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Utilization of iron (III)-doped nanoshells for in vivo marking of nonpalpable tumors using a VX2 rabbit model

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Abstract

BACKGROUND: We aimed to evaluate the potential for ultrasound (US) visible biodegradable nanoshells (NS) as an alternative to wire-guided localization for nonpalpable tumors in vivo.

METHODS: VX2 tumor was injected in bilateral thighs of 22 New Zealand rabbits and after 5 to 10 days, 1 tumor was marked with a wire as a control and the contralateral tumor was injected with 1 mL of 500 nm gas-filled silica NS under Doppler US. Tumors were excised after 24 hours. Chi-square was used for significance, $P = .05$.

RESULTS: One rabbit was excluded on postoperative day 1 due to equipment failure, no ill effects were observed from the NS. The NS were used to localize and resect 100% of marked tissue, 4/21 wires were displaced ($P < .05$).

CONCLUSIONS: We have shown that preoperatively injected US visible silica NS can be successfully used to mark nonpalpable tumors in vivo more consistently than WL.

Breast conserving surgery is the standard treatment for early-stage breast cancer and to 25%–30% of the diagnosed and treated breast cancers are nonpalpable requiring preoperative localization for successful resection.[1–4] Current strategies for marking these tumors include using a wire or implanted radioactive seeds to guide the surgeon to the biopsy-proven tumor.

The current gold standard and most frequently used method for localization is image-guided wire localization (WL). This technique relies on a thin hooked wire that is preoperatively inserted into the tumor under ultrasound or stereotactic guidance by a radiologist to guide the surgeon to the lesion. WL has inherent limits to precise localization

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because the external portion is vulnerable to displacement, and it only provides a single dimension of reference for a 3D tumor volume; consequently, reported rates of positive margins range from 14% to 47%.[5] To minimize the risk of displacement, the wire must be placed shortly before the procedure. Patients also report high levels of discomfort even associated with the wire[6]. Other techniques that address some of these concerns include radio-guided seed localization (RSL) with an internally placed seed. Unfortunately, RSL has been shown to have similar high rates of positive margins requiring re-excision and or mastectomy[5,7,8] Just as with "wire only", with "RSL only" a single plane of the tumor can be marked with the seed consistent with the continued high rates of positive margins[9] The irregular growth of nonpalpable tumors is postulated to contribute to the high rates of positive margins for both techniques.[9,10]

Intraoperative ultrasound-guided lumpectomy has demonstrated excision rates comparable to WL.[11] Studies reveal that up to 95% to 100% of the nonpalpable lesions can be successfully located with ultrasound.[12] This present study demonstrates the utility of localizing nonpalpable tumors with ultrasound visible gas-filled nanoshells (NS) to allow for rapid intraoperative localization and excision of nonpalpable tumors. This technique allows for the tumors to be marked multiple days prior and does not rely on radioactive seeds or vulnerable external wires.[13,14]

Methods

Nanoshells

The 500-nm hollow ultrathin iron (III)–doped silica NS were synthesized as previously described.[15–17] The hollow silica NS are subjected to at least 3 cycles of vacuum desorption followed by perfluoropentane filling. The perfluoropentane gas filled NS are suspended in degassed water at a concentration of 4 mg/mL.

Tumor model

The VX2 rabbit model was employed as rabbits were deemed the smallest species available (a) with an area of tissue of sufficient size to be able to implant and excise a tumor without severely impacting the mobility of the animal, (b) have tumors which were nonpalpable but visible with ultrasound, and (c) have sufficient muscle mass to allow for negative margins to potentially be achieved. The VX2 rabbit model has the additional advantage that it can be introduced and grown in a consistent time line rather than forming spontaneously. This allows for multiple rabbits to have the same time line for injection, marking, excision, and evaluation for regrowth.

This study was approved by the Institutional Animal Care and Use Committee of the University of California San Diego. Twenty-two female New Zealand White rabbits age 10 to 12 weeks were housed in an approved animal housing facility and kept at 20°C with a 12-h light/dark cycle. The animals were fed a commercially pelleted diet (Harlan Tecklad) ad libitum. Rabbits were anesthetized with isoflurane gas with oxygen during the procedures and excisions of primary tumors.

