Objective: To investigate the feasibility of ultrasonographic (US) imaging of head and neck cancer with targeted contrast agents both in vitro and in vivo. We hypothesize that conjugation of microbubble contrast agent to tumor-specific antibodies may improve US detection of head and neck squamous cell carcinoma (HNSCC).

Design: Preclinical blinded assessment of anti-EGFR and anti-CD147 microbubble contrast agents for US imaging of HNSCC.

Setting: Animal study.

Subjects: Immunodeficient mice.

Intervention: Injection of targeted microbubbles.

Main Outcome Measure: Microbubble uptake in tumors as detected by US.

Results: In vitro assessment of anti–epidermal growth factor receptor (EGFR) and anti-CD147–targeted microbubbles in 6 head and neck cancer cell lines yielded a 6-fold improvement over normal dermal fibroblasts (P < .001). Binding of targeted agents had a positive correlation to both epidermal growth factor receptor (EGFR) (R² = 0.81) and CD147 (R² = 0.72) expression among all cell lines. In vivo imaging of flank tumors in nude mice (N = 8) yielded enhanced resolution of anti-EGFR– and anti-CD147–targeted microbubble agents over IgG control (P < .001), while dual-targeted contrast agents offered enhanced imaging over single-targeted contrast agents (P = .02 and P = .05, respectively). In a blinded in vivo assessment, targeted contrast agents increased intratumoral enhancement of flank tumors over controls. Targeted US contrast agents to both EGFR and CD147 were 100% sensitive and 87% specific in the detection of flank tumors.

Conclusion: This preclinical study demonstrates feasibility of using molecular US to target HNSCC for contrast-enhanced imaging of HNSCC tumor in vivo.


Each year more than half a million people are diagnosed as having head and neck squamous cell carcinoma (HNSCC) worldwide.¹ Beyond visual inspection and palpation, the clinical tools with which a surgeon may assess head and neck cancer are often found lacking in improving patient outcomes. The result is an increasing reliance on computed tomography and magnetic resonance imaging studies that still may not discover small foci of metastatic disease. Furthermore, these modalities do not permit cancer-specific detection techniques. Ultrasound (US) is a cost-effective imaging tool that is already used to broadly assess head and neck cancers and to aid in fine-needle biopsy. Ultrasonography offers immediate feedback that could greatly aid in surgical decision making, but poor resolution often renders it unsuitable as a stand-alone diagnostic test.² Recently, microbubble (MB) US contrast agents have been shown to dramatically improve US resolution through imaging of tumor microvasculature.³ Microbubbles have been approved by the US Food and Drug Administration for use in cardiology, and owing to the superficial location of many head and neck lesions, will likely play an important role in the clinical detection of head and neck disease.

Ultrasonographic contrast agents are gas-filled MBs that are administered intravenously and act as intravascular tracers during US imaging. Contrast MBs are typically about one-hundredth of a millimeter in size and will enter tumor microvasculature relatively unimpeded.⁴ The MB shell can be made of protein, polymer, lipid, or carbohydrate surfactant, and to oppose in vivo Bernoulli forces from blood vessels, a poorly soluble biologically in-
ert perfluorinated gas is frequently used as the particle core. Microbubble contrast agents offer excellent detection sensitivities, can be imaged routinely with most current US systems, and are currently being studied as potential diagnostic agents in thyroid cancer. However, so far they have suffered from poor sensitivity and specificity in differentiating benign from malignant lesions. To further improve tumor resolution using MBs, we hypothesize that monoclonal antibodies can be linked to the MB shell via protein conjugation for molecular targeting of ligands specific to HNSCC.

