Contrast-Enhanced Ultrasound With Perflubutane for Sentinel Lymph Node Mapping in Cutaneous Melanoma: A Pilot Study

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Objective: To study the feasibility of contrast-enhanced ultrasound (CEUS) for identification of SLN associated with cutaneous melanoma.

Study Design: Single arm pilot study in a swine animal model.

Methods: One milliliter of perflubutane (Sonazoid, GE Healthcare, Milwaukee, WI) was injected into the peritumoral dermis in five swine with cutaneous melanoma. Ultrasonography was used to follow enhancing lymphatic channels to lymph nodes (LN). Intradermal injection of vital blue (VB) dye was used as a positive control. LN identified by either method were excised and examined histologically.

Results: There were five primary cutaneous melanomas with mean area of 4.36 ± 4.75 cm² and Breslow depth of 3.6 ± 1.5 mm. Six possible sentinel lymph node (SLN)s were identified with CEUS, and nine were identified with VB. SLN averaged 12.44 ± 6.15 cm from the primary tumor. Four of six (67%) SLNs identified by CEUS and four of nine (44%) candidate SLNs identified by VB contained histologically confirmed metastatic melanoma. All six CEUS-identified SLNs were also identified with VB. Two LN not containing melanoma were identified by CEUS; three were identified with VB. In all SLN with metastases, metastatic cells were scattered throughout the LN and not clustered in a discrete mass.

Conclusion: CEUS with perflubutane feasibly identifies SLN associated with cutaneous melanoma and may be a useful adjunct technology in facilitating precise SLN dissection. Our work supports a clinical trial investigating the use of CEUS for this application.

Key Words: Sentinel lymph node biopsy, cutaneous melanoma, radiology.

Level of Evidence: NA

INTRODUCTION

Cutaneous melanoma is the fifth most common malignancy in the United States and is one of the few cancer types with an increasing incidence over the past three decades.1 Due to the tendency to affect younger patients, cutaneous melanoma also has a disproportionate impact in terms of life-years lost. It is an aggressive malignancy with a 20% rate of spread to regional lymph nodes (LN) in patients with tumor depth greater than 1 mm.2,3 The presence of nodal metastases is the most important prognostic factor in cutaneous melanoma,7 and the number of nodes involved is also highly correlated with prognosis among patients with metastases to regional LN.5

Several trials2,6 have shown no survival benefit to performing elective lymph node dissections if regional LNs are not known to harbor metastatic disease. As such, precise identification of draining sentinel lymph nodes (SLN) and removal of basins involved with tumor are of paramount importance in disease control. Several studies, including the large phase III Multicenter Selective Lymphadenectomy Trial,7 have demonstrated improved melanoma-specific survival with sentinel lymph node biopsy (SLNB)-based management. As such, current guidelines for treatment of cutaneous melanoma recommend SLNB for melanomas with Breslow depth greater than 1 mm or tumors of less than 1 mm depth with certain high-risk features, followed by completion lymph node dissection (LND) if the SLN is positive for tumor.8

Current standard protocol for SLNB involves preoperative lymphoscintigraphy using an injection of a technetium sulfur colloid radiotracer, followed by intraoperative lymph node mapping using vital blue (VB) dye. A gamma probe is used to identify radioactive lymph nodes, and SLNs are identified via the colocalization of radiotracer and VB dye uptake.

This method of SLN detection has several shortcomings. First, technetium sulfur colloid can actually identify

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secondary echelon LNs, leading to dissection of LNs outside the primary sentinel nodal basin and resulting in increased operative time and morbidity. Second, the close proximity of the primary lesion to the draining nodal basins in regions with a dense lymphatic supply, such as the head and neck, can have a “shine-through” effect, which may make use of a hand-held gamma probe more difficult during the SLNB procedure. In addition, the gamma probe does not provide depth information for anatomic localization of the SLN; thus, the surgeon relies on removing nodes and testing them ex vivo with the probe.

Contrast-enhanced ultrasound (CEUS) is a relatively new imaging modality that may be used for identification of SLN associated with cutaneous melanoma. Perfluorobutane (Sonazoid; General Electric (GE) Healthcare, Milwaukee, WI) is a new sonographic contrast agent consisting of microbubbles of perfluorobutane gas that are stabilized by hydrogenated egg phosphatidylserine. Perfluorobutane microbubbles can be taken up by reticuloendothelial cells in LN and can provide enhancement for at least 4 hours. Prior research has suggested that perfluorobutane may be used as a contrast agent in a variety of ultrasonographic applications including the identification of SLN associated with cutaneous melanoma. Use of this agent is well suited to head and neck applications because there is no shine-through effect, as seen in lymphoscintigraphy, and the ultrasound (US) probe is able to give the surgeon more precise depth estimation compared to the gamma probe. Both methods may be used in conjunction with blue dye to complement the SLN localization. Finally, although lymphoscintigraphy may unpredictably identify LN outside the primary nodal basin, CEUS has been shown to be useful in systematically identifying secondary echelon LNs by injection of contrast directly into the SLN after the SLN is identified.