After the prescribed 72-hour acclimation period, the rabbits were injected with 1 cc of VX2 slurry in bilateral rear thighs. The tumors were allowed to grow between 5 and 10 days to a nonpalpable tumor approximately 2 to 5 mm in size. Twenty-four hours before excision, each rabbit had 1 tumor marked using B mode ultrasound-guided wire placement to serve as the control. The contralateral thigh was marked with approximately .5 to 1.0 cc of 500-nm gas-filled silica NS with Doppler-guided ultrasound (Fig.1). During imaging of the NS, the US parameters were optimized to maximize signal from particles while reducing background in all cases, as is done clinically. A frequency of 7 MHz and a mechanical index of 1.9 were chosen for Doppler imaging parameters, as in previous studies.[13] The US machine employed was the Siemens Acuson Sequoia 512 with the 15L8 transducer which is traditionally used for breast US imaging.

The marking of the tumors in this model requires general anesthesia for the animals and recovery time was required before the second general anesthesia for tumor excision, thus the wires were implanted 24 hours before tumor excision. This approach allowed for both marking strategies to be evaluated in an equal fashion and although more time was allotted to pass between marking and excision than is clinically standard, the transfers and potential manipulation of the wire was limited to minimize the potential risk of wire displacement. In addition, this necessary strategy allowed evaluation of the NS as a potential tumor marker that could be decoupled with the day of surgery.

The tumors were excised under closely monitored isoflurane anesthesia. The wire side was excised first to minimize any manipulation of the wire. After the wire side was closed, the nanoparticle side was imaged with the optimized Doppler parameters. The tumor was excised based on the Doppler image. The excised tissue was imaged and evaluated for evidence of NS in the sample, as was the tumor cavity. All tissue with evidence of remaining NS was excised at that time (Fig. 2).

Post operatively, the rabbits were allowed to recover for approximately 3 weeks until the animals were sacrificed to allow for evaluation of regrowth in the separate tumor beds. All pathology specimens were evaluated by a board-certified pathologist and tissue from the surgeries were evaluated for evidence of excised tumor and positive margins, which was defined as tumor touching ink (Fig. 3).

Statistics

Chi-square statistics were employed to compare the success of excision between the wire and the NS as well as the percent regrowth at a P value of .05.