Epidermal growth factor receptor (EGFR) and extracellular matrix metalloproteinase inducer (CD147) are both known to be overexpressed by epithelial tumor cells and contribute to tumor progression. Up-regulated expression and subsequent activation of EGFR is known to be associated with tumor proliferation, and CD147 is a membrane glycoprotein greatly enriched on the surface of cancer cells that stimulate neighboring stromal cells to increase the synthesis of matrix metalloproteinases necessary for metastatic spread. Though these cell surface proteins are predominantly expressed on tumor cell membranes, there is evidence that tumor-derived endothelial cells express these ligands as well, making them superb objectives for a targeted MB contrast agent. Subsequently, we hypothesize that MBs conjugated to antibodies against these cell surface ligands may improve US detection of HNSCC. Targeted contrast-enhanced US is an emerging imaging modality that has yet to be explored in head and neck cancer. Herein, we report on the feasibility of US imaging of head and neck cancer with targeted contrast agents both in vitro and in vivo. Because of the widespread clinical availability and relative ease of use, targeted contrast-enhanced US may have a growing role in the clinical assessment of head and neck cancer.

**METHODS**

**CELL CULTURE**

Four head and neck cancer cell lines (SCC-1, SCC-5, FaDu, and Cal27; ATTC) were obtained, and normal dermal fibroblasts (NDFs) were isolated from primary culture as described previously. Cells were maintained in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. Cells were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. All cells were cultured to 70% to 90% confluence before passage.

**ANTIBODY/MB CONJUGATION**

Ultrasonographic contrast MBs with biotinylated shells (Targetstar-B; Targetson) were conjugated to human IgG (Sigma), anti-CD147 monoclonal antibody CNOT3899 (Centocor Inc), and the anti-EGFR antibody cetuximab (Imclone Systems Inc). Preparation of MBs for conjugation involved incubating 0.15 mL of streptavidin with 1 mL of biotinylated MBs (Ultrasonix Medical Corp) at a speed of 25 rpm. Wells were incubated for 30 minutes at room temperature while mechanically rocked (Boekel Scientific) at a speed of 25 rpm. Wells were then washed (×3) and rehydrated with 1 mL of phosphate-buffered saline. Cell counts were made using a light microscope (Olympus IX70; Olympus America Inc) in darkfield mode. For each group, 4 areas of highest MB attachment were identified (original magnification ×200), and the number of attached MBs and cells within each field of view were counted. Attached MBs were normalized by cell count and recorded as MBs per well.

**FLOW CYTOMETRY**

All human cancer cells were aliquoted (100,000 cells per tube) and stained with fluorescein isothiocyanate conjugated to primary antibodies against either human IgG control, EGFR, or CD147 (BD Pharmingen). Following appropriate washes, cells were analyzed for fluorescent counts (50,000 events) using an Accuri C6 flow cytometer (Accuri Cytometers Inc). Baseline fluorescence was measured from cells and human IgG control. All experimental groups were analyzed in triplicate.

**In Vitro Assay**

Cells were plated 100,000 cells per well in 6-well dishes (Costar; Corning Inc) 24 hours prior to the binding assay. Antibody-labeled MB groups were added (5 × 10^8 MBs per well), and plates were incubated for 30 minutes at room temperature while mechanically rocked (Boekel Scientific) at a speed of 25 rpm. Wells were then washed (×3) and rehydrated with 1 mL of phosphate-buffered saline. Cell counts were made using a light microscope (Olympus IX70; Olympus America Inc) in darkfield mode. Baseline fluorescence was measured from cells and human IgG control. All experimental groups were analyzed in triplicate.