We performed a pilot study in a swine animal model to demonstrate safety and feasibility of perfluorobutane-enhanced ultrasonography for identification of SLN associated with cutaneous melanoma. This method may be especially relevant for nodal dissection in the head and neck given variable lymphatic drainage pathways, as well as the shine-through effect observed with the gamma probe due to close proximity between primary tumors and their SLNs.

**MATERIALS AND METHODS**

In accordance with a protocol approved by the institutional review board (IRB) and the Institutional Animal Care Use Committee (IACUC) at the University of Southern California (USC), a pilot experiment examining the feasibility of detection of metastases from cutaneous melanoma in a swine model was performed (IACUC protocol 20476-AM003; IRB study HS-16-00273-AM004). Swine with spontaneously occurring melanoma were identified for this study. Swine were chosen as the pilot animal given that the swine model of melanoma is known to show histologic types and patterns of spread similar to cutaneous melanomas in humans.

Under general endotracheal anesthesia, 1 mL of perfluorobutane (Sonazoid, GE Healthcare) was injected into the peritumoral dermis in five swine with cutaneous melanoma. Peritumoral regions were surveyed via ultrasound continuously in order to identify enhancing lymphatic channels and nodes. Ultrasonography was performed with a GE LogiqE9 unit, using a contrast-specific mode with low mechanical index (≤ 0.1), by two radiologists (V.D. and K.O.K.) with 16 and 4 years, respectively, of postfellowship experience in ultrasound. Times required for enhancement of lymphatic channels and nodes were noted. After 10 minutes, locations of SLN identified by CEUS were marked on the skin to guide surgical resection. Vital blue dye was also injected into the peritumoral dermis as a positive control. Lymph nodes identified by either method were excised and examined histologically. Surgical technique is depicted in Figure 1. Excised LN were examined with ultrasonography after resection to confirm that the excised tissue in fact contained the contrast-enhancing LN. The areas around the excised LN were also examined with ultrasonography after node excision to ensure that no residual contrast-enhancing tissue was present and that no areas of pathological lymph node enlargement were overlooked.

For each swine, location of the primary tumor, location and numbers of excised LN, and distance of the excised LN from the primary tumor were recorded. Specimens were stained with hematoxylin and eosin (H&E) and examined histologically to confirm that the resected specimen represented a lymph node, as well as for the presence of metastatic melanoma. Although the full battery of immunohistochemical testing used in the micrometastatic melanoma workup was beyond the scope of this work, performing histologic analysis allowed confirmation of the presence of nodal tissue in the resected specimens and to prove that nodes containing metastatic melanoma were able to be identified using this method. Histological examination was performed by the pathology department at our institution. Pathologists were blinded with regards to whether a given tissue specimen enhanced with perfluorobutane, VB, or both. Distribution of metastatic cells for each lymph node was recorded.

During the procedure, animals were connected to cardiopulmonary monitors and were observed throughout the procedure. All swine were euthanized after the completion of the LN resections.

**RESULTS**

There were five primary cutaneous melanomas. Mean tumor area was 4.36 ± 4.75 cm² (range, 0.78–12 cm²), and mean Breslow depth was 3.6 ± 1.5 (range, 2–5) mm. Two swine had primary tumors located in the head and neck; one had a primary tumor on the flank; one had a primary tumor in the lower abdomen; and one had a primary tumor of the lateral thigh.

After injection of perfluorobutane, enhancing lymphatic channels were detectable by ultrasound after 4 to 5 minutes and were traceable to LN within 10 to 11 minutes. In this model, performance of ultrasonography by radiologists expedited the process, facilitating dissection by the surgical team and ensuring accurate identification of contrast-enhancing lymph nodes. Figure 2 depicts a grayscale image of the first draining LN from a cutaneous melanoma of the left flank (Fig. 2A, arrow), as well as perfluorobutane-enhanced US demonstrating the enhancing lymphatic channel (Fig. 2B, small arrows) leading to the enhancing LN (Fig. 2B, large arrow). Figure 3 depicts grayscale and CEUS images of the primary tumor ex vivo (Fig. 3A), SLN in vivo (Fig. 3B), and SLN ex vivo (Fig. 3C) from a swine with melanoma of the right posterior neck.