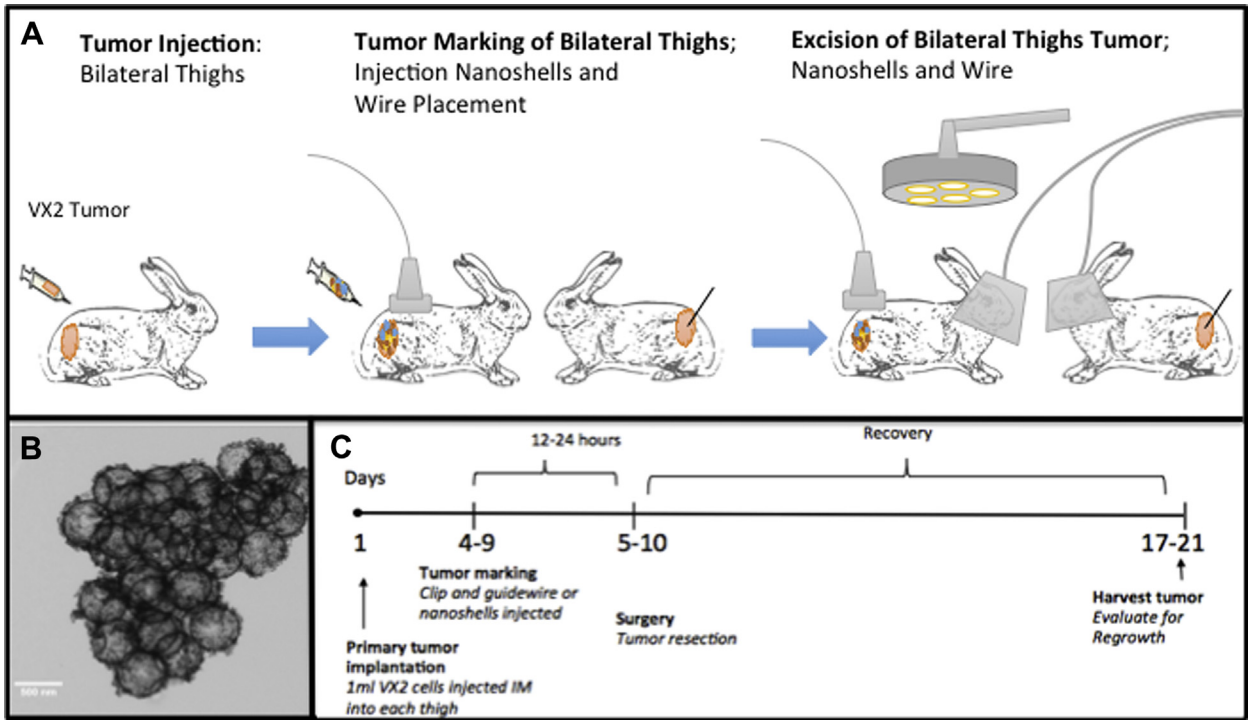

Figure 1 Experimental design, nanoshells, and timeline (A) Schematic of experimental design. Each New Zealand White rabbit was injected with 1 cc of VX2 tumor in bilateral thighs. Tumors were marked with NS or wire for subsequent surgical removal under ultrasound guidance. (B) TEM image of the 500-nm NS before injection. (C) Timeline for tumor injection, growth, marking, and recovery of the VX2 model in 21 White New Zealand rabbits.

Results

All 22 rabbits survived the initial surgery; one rabbit was euthanized on postoperative day 1 secondary due to a burn from equipment failure and was excluded from the study. No direct ill effects were observed from the injected NS. After marking the tumors, the rabbits were allowed to recover for approximately 24 hours before the excision of the tumor. Despite extensive efforts to minimize the displacement of the wire, the wire was displaced in 4 of the 21 surgeries. The NS that were injected in the tumor were successfully visualized through the skin and within the tumor cavity in 100% of the surgeries, significantly more than for the WL ($P < .05$; Fig. 4).

NS were confirmed within the initial tissue excision in 100% of NS-marked tumors. After excision of the specimen on the particle side, the cavity was explored with the Doppler ultrasound to evaluate for any additional visible NS and an additional margin was taken to remove the any ultrasound positive marked. (see Fig. 2). At the conclusion of the procedure, the cavity was verified to be clean of all visible NS in 21/21 NS-based excisions, as there is currently no way to preform such confirmation with WL, this was significantly different from the WL ($P < .05$).

After the excision, the specimen was evaluated for both presence of tumor within the specimen and evidence for positive margins. Of the 21 WL-based excisions, tumor was identified in 17 samples and margins were negative for 5 of the 17 successfully excised tumors. In contrast, 19 of the 21 NS-marked tumors were excised and of these 9 had negative margins. While the NS overall had a higher percentage of negative margins on excision than the WL, addition rabbit experiments will be needed for definitive statistics since the P value was modest. ($P = .27$).

After 17 to 21 days after the surgery, the animals were euthanized to allow for exploration of bilateral tumor cavity for regrowth. A pathologist reviewed the excised tissue for evidence of regrowth. Overall 17 of the 21 WL tumors regrew and 16 of the 21 NS-marked tumors regrew. This had no statistical difference. ($P = .71$).

Comments

Breast cancer accounts for 1 in 3 of all cancers in the US and current statistics support that 1 in 8 American women will develop breast cancer during their lifetime.[18] Screening mammography and public awareness has led to an increased number of breast cancer diagnoses and a shift toward diagnosis at an earlier stage.[3] The primary approach to early stage, small breast cancers is breast-conserving treatment with adjuvant radiation and surgeons rely on preoperative localization to obtain negative margins when resecting nonpalpable tumors.[4] Currently the gold standard is WL, but positive margin rates are reported as high as 47% in reports.[19]

A VX2 tumor in a rabbit model was employed to compare the efficacy of 500-nm silica NS as an ultrasound visible marker for nonpalpable breast cancer localization to the gold standard WL. Nineteen percent of the wires were displaced

