentially expressed on the neovasculature of cancer compared with normal tissue, benign, and precursor breast lesions. Extensive research is under way aimed at identifying such cancer-specific vascular markers using various discovery techniques for both imaging and therapeutic purposes (20).

Using a serial analysis of gene expression technique on isolated vascular endothelial cells, the transmembrane protein B7-H3, also known as CD276, was discovered as a novel tumor neovasculature-associated marker differentially expressed in murine and human colon, breast, and lung cancer xenografts grown in mice (21). Recently, the B7-H3 protein was shown to be expressed in human breast cancer tissues (22). However, it is not known whether B7-H3 is differentially expressed on the neovasculature of breast cancer compared with benign, or precursor breast pathologies and normal breast tissue, which would make B7-H3 an attractive novel molecular imaging target for breast cancer detection using ultrasound.

The purpose of our study was twofold (Fig. 1): First, to evaluate B7-H3 expression on the tumor neovasculature of breast cancer versus normal tissue, benign, and precursor breast lesions in a large-scale human IHC analysis study and, second, to assess feasibility of ultrasound molecular imaging using new B7-H3-targeted contrast microbubbles for breast cancer detection in a genetically engineered mouse model.

## Materials and Methods

Figure 1 summarizes the overall study design.

### Collection of human breast tissues

Human breast tissue samples were obtained retrospectively and were selected under an HIPAA compliant, Institutional Review Board-approved protocol to represent a range of normal tissue, benign and precursor lesions, and cancer tissues. A total of 248 samples were obtained, including 101 breast cancer samples, 100 benign or precursor pathologies, and 47 normal breast tissues (Table 1). Two hundred and nine samples were processed into a breast tissue microarray (TMA) using standardized protocols (23). In brief, TMA cases were constructed from patient resection (surgical) tissues after characterization by a dedicated breast pathologist. Lesional areas were circled and 0.6 mm blocks were cored out from formalin-fixed paraffin-embedded tissue blocks by using a Beecher Tissue Microarrayer, and then slotted in a regular grid pattern into a blank recipient paraffin wax block. Thirty-nine whole-tissue samples of breast cancer were obtained from diagnostic large core needle biopsies. In these 39 whole-tissue cancer samples, benign tissues adjacent to breast cancer were used as intra-individual benign control tissues.

### IHC staining and analysis of B7-H3 expression in human breast tissue samples

IHC was performed on standard serial 5  $\mu$ m sections of paraffin-embedded breast tissues using the Leica Bond Max automated platform (Leica Microsystems Inc.). This platform was used in conjunction with a heat-induced epitope retrieval program using an epitope retrieval solution (2, ER2; Leica Microsystems Inc.) at pH 9.0. Antibodies to both human CD31 (clone JC70A at a 1:150 dilution; to confirm presence on tumor vessels) and to human B7-H3 (AF1027, at 1:200 dilution; R&D systems)

were used on the same automated platform. Slides were imaged using a digital slide scanner (Nanozoomer). All immunohistochemically stained sections were analyzed by a dedicated breast pathologist. B7-H3 expression on tumor-associated vascular endothelial cells was analyzed using adjacent CD31-stained slices for anatomical guidance to determine presence of tumor vessels. Immunostaining of vessels was scored using a 4-point grading scale: 0 = no staining; 1 = weak; 2 = moderate; and 3 = strong vessel staining. Vessel staining was further analyzed for percentage positive vessels using a 5-point grading scale: 0 = no positive staining vessels; 1 = 1%–10%; 2 = 10%–33%; 3 = 33%–66%; and 4 = 66%–100% of positive staining vessels. The results obtained by these two scores were then multiplied together yielding a single value as described (24). In addition, microvessel density (MVD) was calculated on all sections using standard techniques (25).

### Cell culture experiments

Wild-type MS1 (MS1<sub>wt</sub>; ATCC) vascular endothelial cells were transfected with B7-H3 expression vector using Lipofectamine 2000 to generate stable MS1 clones (MS1<sub>B7-H3</sub>) and were maintained in culture under sterile conditions in a 5% CO<sub>2</sub>-humidified atmosphere at 37°C in DMEM and supplemented with 10% FBS and 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. Cells were harvested by using trypsinization at 70% to 80% confluence. Routine morphologic analysis under microscope and growth curve analysis were performed to ensure consistent growth properties and authentication according to the ATCC cell line verification test recommendations. The expression of B7-H3 in transfected cells was tested by immunofluorescence imaging with anti-B7-H3 antibody.

### Preparation of targeted and control microbubbles

Commercially available streptavidin-coated microbubbles (VisualSonics) were used to generate B7-H3-targeted microbubbles (MB<sub>B7-H3</sub>) and control microbubbles (MB<sub>Control</sub>). For further details, please refer to Supplementary Methods.