**In Vivo Assay**

Female athymic nude mice (N = 8) were obtained from Frederick Cancer Research. SCC-1 (2 × 10^6) cancer cells were implanted in the left flank of each mouse in part because of moderate overexpression of both CD147 and EGFR observed in our in vitro studies. Three weeks after implantation, targeted MBs (1 × 10^9) were intravenously injected and allowed to circulate systemically for 2 minutes. Thereafter, US imaging of tumor perfusion along the largest transverse dimension was performed continuously for 1 minute. A custom water bath setup was used for all imaging sessions while animals were maintained under isoflurane anesthesia at 37°C. Ultrasonographic imaging was performed using an MB-sensitive harmonic imaging mode implemented on a SONIX RP system (Ultrasonix Medical Corp). Following a 1-hour period to allow MB clearance, each mouse was injected with a fresh batch of MBs, and the previously described imaging procedure was repeated. Microbubbles were administered in the following order: control (MBIgG), single-targeted MBs (MBEGFR and MBCD147), followed by MBDUAL. Mice were then humanely killed, and tumors were removed for histological analysis. Quantitative region of interest analysis of intratumoral US image intensity was performed with ImageJ (National Institutes of Health) software using representative images acquired 10 seconds after contrast-enhanced US imaging began. Matching regions of interest were compared for mean intensity per pixel. For inter-
group comparison, average image intensity in single-targeted injection groups (MB_{EGFR} and MB_{CD147}) in all 3 mice was compared with the average image intensity of the MB_{igG} and MB_{DUAL} injections.

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS

Serial sections of 5-µm thickness were cut from the formalinfixed, paraffin-embedded tissue blocks and floated onto charged glass slides (Super-Frost Plus; Fisher Scientific) and oven dried overnight at 60°C. An hematoxylin-eosin–stained section was obtained from each tissue block. All sections subject to immunohistochemistry were deparaffinized and hydrated to deionized water. The tissue sections were heat treated with 0.01M TRIS–1mM EDTA buffer (pH 9) using a pressure cooker (CEPC 800; Cook’s Essentials) for 5 minutes at maximum pressure (15 pounds per square inch). Following antigen retrieval, all sections were gently washed in deionized water and then transferred to TBST (0.05% TRIS-based solution in 0.15M sodium chloride with 0.1% volume/volume Triton-X-100, pH 7.6). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes. To further reduce nonspecific background staining, slides were incubated with 3% normal goat or horse serum for 20 minutes (Sigma). Following washes with TBST, peroxidase-conjugated goat antirabbit IgG (dilution 1:5000) (Jackson ImmunoResearch) was applied to the sections for 30 minutes at room temperature. Diaminobenzidine (Sey Tek Laboratories) was used as the chromagen, and hematoxylin (7211; Richard-Allen Scientific), as the counter stain.

STATISTICAL ANALYSIS

Data from both in vivo and in vitro studies are reported as the mean (SE). Linear regression was used to correlate receptor expression with MB binding. Sensitivity and specificity values were calculated within 95% confidence intervals. In all analyses, P < .05 was considered statistically significant.

RESULTS

CORRELATION OF TUMOR CELL EXPRESSION OF EGFR AND CD147 WITH TARGETED MB BINDING IN VITRO

In vitro analysis of SCC-1, SCC-5, FaDu, Cal27, and NDF cells demonstrates the specificity of conjugated MBs targeting specific tumor surface proteins, allowing for the US detection of cancer specific molecular markers. To quantify the targeted ligand expression of these cells, characterization was established with fluorescein isothiocyanate–labeled human IgG control, anti-EGFR, or anti-CD147 antibody using flow cytometry analysis (Figure 1). There was a significant difference between CD147 expression between SCC-1 and SCC-1/SiE (P < .001) and between SCC-5 and SCC-5/SiE (P < .001). There was also a significant difference between EGFR expression in Cal27 and FaDu cell lines (P < .001). The Cal27 cells had the highest expression of EGFR, while FaDu cells had the lowest. SCC-5 cells had the highest expression of CD147, while SCC-1/SiE had the lowest. Cal27 had higher levels of binding to MB_{EGFR} (3.3 MBs per cell), MB_{CD147} (3.5 MBs per cell) and MB_{DUAL} (6.7 MBs per cell), while SCC-5 had binding levels similar to SCC-1 (3.6, 3.9, and 7.1 MBs per cell, respectively). Among all cell lines, binding of MB_{EGFR} (Figure 2A) and MB_{CD147} (Figure 2B) was strongly correlated with cell line expression of CD147 and EGFR (R^2=0.81 and R^2=0.72, respectively; P < .001). Similarly, MB_{DUAL} (Figure 2C) binding correlated with combined receptor expression (R^2=0.67; P < .001). The strong correlation between these values indicates a high degree of fidelity in targeted US imaging.