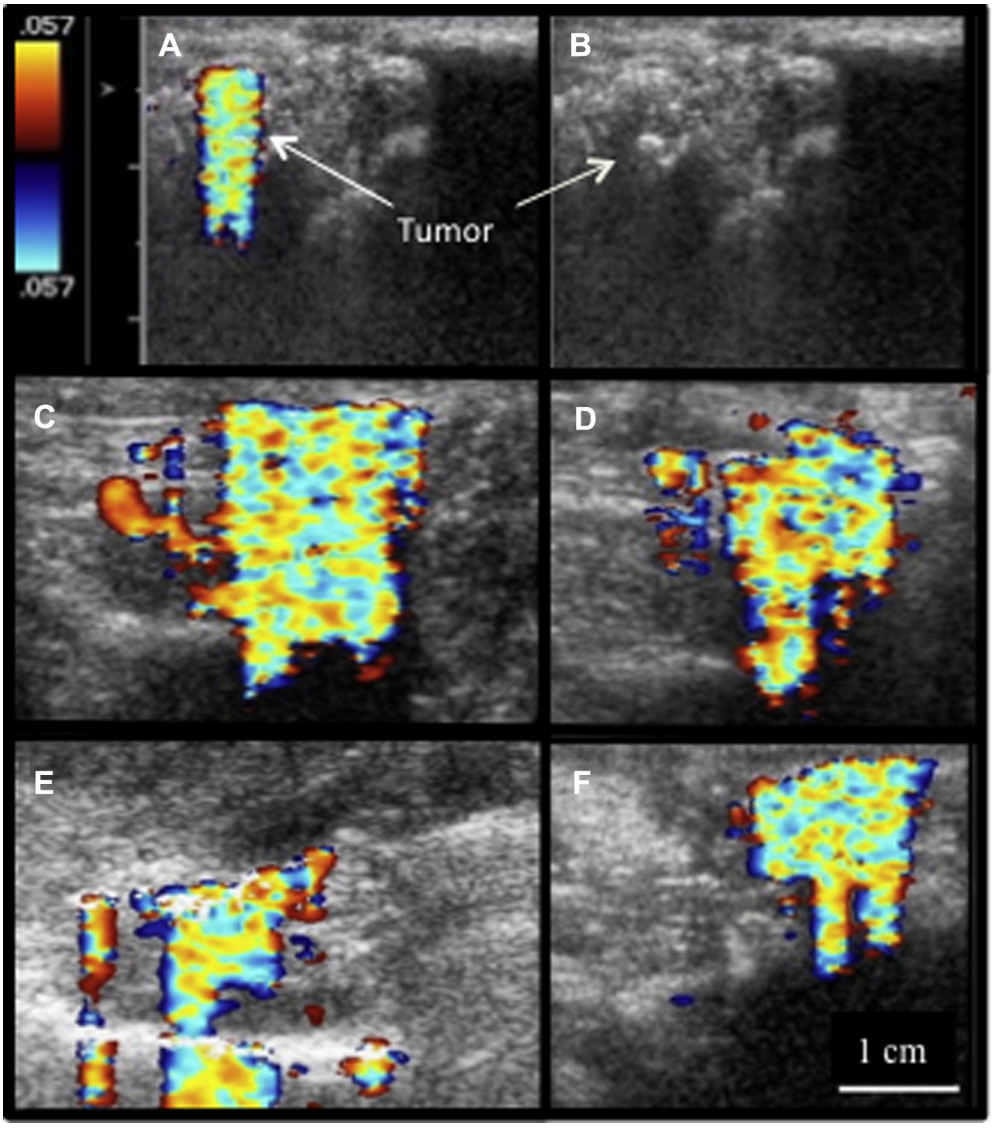

Figure 2 Color Doppler in-vivo nanoshell imaging: intraoperative ultrasound image of nonpalpable tumor (arrows) after marking with 500-nm silica nanoshells 24 hours before excision in (A) Doppler mode and (B) simultaneous B mode. (C) Intraoperative image of nanoshells (NS) in tumor through skin. (D) Same tumor with probe directly on the muscle surrounding marked tumor. (E) Representative image of excised tumor with NS. (F) Representative image of NS found in cavity of excised tumor for margin removal.

and were unable to be used for intraoperative localization. Conversely, using the same localization procedure, a 24-hour delay after the injection of the NS did not cause displacement of the NS allowing for 100% of all marked tissue to be identified intraoperativley for the 21 NS-marked tumors.