### Flow chamber experiments

Binding specificity of MB<sub>B7-H3</sub> to the target B7-H3 was first assessed in cell culture experiments under flow shear stress conditions simulating flow in blood capillaries by using a flow chamber experimental set-up. Detailed description of experimental protocol is provided under Supplementary Methods.

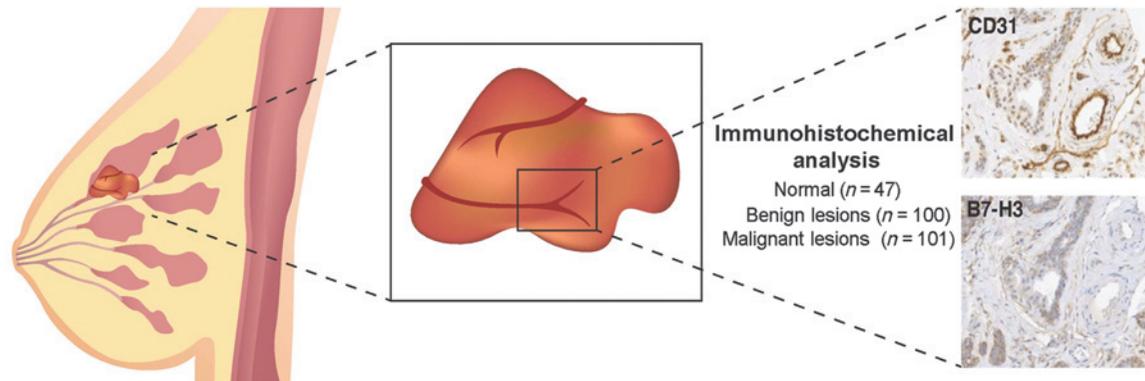
### Transgenic mouse model

All procedures involving the use of laboratory animals were approved by the Institutional Administrative Panel on Laboratory Animal Care. The well-established transgenic mouse model of breast cancer (FVB/N-Tg(MMTV-PyMT)634Mul) was used for all imaging experiments (16, 26). Breast tissue from control litter mates and normal mammary glands from transgenic mice were used as control normal tissue.

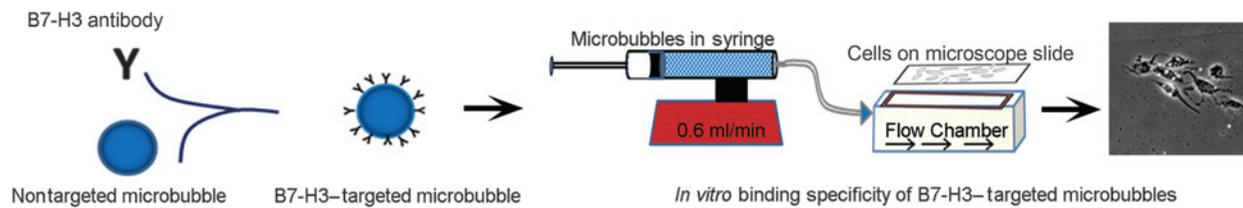
### B7-H3-targeted contrast-enhanced ultrasound imaging of mice

**Imaging protocol.** Mammary glands of transgenic mice bearing tumors ( $n = 146$ ) and normal control glands ( $n = 37$ ) were imaged. A detailed description of ultrasound molecular imaging protocol is provided in the Supplementary Materials. Images representing signal from adherent MB (molecular imaging signal)

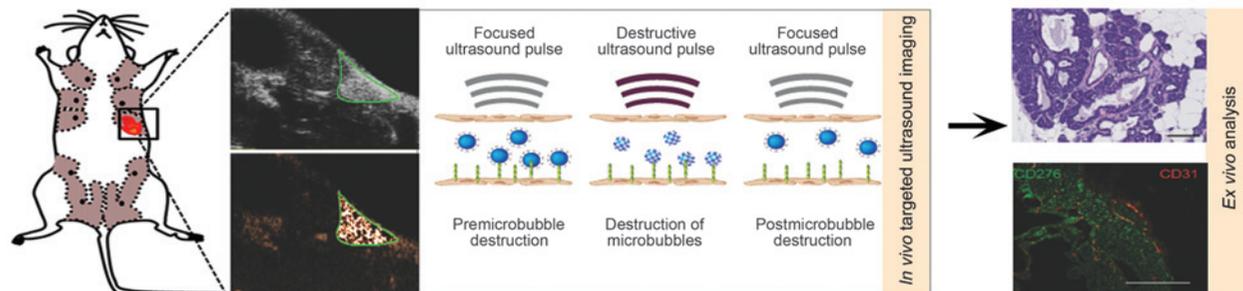
### Validation of vascular B7-H3 expression in human samples



### Development of contrast agent specific to B7-H3



### B7-H3-targeted ultrasound molecular imaging in transgenic mice



**Figure 1.**

Summary of the overall study design. Differential expression of B7-H3 on breast cancer-associated neovasculature was first assessed on a panel of normal, benign, premalignant, and malignant breast lesions obtained from women undergoing biopsy or surgical resection. B7-H3-targeted contrast microbubbles were then generated, followed by testing both in cell culture and *in vivo* in a transgenic mouse model of breast cancer.

were displayed as color maps on contrast-mode images, automatically generated by using commercially available Vevo CQ software (VisualSonics). The scale for the color maps was kept constant for all images.