SIGNIFICANTLY GREATER BINDING OF SINGLE- AND DUAL-TARGETED MICROBUBBLES IN VITRO vs CONTROLS

In vitro representative light microscopy images (original magnification ×200) illustrate targeted MB binding to SCC-1, FaDu, and NDF cells (Figure 3A). The control MB_{igG} group had little affinity for any of the cells during the in vitro binding assay. Targeted MB binding on SCC-1, SCC-5, FaDu, Cal27, and NDF cells was assessed using dark-field microscopy, and these data are presented in graphic representation (Figure 3B). There was a statistically significant difference in adherent MBs per cell between the single EGFR (3.64 MBs per cell) and single CD147 (4.17 MBs per cell) groups compared with MB_{igG} (0.65 MBs per cell) in SCC-1 cells (P < .001). For SCC-1 cells, there was not a significant difference found during intragroup comparison between single-targeted groups (P=.70). For intergroup comparisons of SCC-1 cells, the MB_{DUAL} were shown to bind to cancer cells significantly greater than both MB_{igG} Control (P<.001) and MB_{EGFR} and MB_{CD147} (P=.05 and P=.02, respectively). Likewise, for FaDu cells there was a significant difference in binding between MB_{igG} (0.73 MBs per cell) and MB_{EGFR}
(2.65 MBs per cell; \( P = .01 \)) and MB\(_{CD147} \) (4.41 MBs per cell; \( P < .001 \)). In FaDu cells there was, however, a significant difference in binding between anti-EGFR\(_{MB} \) and anti-CD147\(_{MB} \) (\( P = .05 \)). Binding of MB\(_{Dual} \) (5.82 MBs per cell) in FaDu cells was significantly greater compared with MB\(_{EGFR} \) (\( P = .02 \)), but not with MB\(_{CD147} \) (\( P = .14 \)).

**IMPROVED DETECTION OF HNSCC WITH MOLECULAR US IN VIVO AND CORRELATION OF CALIPER- AND US-MEASURED TUMOR SIZE**

Ultrasonographic images of xenograft flank tumors were paired and assessed by a blinded reviewer (J.A.K. and K.H.) to determine the greatest degree of intratumoral MB enhancement (Figure 4). According to the paired animal data, it was determined that 75% of the US images from the single-targeted MB\(_{EGFR} \) exhibited increased enhancement over MB\(_{IgG} \) (\( P = .06 \)). Single-targeted MB\(_{CD147} \) had significantly greater enhancement (82.5%; \( P = .05 \)) compared with controls. MB\(_{Dual} \) exhibited the greatest enhancement over control (100%; \( P < .001 \)) and offered an improvement over single-targeted agents as well (76% [\( P = .05 \)] and 88% [\( P = .03 \)] for MB\(_{EGFR} \) and MB\(_{CD147} \), respectively). In Figure 4, digital imaging software (ImageJ; http://rsbweb.nih.gov/ij/) was used to quantify intratumoral echogenicity. Contrast values were averaged over the region of interest and were significant greater in MB\(_{EGFR} \) (\( P = .001 \)) and MB\(_{CD147} \) (\( P = .003 \)) vs MB\(_{IgG} \) control. Analysis of in vivo images with MB\(_{Dual} \) yielded significantly improved echogenicity over MB\(_{EGFR} \) (\( P = .001 \)) and MB\(_{CD147} \) (\( P = .002 \), respectively) and control (\( P < .001 \)). Tumor sizes were measured with digital calipers and correlated with size measured with molecularly targeted US (\( R^2 = .93 \), \( P < .001 \)). Ultrasonographically measured tumor size was not significantly different among any of the MB-conjugated treatment groups (\( P = .80 \)).
A cancer-specific US contrast agent has the potential to significantly improve detection of subclinical disease. Microbubbles conjugated to anti-EGFR and anti-CD147 antibodies led to binding of MBs to multiple head and neck cancer lines in vitro that positively correlated with cell surface expression of cell surface ligands measured by flow cytometry. Normal dermal fibroblasts that have low expression of CD147 and EGFR exhibited very little MB binding, indicating a low background binding level of targeted MBs. The silenced CD147 cell lines SCC-1/SiE7 and SCC-5/SiE7 both had lower levels of MBCD147 binding compared with vector controls and receptor profiles of EGFR correlated with MB binding in vitro. Although differential expression of cell proteins appears to result in differential binding of targeted MBs in vitro, the intragroup difference in binding was still several times greater than that observed in NDFs. Though this technique may be limited by marker expression in individual patient tumors, there is still significant potential for targeted US to quickly assess tumors with even modestly expressed head and neck cancer cell markers.