The inherent flaws of WL include the propensity of the wire to migrate from the initial placement and that wire is limited in its ability to represent a 3D structure that may be irregular in shape as it only identifies one segment of the lesion.[9,10] While lesion bracketing with multiple wires has be used to try to overcome this challenge, Liberman et al[20] did not find improvement in rates of negative margins.

In clinical settings, wires must be placed on the day of surgery and all efforts must be made to minimize the delay in excision after wire placement leading to significant scheduling difficulties and often operating room delays. Studies comparing the RSL to WL have shown that because the radioactive seed can be placed before the day of the surgery, waiting times, patient satisfaction, and scheduling difficulties are significantly reduced with RSL.[8]

The NS as a marker overcomes many limitations as the NS are injected intratumorally and previous studies have demonstrated that not only are the NS stationary, they are also visible up to 10 days after initial injection.[13,14] The NS have been shown to be nontoxic and biodegradable in previous animal studies.[13,14] The NS are less restrictive in the way a tumor is marked as it allows for a multidimensional marking. The injection of the particles is flexible in that a radiologist could inject NS to mark multiple tumors and fill irregular shaped tumors, minimizing the amount of interpretation that must be done intraoperatively. This ability to fill the entirety of the tumor may allow the

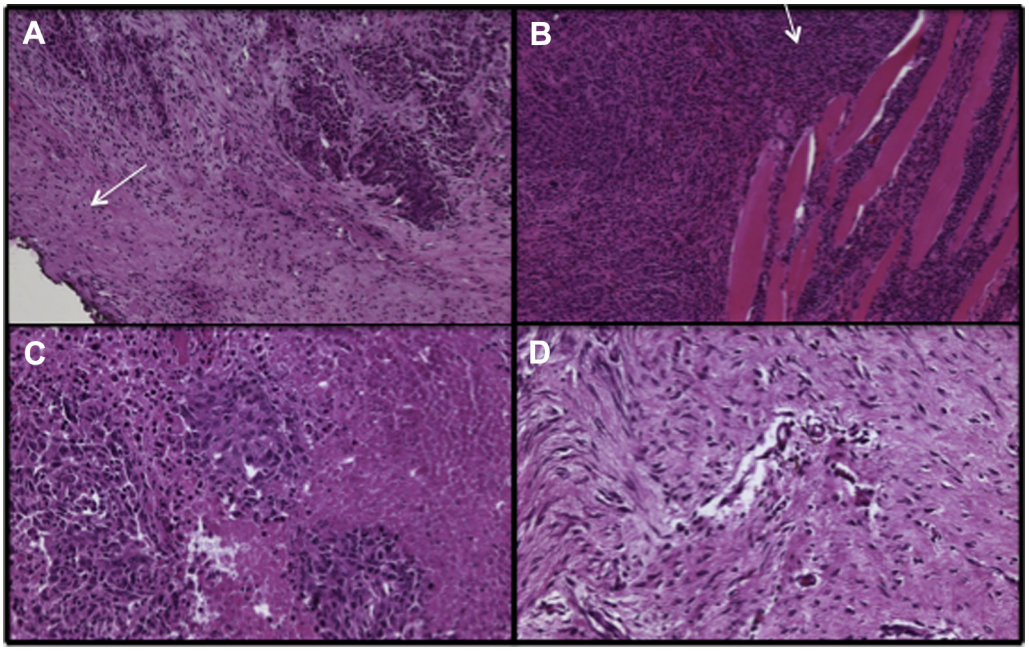

Figure 3 Tissue histology of wire-guided (WG) and nanoshell (NS)-localized surgeries. (A) WG excised tumor with positive margins at the arrow (100×). (B) Infiltrating tumor seen in NS-marked sample invading the surrounding muscle at the arrow (100×). (C) Post-mortem example of regrowth after excision in WG surgery (200×) and (D) Post-mortem example of a tumor bed without evidence of regrowth (200×).

NS to outperform RSL in future studies. The NS ability to remain stationary, even in the face of extreme movement of this animal model supports that this technique is immune to the traditional vulnerability of displacement of the wire. In addition as the NS can be injected days before the procedure, it can be assumed that scheduling benefits seen with the RSL could also be generalized to this strategy.