**Assessment of binding specificity of B7-H3-targeted microbubbles *in vivo*.** To confirm binding specificity of  $MB_{B7-H3}$  to B7-H3 expressed on the tumor neovasculature in transgenic mice, an intra-animal comparison of ultrasound imaging signal following intravenous injection of both  $5 \times 10^7$   $MB_{B7-H3}$  and  $5 \times 10^7$   $MB_{Control}$  in the same session was performed. For this purpose, mammary glands with breast cancer ( $n = 10$ ) were imaged using both  $MB_{B7-H3}$  and  $MB_{Control}$  in random order to minimize any bias from the injection order, and injections were separated by at least 30 minutes waiting time to allow clearance of microbubbles

from previous injections (27). To further confirm binding specificity of  $MB_{B7-H3}$  to B7-H3 in the same mice, targeted ultrasound imaging using  $MB_{B7-H3}$  was repeated 5 hours after intravenous injection of 125  $\mu$ g purified rat anti-mouse B7-H3 antibody (eBiosciences) to block B7-H3 receptor sites *in vivo*.

**Data analysis of *in vivo* imaging datasets.** Imaging datasets of all mice were analyzed offline in random order using a dedicated workstation with commercially available software (Vevo 2100, VisualSonics). Analysis was performed in a blinded fashion by one of the authors. Because the transgenic mice used in this study can develop cancer as early as 4 weeks of age and morphologic changes for these early invasive cancers are not visible on conventional B-mode ultrasound imaging (Fig. 6; ref. 28), this author was blinded to the mammary gland pathology (normal or cancer). The

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**Table 1.** Histologic subtype and sample size. Summary of various breast pathologies collected and analyzed by IHC

| Histology                           | Subtype         | n  |
|-------------------------------------|-----------------|----|
| Normal breast tissue                | N.A.            | 47 |
| Benign and Precursor Breast Lesions | Adenosis        | 4  |
|                                     | ADH             | 1  |
|                                     | ALH             | 4  |
|                                     | ApoM            | 4  |
|                                     | CCL             | 57 |
|                                     | DCIS            | 10 |
|                                     | FA              | 1  |
|                                     | FEA             | 7  |
|                                     | NPFCC           | 2  |
|                                     | Radial scar     | 2  |
|                                     | UDH             | 8  |
| Breast cancer                       | Luminal A       | 45 |
|                                     | Luminal B       | 16 |
|                                     | Her2            | 19 |
|                                     | Triple negative | 21 |

Abbreviations: ADH, atypical ductal hyperplasia; ALH, atypical lobular hyperplasia; ApoM, apocrine metaplasia; CCL, columnar cell lesion; DCIS, ductal carcinoma *in situ*; FA, fibroadenoma; FEA, flat epithelial atypia; NPFCC, non-proliferative fibrocystic changes; UDH, usual ductal hyperplasia; Her2, human epidermal growth factor receptor type 2 positive cancer; Luminal A, estrogen receptor and/or progesterone receptor-positive cancer; Luminal B, estrogen receptor- and/or progesterone receptor-positive and Her2-positive cancer; Triple negative, estrogen, progesterone, and Her2-negative breast cancer.

reader was also blinded to the microbubble type (MB<sub>B7-H3</sub> or MB<sub>Control</sub>). Regions of interest (ROI) were drawn over the mammary glands and the magnitude of imaging signal (expressed in arbitrary units, a.u.) from attached microbubbles was assessed by calculating an average for pre- and postdestruction imaging

signals and subtracting the average postdestruction signal from the average predestruction signal as described previously (19, 27, 29).

#### Ex vivo analysis of mammary glands from transgenic mice

*Ex vivo* histopathological and quantitative immunofluorescence analysis was performed using standard techniques (see Supplementary Materials).