There were nine candidate lymph nodes identified for resection by either CEUS or VB prior to skin incision. On average, SLN were located 12.44 ± 6.15 cm from the
primary tumor. There were six total candidate LNs identified by CEUS—and the same six plus three additional candidate LNs identified by VB. On histologic analysis, all six potential LNs identified by CEUS and seven of nine potential LNs identified by VB contained actual lymph nodes nor metastatic melanoma. Four of the six (67%) SLN identified by CEUS and four of nine (44%) candidate SLN identified by VB contained histologically confirmed metastatic melanoma, with no metastatic melanoma detected in LNs that were not identified by CEUS. Table I summarizes locations, enhancement characteristics, and histologic findings of SLN associated with each primary tumor.

Histologically, none of the positive SLNs showed extranodal extension of tumor. In all of the SLNs with metastatic disease, tumor cells were scattered throughout the node in both the subcapsular and deeper portions of the node. None of the nodes showed a focal mass of metastatic tumor cells. The distribution of metastatic cells within the LN did not correlate with the apparent pattern of sonographic enhancement.

Throughout the procedure, animals were observed for cardiac and ventilatory abnormalities as well as for infusion site reactions to the contrast agent itself and embolic phenomena. There were no acute adverse events associated with the use of perfluorbutane. All swine were uneventfully euthanized at the completion of the procedure.

**DISCUSSION**

In this preclinical pilot study, we demonstrate the feasibility of SLN detection with perfluorbutane-enhanced ultrasonography in swine with cutaneous melanoma. Previous studies demonstrating safety and feasibility of perfluorbutane-enhanced ultrasonography for identification...
of SLN associated with cutaneous melanoma have been undertaken primarily by a single research group.\textsuperscript{10,13} We sought to add to the existing body of evidence supporting use of this method and to validate the findings of Goldberg et al.\textsuperscript{10} In our experiments, we found that enhancing lymphatic channels were easily detectable and traceable to lymph nodes within minutes of peritumoral injection of the contrast agent, and there was overall good agreement between SLN identified with CEUS and with VB-dye controls. In all cases, dissection was straightforward and quick due to the use of ultrasonography to identify the precise location of the nodes to be dissected. Ultrasonography may be performed continuously after injection of perflubutane (as was the case in our experiments) or 10 to 15 minutes after injection of the contrast agent to allow diffusion of perflubutane into lymph nodes. CEUS thus adds minimal time to the SLNB procedure because some of the time required for the CEUS procedure is offset by the time saved in the SLN dissection.

Perflubutane (Sonazoid, GE Healthcare) is a new ultrasound contrast agent that is safe and has a low risk of adverse events in both animals and humans.\textsuperscript{15} It has been increasingly used for detection of lymph node and liver metastases in multiple tumor types\textsuperscript{16–18} due to preferential uptake by cells of the reticuloendothelial system. Our results are consistent with the findings of Goldberg et al.\textsuperscript{10} that CEUS with perflubutane can identify SLN associated with cutaneous melanoma. Our finding that SLNs with metastatic melanoma had metastatic cells scattered throughout the SLN in a pattern not correlated with the pattern of enhancement on US is consistent with the conclusions of Goldberg et al. that sonographic characteristics alone cannot yet be reliably used for identification of malignant SLNs.\textsuperscript{10}

However, perflubutane’s predilection for uptake by the reticuloendothelial system provides a mechanism by which perflubutane-enhanced US can provide further insight into tissue characteristics. This possibility has been explored most thoroughly in hepatic imaging, for which recent work has demonstrated utility of perflubutane-enhanced US not only in identifying location of focal liver lesions but in characterization as benign or malignant.\textsuperscript{19} Another study showed that use of perflubutane-enhanced US improves the accuracy of diagnostic liver biopsy by differentiating viable from necrotic tissue.\textsuperscript{20} Finally, there is evidence from a mouse cholangitis model that perflubutane-enhanced US can detect impaired phagocytic activity associated with hepatic inflammation and hypoxia.\textsuperscript{21} Recently, modifications to the microbubble ligand have demonstrated the ability to label metastatic disease-containing SLN with a higher signal intensity than benign SLN.\textsuperscript{22} Although additional studies are needed to validate this methodology, it is possible that further innovations in molecular labeling techniques will increase the sensitivity of CEUS for malignant SLN detection and thus the overall clinical utility of this technique.