Although other US contrast agents have been developed, the longevity and the ease of visualization are not

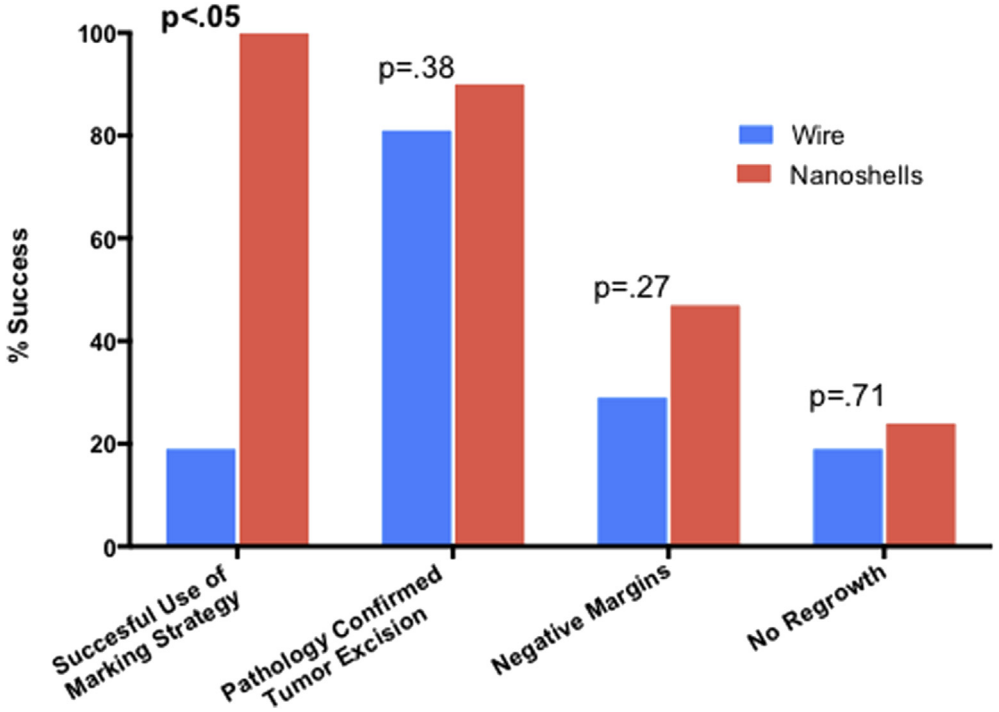

Figure 4 Histogram comparison between wire and nanoshell (NS) strategies for ultrasound-guided tumor marking and resection. The tumors marked with nanoshells (NS) were able to be successfully seen in 100% of all excisions, whereas the wire was displaced and unable to be utilized in 4 surgeries ($P < .05$). The tumor was successfully excised in comparable numbers when comparing the wire to the NS. Margin analysis and regrowth analysis found that NS were similar for both types of marking strategies.

comparable to the NS.[13] With NS, the surgeon is able to visualize the particles within the tumor initially through the skin and then again when imaging directly in the tumor bed with ultrasound, as was demonstrated in this model. This model also demonstrated the potential to be able to use the US within the tumor cavity after excision to verify that all the tumors those were marked has been excised and verify that there are visible NS within the tissue excised.

The rates of positive margins in this model for both the WL- and NS-based excision is likely secondary to the limitations of the model because the model has inherent limitations for achieving successful complete excision with negative margins. Unlike in human breast lumpectomies where cosmetic outcome is often the main factor for consideration in limiting total tissue resected, in this model the amount of tissue excised is limited due to concerns for mobility. To allow for survival surgery, the animal must be able to ambulate postoperatively, which limits the extent of the excision. Therefore, the rate of positive margins for both the NS and the WL was likely influenced by the model in addition to the technique used to mark the tumors. The wire was secured as much as possible using multiple tools but again the model requires the placement of the wire in the thigh of a mobile animal likely contributed to the relatively high rate of loss the wire in 19% of the WL excisions. In the same circumstances, the NS were found to be unaffected by the animal's mobility before the excision of the marked tumor. The elevated rates of regrowth in both WL and NS may also be secondary to the fact that the rabbits did not undergo postoperative radiation that is known to be critical to treat the micro-disease.[10]

The technique of injecting the tumor is a relatively facile procedure that does only requires equipment that is readily available to both surgeons and radiologists. The NS are easily visualized using Doppler US on clinical US machines and allow the radiologist marking the tumor to communicate through a stable mechanism a 3D structure up to 10 days before a planned OR procedure eliminating many of the inherent flaws of WL.

