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Sentinel Lymph Node Mapping of Invasive Urinary Bladder Cancer in Animal Models Using Invisible Light

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Abstract

Objectives—With conventional methodology, sentinel lymph node (SLN) mapping of invasive urinary bladder cancer is technically challenging. This study was performed to determine the utility of invisible, near-infrared fluorescent (NIRF) light for patient-specific SLN mapping, in real time under complete image guidance.

Methods—Lymphatic tracers, injection volume, NIRF excitation fluence rate, light collection of emitted fluorescence, and degree of bladder distension were systematically optimized in normal dogs and pigs. SLN mapping was then performed in pet dogs with naturally occurring invasive transitional cell carcinoma (InvTCC) of the urinary bladder, which closely mimics the human disease.

Results—NIRF albumin (hydrodynamic diameter [HD], 7.4 nm) and NIRF quantum dots (15–20 nm HD) injected into the bladder wall resulted in identification of draining lymph nodes (LNs) in under 3 min. In both species, considerable variability in the lymphatic drainage was observed among individuals. Optimal SLN mapping was achieved with the use of superficial, serosal injection of NIRF tracer, with the bladder distended to an intraluminal pressure of 20–40 cm H₂O. In dogs with InvTCC, NIRF tracers identified SLNs that were confirmed histologically to harbor metastases.

Conclusions—The use of invisible NIRF light permits real-time, patient-specific identification of SLNs that drain bladder cancer. Intraluminal bladder pressure is a key parameter that needs to be controlled for optimal results.

Keywords

Bladder cancer; Fluorescence; Intraoperative imaging; Sentinel lymph node mapping

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Take-home message

This article describes successful use of intraoperative near-infrared light to identify sentinel lymph nodes (SLNs) draining invasive urinary bladder cancer in animals. This technology will permit studies of a new approach to identify SLNs at the time of cystectomy.

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1. Introduction

Cystectomy is frontline treatment for invasive transitional cell carcinoma (InvTCC) of the urinary bladder [1]. Lymphadenectomy is performed at the time of cystectomy. Lymph node (LN) metastases, typically found in 20–25% of patients, are associated with increased local recurrence and disease progression, and may serve as an indicator of the need for further therapy [2–5].

Lymphadenectomy provides prognostic information and contributes to long-term cancer control and possibly cure of InvTCC [2–7]. The extent to which LN dissection should occur, however, has not been completely defined [8,9]. The standard approach is to remove the major regional bladder-draining nodes [4]. However, the extent of LN dissection varies considerably from patient to patient (less than 5 nodes to greater than 75 nodes removed) [9]. This variation is partly due to lack of agreement in proper anatomical boundaries for the dissection, differences in surgical technique (en bloc vs. separate LN packet), variability in the meticulousness of node identification by the pathologist, as well as unvalidated concern for morbidity of extensive node dissection [8,10]. Controversy particularly exists as to whether higher, third echelon LNs, around the inferior mesenteric artery and down to the aortic bifurcation, should be routinely dissected. Involvement of these nodes is rare in the absence of lower echelon disease, and dissection is less likely to benefit those patients with extensive lymphadenopathy [8].

A new and still experimental lymphadenectomy approach is to identify, remove, and analyze (intraoperatively) a sentinel lymph node (SLN) or nodes, and to use the presence of cancer cells or molecular markers in the SLN to guide the extent of LN dissection in that patient [11]. Even in the setting of a planned complete anatomical lymphadenectomy, the use of intraoperative SLN identification has permitted resection of otherwise missed positive LNs [8]. To determine the validity of the SLN approach, two important areas of study are required: (1) development of methods to effectively identify SLNs and (2) assessment of the prognostic value of SLN status.

Methods to identify SLNs in InvTCC are under active investigation. Anatomical location cannot be used to identify SLNs owing to wide interpatient variation in LN drainage from the bladder [4]. Refined techniques using preoperative intravesical injection of radioactive tracer and blue dye have shown promise in identifying LNs that drain urinary bladder cancer [11–13]. However, radiotracers, while sensitive and capable of penetrating thicker tissue, are not very suitable for real-time use because the larger colloid (hydrodynamic diameter [HD], 50–100 nm) requires long transit time (typically more than 1 h) to ensure transport to SLNs. Anatomical resolution and localization are also restricted by using a small hand-held intraoperative probe. Still, these agents can provide reliable preoperative imaging with hybrid single-photon emission computed tomography scanning [13]. Blue dyes, while rapidly transported, are obscured by thick tissue, and their blue color is often difficult to distinguish from normal tissue after lymphatic transport. The small HD allows blue dye to readily pass through SLNs into more distal nodes. For this reason, blue dyes and radiotracers are often used in combination.

Recently, near-infrared fluorescence imaging (NIRFI) has emerged as a potential tool for tissue imaging. Light in the NIR wavelength range of 700–900 nm penetrates relatively deeply into tissue, but is invisible to the human eye. We hypothesize that an exogenous NIR fluorophore coupled to a carrier of intermediate size would provide a highly sensitive, high-resolution image of SLNs. Furthermore, using an appropriate imaging system, the surgeon could potentially follow a NIR fluorescent lymphatic tracer from the point of injection to the SLN in real time

[14]. As proof of principle, SLN mapping using NIRFI was first optimized in pigs and dogs, and then tested in dogs with naturally occurring InvTCC that strongly resembles human invasive bladder cancer [15].