In vivo animal experiments confirmed the potential for targeted MB imaging in flank tumors. Significant differences in intratumoral echogenicity were detected in MBEGFR and MBCD147 as well as. Exploiting the differences in cancer cell surface expression of EGFR and CD147 has been a strategy for improving treatment of HNSCC. To the extent that these markers correlate with tumor proliferation and metastatic potential, the ability to identify them in vivo could provide important diagnostic information regarding disease progression and response to treatment. The in vitro analysis exhibited in Figure 3 supports a role for targeted contrast-enhanced US in imaging of HNSCC.

The emergence of monoclonal antibodies as adjuvant therapies in HNSCC has led to an increased demand for a method to monitor and predict tumor response to treatment. Recently, contrast-enhanced US has been validated as a powerful tool for monitoring antiangiogenic therapy in a preclinical cancer study. In addition, clinical trials have demonstrated contrast-enhanced US may differentiate tumor vascular differences both of these (100% and 87%, respectively), but improvement was not statistically significant (Table).

### Table. Comparison of the Sensitivity and Specificity of Single- and Dual-Targeted Microbubbles (MBs) in SCC-1 Flank Tumors

<table>
<thead>
<tr>
<th>Variable</th>
<th>MBEGFR</th>
<th>MBCD147</th>
<th>MBDUAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity, % (95% CI)</td>
<td>91 (88-95)</td>
<td>93 (81-98)</td>
<td>100 (100-100)</td>
</tr>
<tr>
<td>Specificity, % (95% CI)</td>
<td>83 (77-91)</td>
<td>79 (75-89)</td>
<td>87 (83-95)</td>
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<tr>
<td>PPV, %</td>
<td>91</td>
<td>93</td>
<td>100</td>
</tr>
<tr>
<td>NPV, %</td>
<td>85</td>
<td>82</td>
<td>89</td>
</tr>
</tbody>
</table>

Abbreviations: MBEGFR, MBCD147, and MBDUAL indicate anti-EGFR–, and anti-CD147–targeted MBs and dual-conjugated MBs, respectively; NPV, negative predictive value; PPV, positive predictive value.

### SENSITIVITY AND SPECIFICITY OF TARGETED US CONTRAST AGENTS

To demonstrate that enhanced binding of the dual-targeted construct was not due to the preferential binding of a single target, histological analysis was performed to qualify targeted ligand expression in vivo. Representative images of histological staining demonstrate ubiquitous expression of head and neck squamous cell carcinoma used to confirm tumor pathology (original magnification ×200).

**Figure 4.** Representative ultrasonographic (US) images of SCC-1 flank tumors after injection with MBIgG, MBEGFR, MBCD147, and MBDUAL. A, White dashed ovals represent targeted contrast agent in tumor microvasculature. B, Images were analyzed using ImageJ, and average intratumoral dashed ovals represent targeted contrast agent in tumor microvasculature. Error bars indicate SE.