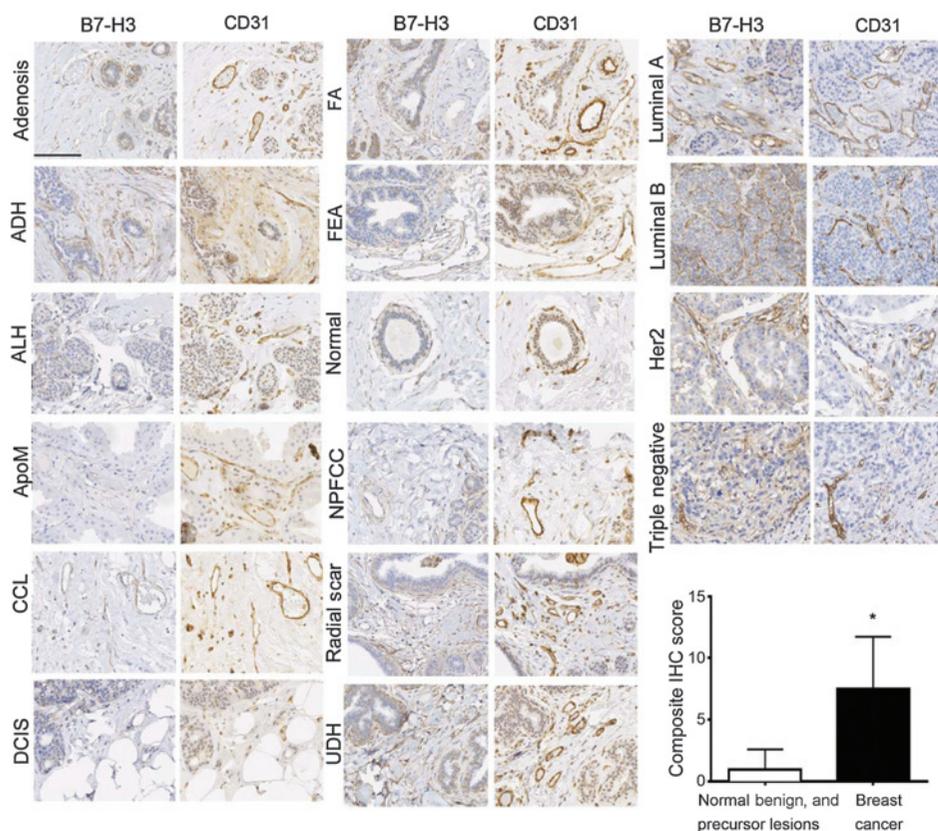
#### Statistical analysis

All data were expressed as mean  $\pm$  SD. For details on the statistical analysis, please refer to Supplementary Methods

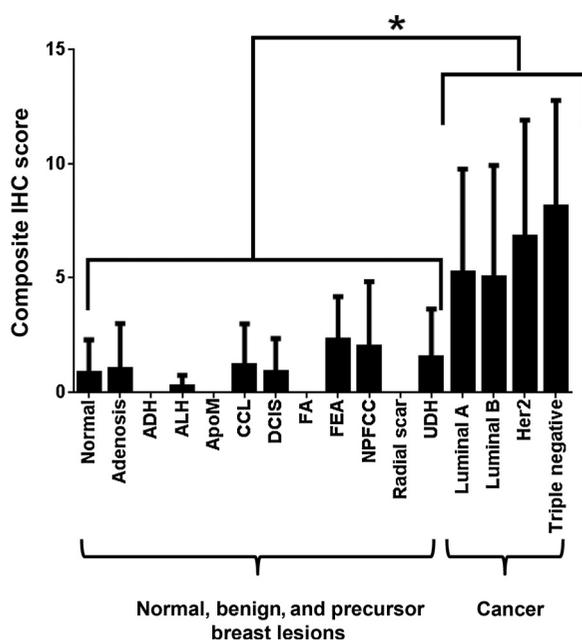
## Results

### Validation of B7-H3 expression in human breast tissues

To assess B7-H3 expression in breast cancer-associated neovasculature in humans, IHC analysis was performed on breast tissues from a total of 248 women with normal breast tissue ( $n = 47$ ), 11 different benign and precursor breast pathologies ( $n = 100$ ), and four different subtypes of breast cancer ( $n = 101$ ; Table 1). B7-H3 expression was detected on the cell membrane and within the cytoplasm of tumor epithelial cells, on fibroblast-like cells within the stroma, as well as on membranes of vascular endothelial cells. Because of the vascular restriction of the ultrasound molecular contrast agent, only vascular staining (guided by vascular marker CD31 staining) was quantified. In 209 samples processed into a breast TMA, B7-H3 expression was significantly ( $P < 0.001$ ) higher in breast cancer (mean composite score, 7.7) compared with normal tissue, benign, and precursor breast lesions (mean composite score, 1.3; Fig. 2). Individual

