Robotic Real-time Near Infrared Targeted Fluorescence Imaging in a Murine Model of Prostate Cancer: A Feasibility Study

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OBJECTIVE To evaluate the detection of near-infrared fluorescence from prostate tumors stained with a prostate-specific membrane antigen (PSMA)–targeted tracer developed in our institution with a novel robotic imaging system.

METHODS Prostate cancer cell lines PC3-pip (PSMA positive) and PC3-flu (PSMA negative) were implanted subcutaneously into 6 immunodeficient mice. When tumors reached 5 mm, a PSMA-targeted fluorescent conjugate was injected intravenously. The first 3 mice underwent near-infrared imaging immediately and hourly up to 4 hours after injection to determine the time necessary to obtain peak fluorescence and were killed. The last 3 mice were imaged once preoperatively and were euthanized 120 minutes later. Excision of the tumors was performed by using a novel robotic imaging system to detect near-infrared fluorescence in real time. Specimens were submitted for pathology.

RESULTS In the first 3 mice, we found 120 minutes as the time needed to observe peak fluorescence from the PSMA-positive tumors. We identified discrete near-infrared fluorescence from 2 of 3 PSMA-positive tumors with the robotic imaging system. Surgical margins were negative for all excised specimens except for one PSMA-negative tumor.

CONCLUSIONS Real-time near-infrared fluorescence imaging of prostate cancer is feasible with a novel robotic imaging system. Further research is needed to optimize the signal intensity detectable from prostate cancer with our tracer. Toxicologic studies are needed before its clinical use. UROLOGY 81: 451–457, 2013. © 2013 Elsevier Inc.

The use of near-infrared (NIR) fluorescence is a promising approach for biomedical imaging in living tissue. NIR fluorescence (700-1000 nm) detection avoids the natural background fluorescence interference of biomolecules, providing a high contrast between target and background tissues. Recently, the Food and Drug Administration (FDA) approved for marketing the Intuitive Surgical da Vinci Fluorescence Imaging Vision System, an integration of the SPY imaging technology (Novadaq Technologies, Mississauga, ON, Canada) into the 3-D high-definition imaging capabilities of the da Vinci Surgical Robotic System.

Use of the scope in urology has so far been limited to renal surgery, as kidney fluorescence is intense after intravenous administration of a nontargeted agent such as indocyanine green. Conversely, the prostate does not fluoresce after intravenous administration of a nontargeted agent, and therefore the fluorophore must be modified to specifically bind the prostate.

Prostate-specific membrane antigen (PSMA) is a cell surface glycoprotein with a molecular weight of approximately 100 kDa. It is not expressed in significant amounts in the prostates of mice, dogs, or monkeys.[1] PSMA is expressed in high levels in the human prostate, especially in prostate cancer cells and in the vasculature of primary and metastatic prostate tumors.

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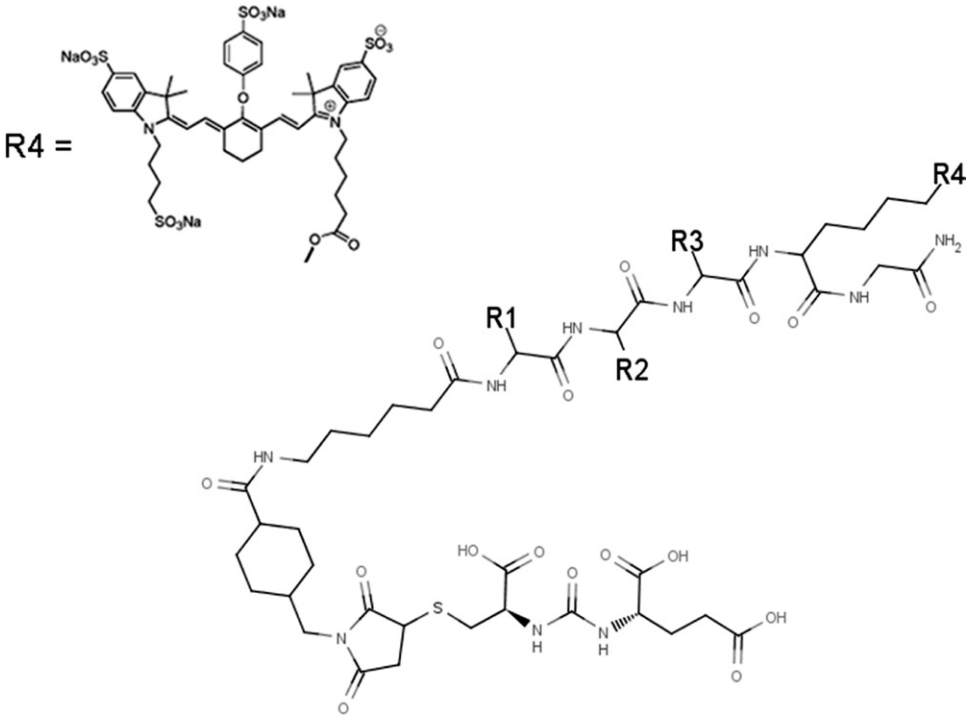