**Figure 5.** Immunohistochemistry staining of FaDu and SCC-1 flank tumors in athymic nude mice. Hematoxylin-eosin staining show tumor cells and anti-CD147 and anti-EGFR immunostaining show ubiquitous expression of head and neck squamous cell carcinoma used to confirm tumor pathology (original magnification ×200).
between benign and malignant thyroid neoplasms. Microvascular imaging using MB contrast agents has been shown to be enhanced by targeting specific markers on tumor-associated endothelial cells. This evolution of targeted contrast-enhanced US has led to improved imaging of tumor vasculature allowing for enhanced visualization and microvessel assessment. Targeted contrast-enhanced US combines traditional US with molecular imaging and a real-time clinical assessment of HNSCC.

In a large melanoma study, noncontrast US was able to detect only 45.8% of positive neck sentinel lymph nodes. Adding MB contrast to US improves effectiveness of sentinel lymph node detection in head and neck animal models, but no one has had yet targeted MB to specific HNSCC markers. We have previously shown that fluorescent optical imaging of EGFR and CD147 ligands in a murine xenograft flank model is an effective method of monitoring lymph node metastasis in HNSCC. In these studies, anti-EGFR and anti-CD147 antibodies were conjugated to fluorescent dye and demonstrated 100% sensitivity and 87% specificity for HNSCC. In our present study, MBs conjugated to anti-EGFR and anti-CD147 antibodies were sensitive enough to differentiate tumor from nontumor tissue (91% and 93%, respectively), and had specificity of 83% and 79%, respectively, for tumor. This is similar to clinical trials using lymphoscintigraphy that were able to detect sentinel lymph node metastasis with 90% sensitivity and 81% specificity. Targeting MBs to both EGFR and CD147 further enhances sensitivity to 100% and specificity to 85%, indicating significant improvement over nontargeted US contrast imaging. There was no increase in image contrast after administration of MBs compared with MB alone. This indicates very little nonspecific binding of targeted MB in vivo.

Although we investigated targeted MBs as only a diagnostic modality, they may one day also find use in delivery of a drug or virus payload to treat locally metastatic HNSCC. One study has already tried to improve specificity by targeting EGFR with micelles containing drugs such as doxorubicin. However, MBs are a superior drug delivery vehicle because they can be ruptured at the tumor site using high-intensity US. This technique is being explored to treat HNSCC with conditionally replicating adenoviruses but may benefit from enhanced sensitivity and specificity of targeted MB.

Developing US technology is important for head and neck cancer because it is a cost-effective imaging tool that allows for clinical assessment of lymph node metastasis beyond the clinician’s physical examination. Ultrasoundography is uniquely portable compared with other imaging modalities, does not carry the risk of irradiation associated with x-ray or computed tomographic imaging studies, and is already used in diagnostic and therapeutic interventions such as fine-needle aspiration and line placement. The emergence of targeted therapies in head and neck cancer should support a trend toward developing targeted imaging modalities that will allow for early detection of HNSCC and improved patient outcomes. This study demonstrates feasibility of using anti-EGFR– and anti-CD147–targeted MB contrast agents for US imaging of HNSCC.

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Author Contributions: Dr Hoyt had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Knowles, Heath, Saini, Warram, Hoyt, and Rosenthal. Acquisition of data: Knowles, Heath, Saini, Umphrey, Warram, and Hoyt. Analysis and interpretation of data: Knowles, Heath, Umphrey, Warram, and Hoyt. Drafting of the manuscript: Knowles, Heath, Saini, Hoyt, and Rosenthal. Critical revision of the manuscript for important intellectual content: Knowles, Heath, Umphrey, Warram, and Hoyt. Statistical analysis: Knowles, Heath, and Hoyt. Obtained funding: Knowles. Administrative, technical, and material support: Knowles, Umphrey, Warram, and Hoyt. Study supervision: Knowles, Saini, Umphrey, Warram, Hoyt, and Rosenthal.

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