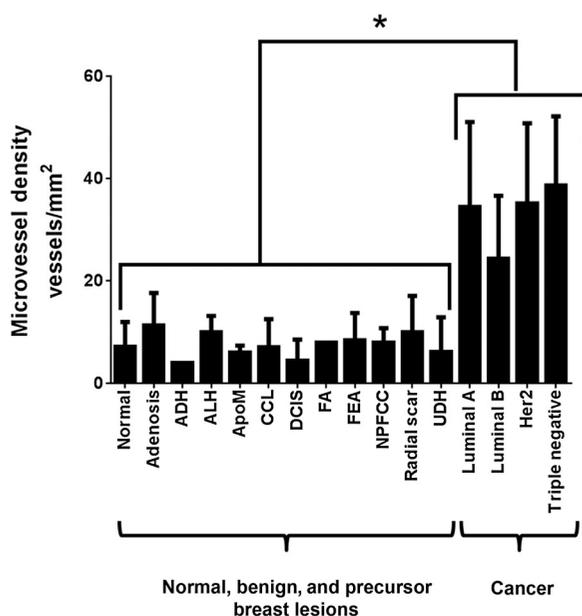
**Figure 2.**

IHC analysis of B7-H3 expression in human breast tissues. Photomicrographs show representative staining results from normal breast tissues, various benign, and precursor breast pathologies, as well as different types of breast cancer obtained from women undergoing biopsy or surgical resection. Graph summarizes composite IHC scores from normal tissue, benign and precursor lesions versus breast cancer. \*,  $P < 0.001$ ; error bars, SD; scale bar, 100  $\mu$ m. ADH, atypical ductal hyperplasia; ALH, atypical lobular hyperplasia; ApoM, apocrine metaplasia; CCL, columnar cell lesion; DCIS, ductal carcinoma *in situ*; FA, fibroadenoma; FEA, Flat epithelial atypia; NPFCC, nonproliferative fibrocystic changes; UDH, usual ductal hyperplasia; Luminal A, estrogen receptor and/or progesterone receptor-positive cancer; Luminal B, estrogen receptor and/or progesterone receptor-positive and Her2-positive cancer; triple negative, estrogen, progesterone, and Her2-negative cancer.



**Figure 3.** Composite IHC score. Summary of composite IHC scores of B7-H3 staining of the vasculature in normal breast tissue, benign, premalignant, and malignant breast lesions. \*,  $P < 0.001$ ; error bars, SD.

composite scores for all benign and malignant subtypes are shown in Fig. 3. A detailed summary of B7-H3 staining intensities and percent positive vessels in all normal, benign, premalignant, and malignant human breast tissues is provided in Supplementary Table S1. MVD was also significantly ( $P < 0.001$ ) increased in breast cancer versus normal, benign, and precursor breast lesions (Fig. 4).



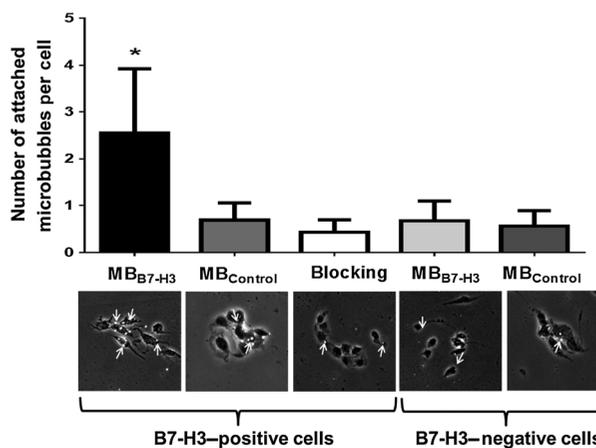
**Figure 4.** MVD analysis. Summary of MVD analysis on CD31-stained normal breast tissue, benign, premalignant, and malignant lesions. \*,  $P < 0.001$ ; error bars, SD.

Considering a composite score of 4 or higher as positive staining, overall 88 of 101 breast cancer, 17 of 100 benign lesions, and 6 of 47 normal tissues stained positive. Receiver operating characteristic (ROC) analysis indicated that B7-H3 neovascular immunostaining could distinguish breast cancer from normal tissue, benign, and precursor lesions with an area under the ROC curve (AUC) of 0.90 (95% confidence intervals; CI, 0.86–0.94).

Because TMA represents only very small tissue samples of the various histologies, a subanalysis of an additional 39 whole-tissue samples of breast cancer was performed containing more representative amounts of respective tumor tissues and using the noncancerous surrounding tissue as intra-individual benign controls. In these samples, the mean composite IHC score of malignant lesions (mean composite IHC score, 9.79) was significantly ( $P < 0.001$ ) higher compared with normal tissue, benign, and precursor breast lesions (mean composite IHC score, 1.67; Supplementary Fig. S1 and Supplementary Table S2). Considering a composite score of 4 or higher as positive staining, 39 of 39 breast cancer, 5 of 9 benign lesions, and 3 of 30 normal tissues stained positive. This corresponds to an AUC of 0.96 (95% CI, 0.92–0.99) in differentiating cancer versus normal, benign, and precursor lesions. Similarly, the MVD was significantly ( $P < 0.001$ ) increased in breast cancer versus normal tissue, benign, and precursor lesions (Supplementary Fig. S2).