Figure 1. General schematic representation of our elongated PSMA binding tracers (Huang, unpublished). For ZJ-MCC-dEdEdEGK(IRDye800cw)G, R1, R2, and R3 are D-glutamic acid residues; IRDye800cw is attached at the R4 position.

In this study, we aimed to evaluate the real-time detection of NIR fluorescence emitted by a PSMA-targeted agent using the Intuitive da Vinci Fluorescence Imaging Vision System.

MATERIAL AND METHODS

PSMA Ligand

The PSMA targeting ligand ZJ-MCC-dEdEdEGK(IRDye800cw)G is synthesized using well-established fluorenylmethyloxycarbonyl (FMOC) solid-phase peptide synthesis chemistry. Using a rink-amide or equivalent resin, peptide synthesis started with a stem containing the DGlu-DGlu-DGlu-Gly-Lys-Gly sequence. At the N-terminal end of the stem, the linker succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate [SMCC], Thermo Scientific) was added. Then a glutamate and cysteine containing urea compound (R)-Cys-C(O)-(S)-Glu was added.[2] After coupling, the entire compound was then cleaved from the solid phase, deprotected with 95% trifluoroacetic acid and purified. An activated ester of IRDye800cw (Li-Cor- *BioScience*, Lincoln, NE), was then reacted with the primary amine on the lysine to create the final tracer (Fig. 1). The final product was purified by high-performance liquid chromatography (HPLC) and reconstituted in 0.2 mL of phosphate-buffered saline, pH 7.

Injection of PSMA-Ligand and Preoperative NIR Imaging

PC3-pip (PSMA-positive) and PC3-flu (PSMA-negative) cells were injected subcutaneously 1.0×10^6 PC3 cells with 200 μL of matrigel into each flank of 6 NOD SCID Gamma (NSG) mice.[3] After the tumors reached a size of ~5 mm, the mice were anesthetized with isoflurane and were administered 2 or 10 nmol of PSMA-binding fluorescent conjugate via tail vein injection. Preoperative NIR imaging was performed using the Maestro in vivo imaging system (Cambridge Research and Instrumentation, Hopkinton, MA). The mice were imaged immediately and at 1-hour intervals after injection of the PSMA targeting fluorescent compound for up to 4 hours. The mice were killed once PSMA+ and PSMA-tumors could be visually differentiated by NIR imaging. The method used was CO_2 asphyxiation followed by cervical dislocation. The procedure was approved by the Institutional Animal Care and Use Committee (protocol # 2010-0340).

NIR Fluorescence-Guided Robotic Surgery

Mouse carcasses were operated on using a da Vinci Si Robot (Intuitive Surgical, Sunnyvale, CA). A portable dark box was used to minimize ambient light and to allow optimal fluorescence detection. Three robotic instruments (the novel endoscope to detect NIR fluorescence, a scissor, and a grasper) were inserted through robotic trocars positioned across the top cover of the dark box. We excised the tumors, using the new robotic camera to detect the fluorescence of our PSMA-binding conjugate. All procedures were done by a single surgeon (R.S.), who had extensive experience in robotic surgery. Tumor specimens were sent for histopathological analysis to assess margin status.