Detection of SLN with perflubutane-enhanced ultrasonography may complement the information provided by nuclear lymphoscintigraphy, thereby facilitating precise SLN dissection. Specific advantages include identification of the target lymph node in three dimensions, as well as avoidance of signal obscuration through the shine-through effect sometimes seen with radiotracer SLN mapping. These features are particularly relevant in the head and neck, where the high density of lymphatics makes a shine-through effect more likely due to the proximity of draining lymphatics to the primary tumor. Additionally, the dense lymphatic tissue of the head and neck increases the variability of drainage patterns of cutaneous lesions, which can increase the difficulty and operative time associated with SLN dissection. Thus, the ability to determine the depth of potential SLNs with CEUS is a benefit especially pertinent in the head and neck. This feature allows improved surgical accuracy in locating SLNs and may decrease the risk of injury to neighboring anatomical structures.

SLN mapping with CEUS may be done quickly and easily in the operating room at the time of surgery to confirm the results of the lymphoscintigraph and provide information on nodal depth. This could be done routinely or can be reserved for use in cases in which the

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Fig. 2. (A) Sentinel lymph node (arrow) associated with left flank melanoma. (B) Perflubutane-enhanced ultrasound demonstrating the lymphatic channel (small arrows) leading to the enhancing lymph node (large arrow).
lymphoscintigraphy is ambiguous or there is difficulty identifying SLNs with the gamma probe. Additionally, previous work with perflubutane describes injection up to 4 hours prior to surgical dissection of SLNs. This duration of enhancement potentially permits performance of CEUS and SLN mapping in the radiology suite prior to surgery, circumventing the need for any additional operative time. Although this possibility adds the risk of discomfort to the patient associated with a contrast injection, it carries the advantages of improved operative planning due to accurate knowledge of SLN locations and allows for more comprehensive preoperative patient counseling. Studies with other sonographic contrast agents describe the administration of contrast on the day

<table>
<thead>
<tr>
<th>Swine</th>
<th>Location of Primary</th>
<th>LN</th>
<th>Location</th>
<th>Distance From Primary (on skin, cm)</th>
<th>CEUS +</th>
<th>VB +</th>
<th>True LN?</th>
<th>Metastatic Tumor +</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>R posterior-neck</td>
<td>1</td>
<td>R cervical</td>
<td>13</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
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<tr>
<td>2</td>
<td>RLQ abdomen</td>
<td>1</td>
<td>R inguinal</td>
<td>18</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>NA</td>
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<td>2</td>
<td></td>
<td>2</td>
<td>R inguinal</td>
<td>18</td>
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<tr>
<td>2</td>
<td></td>
<td>3</td>
<td>RLQ Abdomen</td>
<td>4</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>NA</td>
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<tr>
<td>3</td>
<td>L flank</td>
<td>1</td>
<td>L subiliac</td>
<td>15</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>4</td>
<td>R lateral neck</td>
<td>1</td>
<td>R cervical</td>
<td>4</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
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<tr>
<td>4</td>
<td></td>
<td>2</td>
<td>R cervical (deep)</td>
<td>6</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
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<tr>
<td>5</td>
<td>L lateral thigh</td>
<td>1</td>
<td>L inguinal</td>
<td>19</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
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<td>5</td>
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<td>2</td>
<td>L subiliac</td>
<td>15</td>
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<td>Y</td>
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CEUS = contrast-enhanced ultrasound; L = left; LN = lymph node; Met = metastatic; NA = nonapplicable; R = right; RLQ = right lower quadrant; VB = vital blue.
prior to surgery. It is possible that perfluorobutane also has a longer duration of action, which would further increase the flexibility of the timing of the procedure and convenience for patients and facilities. More studies are needed to needed to clarify the exact duration of action of perfluorobutane to optimize clinical use.

Limitations of this study include the small number of animals used and the lymph nodes excised, which does not permit the assessment of statistical significance of our conclusions. Additionally, the small sample size precludes our ability to draw conclusions regarding any technical features of CEUS for SLN detection specific to cutaneous melanoma of the head and neck. Finally, we are unable to formally compare CEUS with lymphoscintigraphy due to the lack of a comparator group of animals undergoing lymphoscintigraphy. As a result, we do not intend to recommend CEUS as a replacement for, but rather as an adjunct treatment to, lymphoscintigraphy for SLN identification. In this study we used VB, which is able to reliably detect efferent lymphatic vessels and sentinel nodes as a visual aid in nodal dissection. Although this comparison does not represent formal statistical hypothesis testing, the overall agreement observed between VB and CEUS for lymphatic detection allows us to recommend further investigations of CEUS for this purpose.

CONCLUSION

From this pilot study, we conclude that CEUS with perfluorobutane (Sonazoid, GE Healthcare) is a feasible method of identification of SLN associated with cutaneous melanoma. CEUS may be a useful adjunct technology in facilitating precise SLN dissection during the treatment of cutaneous melanoma of the head and neck. Our results support the initiation of a clinical trial investigating the use of CEUS for SLN dissection in the head and neck.

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BIBLIOGRAPHY