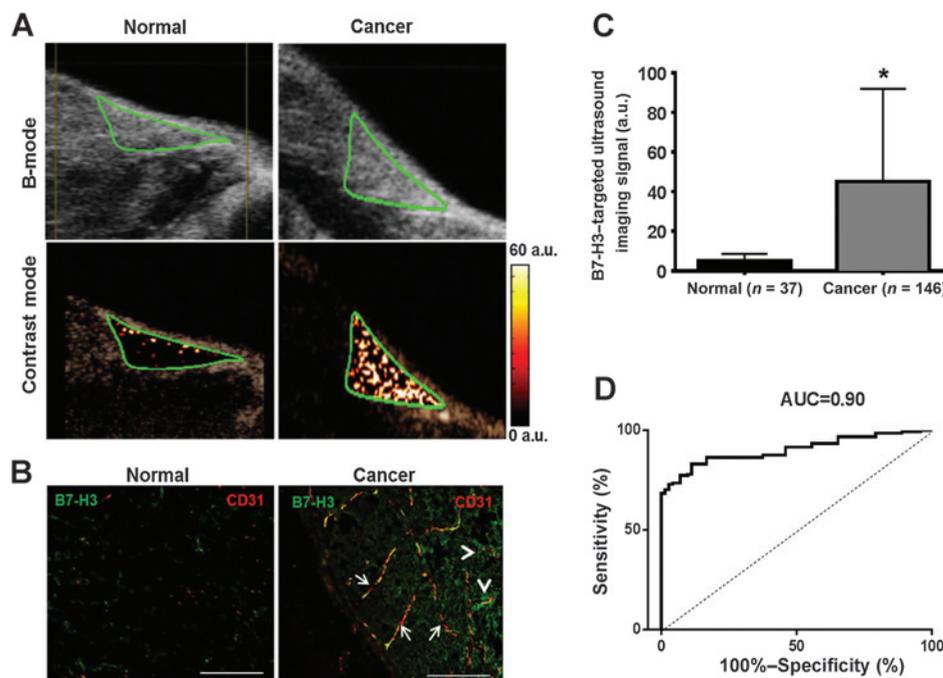
#### Flow chamber experiments

Microbubbles targeted to B7-H3 ( $MB_{B7-H3}$ ) and control nontargeted microbubbles ( $MB_{Control}$ ) were synthesized and binding specificity to B7-H3 was first tested in cell culture experiments. Figure 5 illustrates binding of both  $MB_{B7-H3}$  and  $MB_{Control}$  to B7-H3-positive and -negative mouse endothelial cells under flow shear stress conditions in a flow chamber. Average number of  $MB_{B7-H3}$  attached per cell was significantly higher ( $P < 0.001$ ) on B7-H3-positive compared with negative cells. Blocking of the B7-H3 receptors with anti-B7-H3 antibodies resulted in



**Figure 5.** *In vitro* binding specificity of B7-H3-targeted microbubbles. Representative photomicrographs from cell culture experiments using a parallel plate flow chamber setting with B7-H3-positive and -negative vascular endothelial cells exposed to B7-H3-targeted microbubbles (of  $MB_{B7-H3}$ ) and nontargeted control microbubbles (of  $MB_{Control}$ ). Note specific attachment of  $MB_{B7-H3}$  to B7-H3-positive cells and substantial binding inhibition following administration of blocking antibodies. Microbubbles (arrows) are visualized as white spherical dots. \*,  $P < 0.01$ ; error bars, SD.

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**Figure 6.**

*In vivo* ultrasound molecular imaging. A, representative transverse B-mode and contrast mode ultrasound images following injection of B7-H3-targeted contrast microbubbles show strong signal in breast cancer and only background signal in a mammary gland with normal breast tissue (both outlined by a green region of interest). B, photomicrographs of immunofluorescence images [double stained for both the vascular marker CD31 (red) and B7-H3 (green)] confirm expression of B7-H3 on tumor neovasculature (arrows, yellow signal on merged images) in breast cancer with little to no vascular expression in normal tissue. Note B7-H3 is also expressed on tumor epithelium (arrowheads; green). C, bar graph summarizes quantitative B7-H3-targeted ultrasound molecular imaging signal obtained in normal and breast cancer in a total of 183 mammary glands, with significantly increased imaging signal in breast cancer versus normal tissue. \*,  $P < 0.001$ ; error bars, SD. D, ROC curve in distinguishing normal from breast cancer based on quantitative ultrasound molecular imaging signal.

significantly reduced ( $P < 0.001$ ) binding of  $MB_{B7-H3}$  to B7-H3-positive cells, confirming binding specificity of  $MB_{B7-H3}$  to B7-H3. There was only minimal nonspecific binding of  $MB_{Control}$  to B7-H3-positive cells compared with  $MB_{B7-H3}$  ( $P < 0.001$ ).