RESULTS

All mice were male, with an average weight of 22 g and a mean age of 49 days by the time that they were killed. The mean size of the tumors was 5.4 mm.

In the first 3 mice, we identified 120 minutes as the time to detect peak fluorescence from the PSMA-positive tumors with the Maestro imaging system (Fig. 2). Within

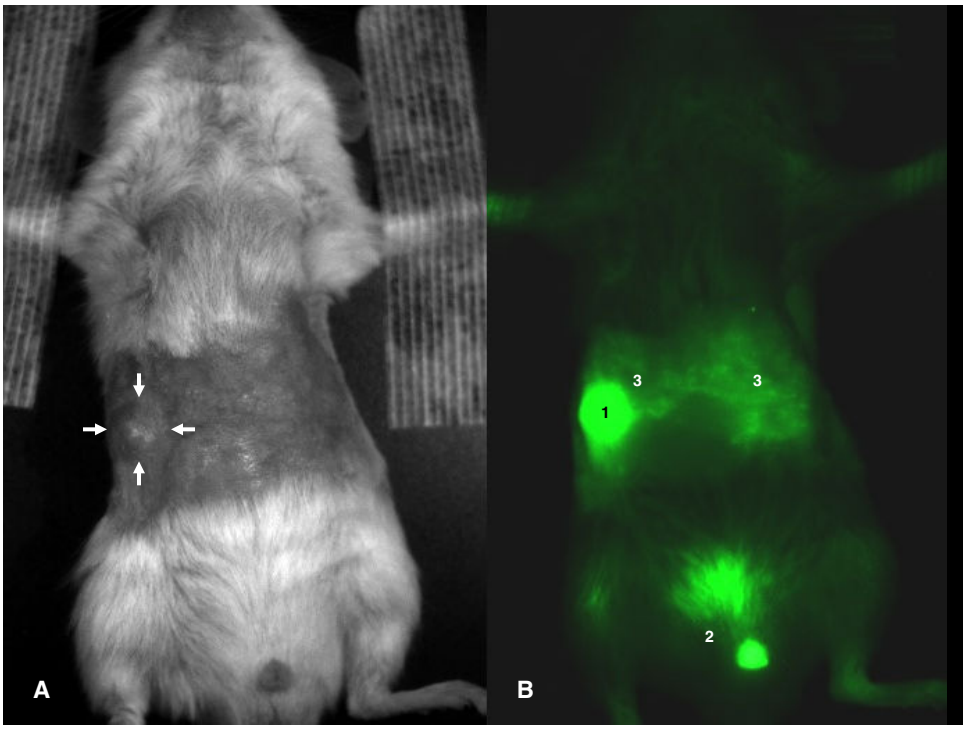

Figure 2. Preoperative white light **(A)** and near-infrared (NIR) fluorescence **(B)** imaging with the Maestro in vivo imaging system. Note the strong fluorescence signal from the tracer in the right flank (PSMA positive tumor), bladder, and urethra, and the background fluorescence signal from the kidneys. Arrows indicate bulging of the tumor in the skin.

240 minutes, the signal intensity from the tumor was already partially decreased. Based on this information, the last 3 mice were killed at 120 minutes after injection of our conjugate and immediately underwent robotic surgery. Two of the 3 mice injected with 10 nmol of fluorescent compound demonstrated proper targeting on presurgical NIR imaging, whereas the third mouse demonstrated minimal targeting.