Conclusions

Localization of nonpalpable tumors remains critical to effective surgery and thus treatment of early-stage breast cancer. It has been demonstrated that preoperatively injected silica nanoparticles can be successfully used to mark nonpalpable tumors in vivo more consistently than the current gold standard wire-based platform. This study establishes a new category of tumor marking strategies using materials based on nanoparticles with the ability to mark 3-D physiological tumors structures that are not always captured well using traditional marking techniques.

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Discussion

Discussant

Dr. Barbara Pockaj (Phoenix, AZ): I want to thank Erin for performing a nice study and a nicely written manuscript that I got on time. Based on the study, I have the following questions.

I think it is obvious that this technique should be superior to wire localization for nonpalpable breast lesions. Since many surgeons can use ultrasound in the OR, how much will this help for those nonpalpable but ultrasound visible lesions? Is this better than the products that are already on the market which deploy things such as 10 polylactic acid/polyglycolic acid pellets, which are Vicryl, and provide 4 to 6 weeks of ultrasound visibility?

Two, in the same vein, as alluded to in your manuscript and in your introduction, do you plan to compare this technique to radioactive seed localization?

Finally, when do you think this is going to be ready for clinical trials? So you have this in the rabbit model. It looks fine. So what's holding you up for patient trials?

Dr. Erin Ward (San Diego, CA): Thank you for your questions. To address the first question in terms of how this is going to change for surgeons who already use the ultrasound in the OR, for the novice surgeon who may not feel as comfortable with identifying tumors, even if they are visible on the ultrasound, we believe that these nanoparticles are much easier to identify and also allows the surgeon to still rely on that radiologist to do the initial marking and interpret multiple types of studies that were done preoperatively, like the mammogram or even an MRI, to try to better target the tumor for excision.

Some of the products that are now available, while they are visible for multiple weeks, we believe that ours are more easily visualized than some of the products that are on the market right now. We would like to compare it to a radioactive seed localization, as it being an internal marker and being able to be placed multiple weeks before makes it a better comparison. Unfortunately, our IACUC has not allowed that. We'd likely jump to the human study.

In terms of when we are going to get this ready for FDA approval, a company is being formed called Nanocyte, and then after that, there will be pursuit for FDA approval and studies in humans.

Dr. Ronda Henry-Tillman (Little Rock, AR): Thank you, Dr. Ward, for your presentation. I have two questions. As alluded to, hematoma is very visible on ultrasound as well. It lasts up to 3 weeks after a biopsy, and 90% of women have hematoma after a biopsy. You did not allude to that at all in your presentation as a way to remove lesions.

Second, what is the estimated cost? Finally, one of the biggest problems with needle localization is timing. How do you estimate where to put these particles to determine margin status?

Dr. Erin Ward (San Diego, CA): In terms of timing with the wire localization, we hope that the nanoshells will kind of fix some of the problems, as it can be placed multiple days prior like radioactive seed.

In terms of the first question with the hematoma, I think you could still use this to outline. So if you didn't want to use just the hematoma to guide what you excised, if there were calcifications that were felt to correlate with under the ultrasound, they could still get a better idea of the 3D tumor. Perhaps it would be in addition.

Dr. Anne Mancino (Little Rock, AR): Along that line, you talk about injecting this around lesions that are visible on ultrasound. Would you see yourself also using this on something that had only been visible on mammogram? And how would they inject it? Also, is there any problem with these particles being present when they are looking at the pathology?

Dr. Erin Ward (San Diego, CA): We have evaluated the pathology. The board-certified pathologist hasn't seen any difference between those that have been injected with the nanoshells or without when we compare with wire localization.

In terms of being able to mark the lesion based on mammogram, I think we would still be limited currently to the ultrasound just because that gives you confirmation of where you have actually injected it. Potentially, you could use a combo of ultrasound and mammography. That could be used in the future.