#### B7-H3-targeted contrast-enhanced ultrasound imaging in transgenic mice

Binding specificity of  $MB_{B7-H3}$  to murine B7-H3 was first tested in 10 breast tumors in transgenic mice. *In vivo* ultrasound imaging signal obtained from  $MB_{B7-H3}$  ( $36.6 \pm 7.9$  a.u.) was significantly higher ( $P < 0.001$ ) compared with the signal from  $MB_{Control}$  ( $8.4 \pm 3.6$  a.u.). Furthermore, *in vivo* B7-H3-targeted ultrasound molecular imaging signal was significantly reduced ( $4.2 \pm 1.6$  a.u.;  $P < 0.001$ ) following administration of blocking anti-B7-H3 antibodies, further confirming *in vivo* binding specificity of  $MB_{B7-H3}$  to the imaging target B7-H3 (Supplementary Fig. S3). We then studied whether ultrasound using B7-H3-targeted contrast microbubbles allows imaging of B7-H3 expression *in vivo* in 146 mammary glands bearing breast cancer and 37 normal mammary glands. Imaging signal in breast cancer following injection of  $MB_{B7-H3}$  ( $49.4 \pm 5.3$  a.u.) was significantly higher ( $P < 0.001$ ) in breast cancer than in normal breast tissue ( $5.0 \pm 0.5$  a.u.; Fig. 6).

#### Ex vivo analysis

Similar to the human staining, B7-H3 expression was observed both on the tumor neovasculature and on tumor epithelial cells in

mice (Fig. 6B). B7-H3 expression on breast cancer-associated neovasculature was significantly ( $P < 0.001$ ) higher (mean intensity,  $53 \pm 28$  a.u.) compared with normal breast tissue (mean intensity,  $1.7 \pm 1.1$  a.u.). *Ex vivo* B7-H3 expression levels as assessed on quantitative immunofluorescence correlated well ( $R^2 = 0.77$ ,  $P < 0.001$ ) with *in vivo* B7-H3-targeted ultrasound imaging signal. MVD was also significantly ( $P < 0.001$ ) higher in breast cancer (mean,  $28 \pm 16$  vessels/ $mm^2$ ) compared with normal mammary tissue (mean,  $3 \pm 4$  vessels/ $mm^2$ ).

## Discussion

Our IHC analysis of normal and a broad spectrum of different benign, premalignant, and malignant breast pathologies in women undergoing surgical resection or biopsy show that vascular endothelial cell expression of B7-H3 allows differentiation of breast cancer from benign entities with high diagnostic accuracy. Ultrasound molecular imaging signal in transgenic mice using B7-H3-targeted contrast microbubbles is substantially higher in breast cancer versus normal breast tissue.

In patients with dense breast tissues, ultrasound is currently being explored as a complementary imaging modality to screening mammography for breast cancer detection (7). Ultrasound is advantageous because it is widely available, cost-effective, does not expose patients to ionizing radiation, and allows real-time guided biopsy of sonographically detected lesions, if needed.

General limitations of ultrasound as a screening tool, such as long imaging times and operator dependency, are already being addressed by the introduction of commercially available automated whole-breast ultrasound imaging systems that allow a time- and cost-efficient as well as more standardized acquisition and interpretation of breast ultrasound exams (30). In recent years, molecularly targeted ultrasound contrast agents have been developed to improve diagnostic accuracy of ultrasound in earlier detection of cancer such as pancreatic (15, 31), ovarian (32), and breast cancer (16, 19). To allow differentiation of cancer from noncancerous tissue using ultrasound and molecularly targeted contrast microbubbles, imaging targets have to be differentially expressed on the neovasculature of cancer compared with vessels in noncancerous tissue. Therefore, the goals of our study were, first, to explore whether a new potential molecular imaging target, B7-H3, is differentially expressed on the neovasculature of human breast cancer and, second, to assess binding specificity of a new B7-H3-targeted ultrasound contrast microbubble both in cell culture and *in vivo*.

B7-H3, a member of the B7 family of immunoregulators, was first identified on human dendritic cells and activated T cells (33, 34). Recently, B7-H3 expression has been shown in several cancer types, including acute leukemia, gastric, pancreatic, renal, liver, lung, bone, colon, prostate, ovarian, endometrial, and breast cancers (35–47). However, its role in immune response, including tumor immunity of different cancer types, remains unclear and controversial (33, 48, 49). Both T-cell costimulatory and inhibitory functions have been shown in various cancer types and B7-H3 expression has been correlated with both favorable and poor prognosis in patients with cancer (33, 35, 50). For example, in human gastric adenocarcinomas, B7-H3 expression was associated with prolonged patient survival compared with receptor-negative tumors (50). In contrast, recent studies showed that B7-H3 tumor expression may be a predictor of poor prognosis and increased risk for metastasis in other cancers such as renal, colon, breast, and ovarian cancers (37, 41, 43, 46). In women with breast cancer, tumor expression of B7-H3 was suggested as a predictor of early regional lymph node metastases (47, 51), advanced stage disease (51), and overall worsened prognosis (43). Whether B7-H3 is expressed on the neovasculature of breast cancer and whether it can be used as new molecular imaging target for breast cancer with ultrasound remains unclear.