During robotic surgery, we were able to detect fluorescence from the PSMA positive tumors in 2 of 3 mice that were injected with 10 nmol of fluorescent compound. However, the intensity of fluorescence was weak (Fig. 3). We did not observe NIR fluorescence in the base of PSMA positive or PSMA negative tumors. In the PSMA negative (control) tumors, no tumor fluorescence was identified in any of the mice. In the subcutaneous tissue, the fluorescence was limited to the PSMA positive tumors, we did not observe loss of contrast with surrounding tissues. Because of the compound's biodistribution and clearance, fluorescence from internal organs (liver, kidneys, and bladder) could also be observed, even without penetrating the abdominal wall musculature, but it did not impair the visualization of the subcutaneous tumor. Given the poor biodistribution of the described fluorescent marker in tissues not directly targeted, loss of contrast compared with surrounding tissues is possible, yet unlikely, with higher concentrations of administered conjugate. Nevertheless, further evaluation is needed to test this hypothesis.

PSMA-positive and -negative tumors had the same gross appearance, except for detectable fluorescence in two of the PSMA positive tumors. We did not perform any microscopic analysis during resection. Microscopy was obtained later at histopathologic examination, and no fluorescence imaging was performed at that time. The microscopic appearance of PSMA-positive and -negative tumors was indistinguishable. Pathologic examination confirmed prostate cancer and identified negative margins in all 3 PSMA-positive tumors. One of the PSMA-negative tumors had a positive margin (Fig. 4).

COMMENT

We were able to detect NIR fluorescence from prostate cancer implanted in mice with 2 different imaging systems after the intravenous injection of a new compound targeted to PSMA. The prostate tumors were then resected using the Da Vinci Si robot equipped with the Fluorescence Imaging Vision System, and tumor fluorescence was noted in 2 of 3 mice. To our knowledge, this is the first description of fluorescence detection from prostate cancer stained with a PSMA-targeted agent by an imaging system developed for minimally invasive surgery.

The real-time intraoperative enhanced visualization of organs and tissues with NIR fluorescent dyes has enormous potential clinical applications, and one of its most promising uses is in oncology. Few NIR fluorophores, such as indocyanine green (ICG) and methylene blue

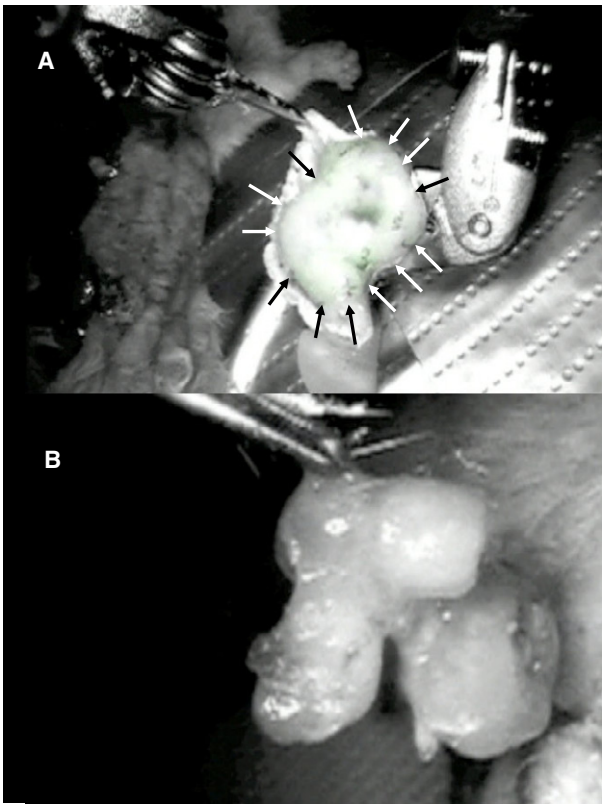

Figure 3. Intraoperative images of prostate tumors excised from mice using the da Vinci Si system with a fluorescence detecting endoscope. **(A)** Fluorescent staining of a PSMA-positive tumor implant using a PSMA binding, fluorescently tagged compound (arrows). **(B)** Nonstaining of a PSMA negative (control) tumor implant.

(MB), are approved by the FDA and available for clinical use. In vivo optical imaging probes aiming to identify prostatic tissue intraoperatively are under intense investigation by several groups. PSMA is a potential target for both imaging and treatment purposes.[4] Chen et al synthesized YC-27, also a PSMA-based imaging agent and conjugated it to IRDye 800CW.[5] Eifler et al identified in vitro and in vivo NIR fluorescence imaging of LNCaP and PC3-PIP cells using the Fluobeam (Fluoptics, Grenoble, France), which can be used to detect NIR fluorescence during open surgery.[6,7] Liu et al developed Cy5.5-CTT-54.2, another PSMA-targeted NIR fluorescent imaging probe, and identified in vitro NIR fluorescence of LNCaP cells.[8] Recently, Nakajima et al synthesized a PSMA-targeted activatable monoclonal antibody fluorophore conjugate (J591-ICG) and detected both in vitro and in vivo fluorescence of PC3-PIP cells.[9] These new imaging probes, as well as our own conjugate, must undergo toxicity studies before initiating clinical investigation.

Gordetsky et al investigated the use of NIR fluorescence lymph node imaging with open surgery in 14 patients with bladder cancer, using ICG injected at the tumor base.[10] They used the SPY imaging system (Novadaq Technologies, Mississauga, ON, Canada) to detect fluorescence in pelvic lymph node specimens after 2-4 hours. They were able to identify from 1 to 14 lymph nodes per patient in more than 85% of the patients. One lymph node was positive for high-grade urothelial carcinoma. Van der Poel et al reported the use of a hybrid multimodal radiocolloid (ICG-^{99m}Tc-NanoColl) that is both radioactive and fluorescent.[11] After preoperative intraprostatic injection of the tracer under transrectal ultrasound guidance and removal of the prostate, they dissected the sentinel lymph nodes guided by a laparoscopic gamma probe (Europrobe, London, UK) and a fluorescence laparoscope (Karl Storz, Tuttlingen, Germany), being able to link preoperative single-photon emission computed tomography/computed tomography (SPECT/CT) guidance with intraoperative NIR fluorescence laparoscopy.

Several NIR fluorescent dyes have been developed, with properties that enable them to be conjugated to ligands or monoclonal antibodies directed to certain targets, producing agents molecularly specific to detect cancer cells. The increased tumor angiogenesis and expression of growth signaling receptors are key features that can be used to identify optimal targets. IRDye 800CW, Cy7, and Alexa Fluor 750 are some of the most commonly used fluorophores. The excitation and emission maxima of the IRDye 800CW are centered at 800 nm, which is the optimal wavelength for in vivo imaging, with minimal tissue absorption, autofluorescence, and scattering, yielding excellent signal to background ratios (SBR).[12] IRDye 800CW is highly water soluble and shows very low nonspecific binding to cellular components. Because of their biodistribution and clearance, most fluorescent agents have a high background signal in the kidneys, bladder, and liver.[13] This was observed in our experiment.

Marshall et al reported no pathologic evidence of toxicity of IRDye 800CW based on hematological, biochemistry, and histopathological analyses.[14] However, linking the fluorescent dye and the targeting moiety produces a new molecule that may have properties different from its original precursors. Thus, NIR contrast agents must undergo toxicity studies of the dye, the targeting ligand, and the final molecule before considering clinical application.[12]

For open surgery, some of the NIR camera systems developed for image-guided procedures are the SPY imaging system (Novadaq Technologies, Mississauga, ON, Canada), the Photodynamic Eye (Hamamatsu Photonics, Hamamatsu City, Japan), the Fluobeam (Fluoptics, Grenoble, France), and the Fluorescence-assisted Resection and Exploration (FLARE) and the Mini-FLARE (Fragioni Laboratory, Brookline, MA). In 2011, Intuitive Surgical received FDA approval to market the da Vinci Fluorescence Imaging Vision System. Tobis et al evaluated the new system in 11 patients who underwent robot-assisted laparoscopic partial nephrectomy (RALP), using intravenous ICG as the NIR fluorophore.[15] Of the 10