In 248 patient samples including normal, 11 different benign and precursor breast pathologies, and four subtypes of breast cancer, processed both in a TMA and as whole tissue samples, we demonstrated that B7-H3 is overexpressed on breast cancer neovasculature compared with normal, benign, and precursor breast pathologies, using a composite IHC score of both staining intensity and percentage of positively staining vessels. Considering a composite score of 4 or more (out of a maximum of 12) as positive staining, B7-H3 allowed differentiation of breast cancer from normal, benign, and precursor lesions with high diagnostic accuracy. Because TMAs only represent a very small sample of tumor or benign tissues, an IHC subanalysis of 39 whole-tissue breast cancer samples was also performed. In this subgroup, all breast cancer types showed positive B7-H3 staining on the neovasculature. Peri-tumoral breast tissues served as intra-individual controls and confirmed substantially

less staining in normal, benign, or precursor breast lesions associated with breast cancer.

After validation of B7-H3 as a potential vascular molecular imaging target for human breast cancer detection, B7-H3-targeted microbubbles were designed and tested both in cell culture experiments and *in vivo*. Flow chamber experiments simulating shear stress flow in tumor vessels confirmed binding specificity of B7-H3-targeted microbubbles to their molecular target. This was further confirmed in breast cancer imaging experiments *in vivo*, which showed substantially higher ultrasound molecular imaging signal in breast cancer following intravenous injection of B7-H3-targeted microbubbles compared with control microbubbles in intra-animal comparison experiments in the same breast cancers. Quantitative immunofluorescence of excised murine mammary tissues further confirmed vascular expression of B7-H3 with excellent quantitative correlation between *in vivo* imaging signal and *ex vivo* expression levels of B7-H3. These results suggest that B7-H3-targeted ultrasound molecular imaging should be further developed as a noninvasive, relatively inexpensive imaging approach for breast cancer detection in patients.

We acknowledge the following limitations of our study. For this proof-of-principle imaging study in mice, we used biotin-streptavidin binding chemistry and commercially available antibodies to generate B7-H3-targeted microbubbles. These were not intended for clinical use and ongoing experiments explore the design of clinical grade contrast microbubbles targeted at B7-H3 using techniques described previously (27, 52). Also, due to the small dimensions of murine breast tissues in the z-plane, we chose to scan mice in two-dimensional planes only in our study. Automatic whole breast scanners are now available in the clinic (30), which will facilitate future translation of volumetric ultrasound molecular imaging for screening purposes in patients. Finally, although we assessed breast cancer-associated B7-H3 vascular endothelial cell expression in human tissue samples in a broad spectrum of benign and malignant breast lesions by IHC, B7-H3-targeted ultrasound molecular imaging was only tested in normal and invasive breast cancer *in vivo*. To the best of our knowledge, no mouse models are available that harbor the spectrum of all the benign diseases tested in the human samples in our study, which would allow modeling the diagnostic accuracy of B7-H3-targeted ultrasound molecular imaging in preclinical studies before translating this approach into the clinic. Therefore, future clinical studies using clinical grade B7-H3-targeted contrast microbubbles are warranted to both confirm our human IHC staining results and to assess diagnostic accuracy of ultrasound molecular imaging in detecting and characterizing breast cancer in patients.

In conclusion, our results suggest that B7-H3 is differentially expressed on the neovasculature of breast cancer compared with normal breast tissue and multiple benign breast pathologies in women undergoing surgical resection or biopsy. Ultrasound molecular imaging signal using contrast microbubbles targeted at B7-H3 is substantially increased in breast cancer versus normal breast tissue in transgenic mice. Future work toward clinical translation will develop clinical grade contrast agents targeted at B7-H3 that will eventually help in improving the diagnostic accuracy of ultrasound screening exams in detection and characterization of breast lesions in women with dense breast tissue.

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### Disclosure of Potential Conflicts of Interest

J.K. Willmann is a consultant for Bracco; this is unrelated to this study. No potential conflicts of interest were disclosed by the other authors.

### Authors' Contributions

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** S.V. Bachawal, K.C. Jensen, K.E. Wilson, J.K. Willmann  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** S.V. Bachawal, K.C. Jensen, L. Tain, J.K. Willmann  
**Writing, review, and/or revision of the manuscript:** S.V. Bachawal, K.C. Jensen, K.E. Wilson, L. Tain, A.M. Lutz, J.K. Willmann

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** J.K. Willmann

**Study supervision:** J.K. Willmann

**Other (provided NIH funding):** J.K. Willmann

### Acknowledgments

The authors thank Ferdinand Knieling, visiting medical student from Erlangen in Germany, for his assistance with ultrasound imaging and Timothy Doyle in the Small Animal Imaging Facility at Stanford University for support.

### Grant Support

This work was supported by the NIH R01 CA155289-01A1 grant (J.K. Willmann) and by a Developmental Cancer Research Award from the Stanford Cancer Center (J.K. Willmann).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 14, 2014; revised March 23, 2015; accepted April 9, 2015; published OnlineFirst April 21, 2015.

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## Breast Cancer Detection by B7-H3–Targeted Ultrasound Molecular Imaging

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*Cancer Res* 2015;75:2501-2509. Published OnlineFirst April 21, 2015.

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