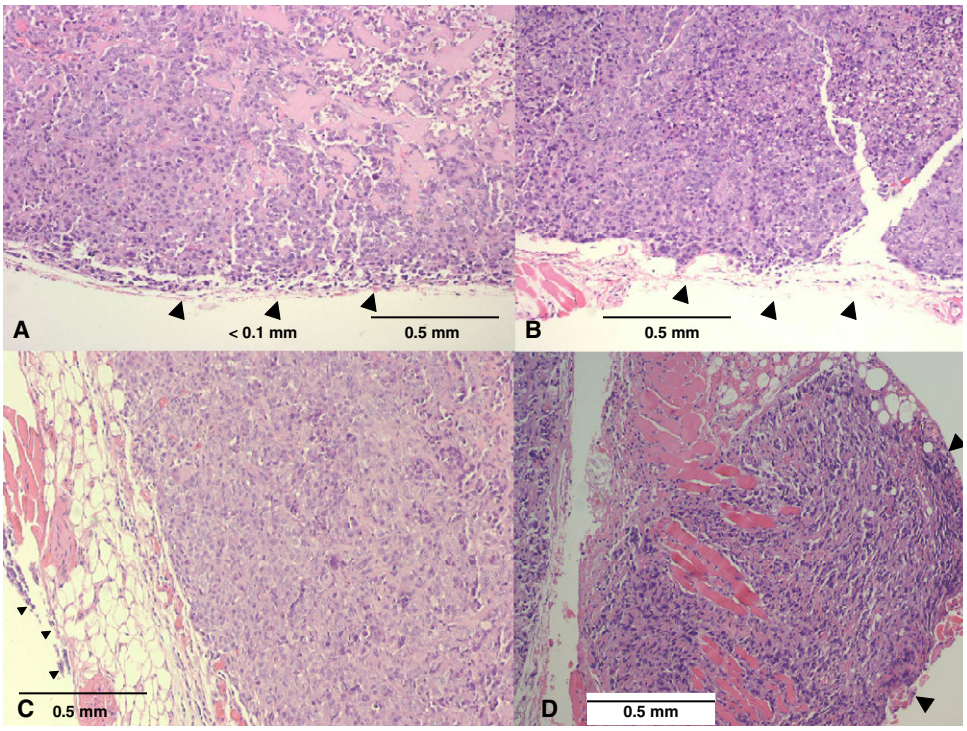

Figure 4. Pathology examination findings. **(A)** PSMA positive, margin negative. **(B)** PSMA negative, margin negative. **(C)** PSMA positive, margin negative. **(D)** PSMA negative, margin positive. Arrowheads indicate margins.

malignant tumors, 70% were hypofluorescent and 30% were isofluorescent in comparison with the normal parenchyma. The vascular anatomy was accurately delineated in all cases with this imaging method.

Fluorescence of organs, such as kidney and liver are straightforward, as nonspecific NIR fluorophores are significantly taken up by these tissues during normal clearance. The prostate, by contrast, does not retain a significant amount of nonspecific fluorescent probe, and therefore intraoperative fluorescence guidance is not possible. We thus sought to develop a targeted fluorophore conjugate that would specifically bind prostatic tissue and lead to detectable levels of fluorescence.

The use of implanted tumors derived from prostate cancer cell lines was chosen, as animals do not express significant levels of PSMA in the prostate. We operated on mouse carcasses instead of live animals, as we were able to observe NIR fluorescence with the Maestro system up to 48 hours after euthanasia in a previous study (Huang, unpublished).

Although we indeed detected NIR fluorescence from prostate tumors with both the Maestro in vivo imaging system and the new robotic scope, the fluorescent signal was considerably less with the robotic system. The fluorescence of the tumor noted by the robotic system was diffuse but with some patchy distribution. Because of the lack of more intense fluorescent staining, it is difficult to completely characterize the staining pattern of individual tissue components. With more intense staining, a complete assessment of the staining pattern should be performed. Several factors could possibly explain the decreased fluorescent signal noted with the robotic system. The camera of the Maestro system can be selected to receive longer periods of exposure to NIR fluorescence, which permits stronger signal detection but is not ideal for real-time image guidance. By contrast, the frame rate of the robotic scope is 30 frames per second, which was adequate for real-time detection of NIR fluorescence from other organs such as the kidney, but may not be optimal to detect fluorescence from prostate tissue with our tracer. Another possible explanation is that the concentration of 10 nmol, used in our experiments, may not be enough for strong real-time NIR fluorescence detection in prostatic tissue. We also cannot exclude the possibility that a certain amount of the tracer did not reach the intravascular space during the tail vein injection, which could have led to less accumulation in the tumor, impairing its visualization. Another limitation is the small sample size. However, this was mainly a proof-of-concept study, as it was the first time that we used this robotic scope to detect fluorescence from our PSMA-targeted conjugate.

Even without detecting fluorescence intensity as strong as that seen in the kidneys and bladder, we believe that our preliminary results are encouraging. Further studies are necessary to more precisely define the role of NIR fluorescence image-guided robotic surgery, identifying which procedures will be the most suitable for its application. We cannot precisely determine the cause for the positive margins in 1 tumor. Although evidence from this study is not intended or adequate to draw conclusions regarding the improvement of margin status, with further refinement of fluorescent visualization, the precision of tumor resection could potentially increase, minimizing damage to normal tissue and ideally

improving tumor margin assessment intraoperatively. Intraoperative robotic fluorescence detection in real time with nonspecific fluorophores, such as ICG, is readily available for clinical use. In the future, other fluorescent dyes suitable for binding to ligands that can be targeted to specific molecules may also obtain approval for clinical use, broadening the spectrum of procedures which can be enhanced by NIR fluorescence-guided surgery.

CONCLUSIONS

In this study, we demonstrated the feasibility of prostatic tissue identification and resection by using a novel robotic fluorescence imaging system in a murine model after the intravenous injection of a PSMA-targeted agent. Further research is warranted to improve real-time fluorescence detection of prostatic tissue and to move this technology toward its potential clinical application.

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EDITORIAL COMMENT

The authors of this work demonstrated, in 2 mice with implanted subcutaneous prostate cancers, that an intravenously injected compound targeting prostate-specific membrane antigen (PSMA) allowed visualization of tumor fluorescence using the da Vinci robotic system with Firefly capability (near-infrared fluorescence). Although this study was limited in animal number due to equipment availability, and although there remain many unanswered questions before clinical application could be pursued, the significance of this work should not be dismissed.

Robotic surgery has become the most common surgical therapy for prostate cancer in the United States and is a rapidly growing modality internationally. Despite earlier detection of prostate cancer, some men with organ-confined disease have positive margins and/or experience local recurrence from incomplete tumor resection. When invasive, the likelihood of positive margins is much higher, even in the hands of the most experienced robotic surgeons. Although this study is only a first step, any strategy intending to potentially have an impact on this is welcome, particularly when attempting to take advantage of the unique surgical environment created by robot-assisted surgery.

Robotic surgical platforms immerse the surgeon in an atmosphere currently built around a camera view that is little more than could be seen with the human eye if equally magnified. Although not without its benefits, the power of robot-assisted surgery is not only this immersion and the precision of miniature mechanical instruments, but rather is the computer between the surgeon and the robotic arms working within the patient. This computer allows the surgeon to seamlessly integrate supplementary information into the operative "field" in a way never available in open surgery.

This study has several limitations, but the concept that it furthers is real. Visualization of tissues and tumors with computer augmentation will come to fruition, whether with real-time fluorescence, preoperative or intraoperative imaging modalities superimposed on the robotic camera view ("augmented reality"), or some other method still in its infancy or perhaps not yet even imagined. The authors are to be congratulated on their initial venture into this arena of study, if for nothing else for encouraging us that what we can imagine can and should be pursued.

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REPLY

We much appreciate the forward-looking thoughts and encouragement provided by the authors of the invited commentary on our article. We completely agree that the small number of animals is a major limitation of our experiment. However, as well pointed out in the comment, this was just a first step toward what we believe is the right direction. Although still in its earlier phases of development, the full integration of image-guidance techniques in minimally invasive surgery bears the potential to benefit prostate cancer patients by increasing the precision of surgical procedures.

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