2. Methods

2.1. Study design

Lymph node mapping was performed in pigs, laboratory dogs, and pet dogs with naturally occurring InvTCC in the Purdue University Clinical Research Laboratory, with approval of the Purdue Animal Care and Use Committee. NIR fluorophores were injected into the bladder wall, and migration through lymphatics observed in real-time with the use of a previously described intraoperative NIRFI system [14]. The number and location of fluorescing LNs were recorded. Technique variables that were optimized are listed in Table 1.

2.2. NIR fluorescent lymphatic tracer and imaging systems

Human serum albumin conjugated with three molecules of IRDye™ 800CW (HD, 7.4 nm; LI-COR; Lincoln, NE, USA) [16], HSA800, and NIR fluorescent quantum dots (NIRQDs) with an oligomeric phosphine coating (HD, 15–20 nm) [17] have been described previously. Peak excitation and emission wavelengths of HSA800 in serum were 784 and 802 nm, respectively, and per fluorophore quantum yield was 12%. The NIRQDs exhibit broad-band absorption, with an extinction coefficient of 581,000 M⁻¹cm⁻¹ at 775 nm, and peak emission at 820 nm. The first-generation NIRFI system [14] is described in Figure 1.

2.3. Laboratory animal studies

Mixed breed dogs (20–35 kg) and pigs (30–50 kg) were anesthetized with 12 mg/kg thiopental (Fort Dodge Labs, Fort Dodge, IA, USA) or 4.4 mg/kg Telazol (Fort Dodge Labs), respectively, and anesthesia maintained with isoflurane gas. A ventral midline abdominal incision was made, and the bladder visualized. Care was taken to avoid trauma to ligaments and soft tissues around the bladder to minimize any disruption of lymphatic drainage.

The NIR lymphatic tracer injections (25-G needle; 0.1–0.5 ml injection volumes studied) were made into the designated quadrant (left cranial, right cranial, left caudal, right caudal) of the bladder. Travel of the fluorophore to draining LN(s) was visualized in real-time. With the interanimal variability in drainage patterns, 12 animals were needed to optimize the methodology. In the first 7 animals, fluorophore injection was made in only one quadrant of the bladder. In subsequent animals, multiple quadrants were injected sequentially. In 3 animals, injection into the mucosal surface was made following incision into the apex of the bladder.

In two animals, the amount of bladder distention was varied by infusing sterile saline through a urinary catheter. Intraluminal pressure (via pressure transducer in the bladder) and fluorophore migration were noted with minimal, moderate, and full bladder distention.

In five animals, following NIRFI, blue dye (0.1 ml isosulfan blue, Lymphazurin 1%; US Surgical Corp, Norwalk, CT, USA) was injected into the bladder wall, and migration of the dye compared with that of the NIR tracer.

Following the imaging procedures, while animals were still under anesthesia, euthanasia was performed (0.22 mg/kg sodium pentobarbital iv; Beuthanasia; Schering). The LNs were then removed and evaluated for fluorescence ex vivo.

2.4. Pet dog studies

Eligibility requirements for participating pet dogs were (1) histopathological diagnosis of InvTCC, (2) pet owner request to have the dog euthanized (owing to unacceptable quality of life resulting from cancer or other conditions), and (3) informed pet owner consent to allow general anesthesia and NIRFI immediately prior to euthanasia. The LN mapping was performed in five pet dogs with InvTCC as described above. In one of the five dogs, initial lymphatic tracer injection was made via cystoscopy, prior to laparotomy. Euthanasia and necropsy were then performed.

3. Results

3.1. Animals imaged

Animals included four pigs (one male, three female), eight laboratory dogs (six male, two female), and five pet dogs with naturally occurring InvTCC (one male, four female). Migration of the NIR fluorescent lymphatic tracers and intraoperative imaging were similar among laboratory dogs, pet dogs, and pigs; results from the two species are combined herein.

3.2. Real-time SLN mapping

The intraoperative NIRFI approach was effective in observing lymphatic drainage in real time. After tracer injection, bright fluorescence was immediately observed in the bladder wall, followed by fluorescence in lymphatics within 10 s, and fluorescence in SLNs within 30 s to 3 min (Fig. 2A). Injection site fluorescence persisted in the bladder, and massaging this site resulted in further fluorophore migration through lymphatics. Fluorescence at the injection site and in SLNs persisted for at least 2 h after tracer injection. Resected samples could be prepared for pathological examination under image guidance (Fig. 2B). Histology confirmed the presence of LN tissue (Fig. 3A).

The degree of dissection at the time of fluorophore injection dictated the extent to which fluorescence was observed to migrate the entire distance from injection site to SLN in real time. We attempted to minimize disruption of soft tissues around the bladder before tracer injection to prevent disruption of lymphatic flow. In some animals, the SLN could not be definitively identified until further dissection was performed because extensive fat and other soft tissues obscured the view of the fluorescence. In these animals, the direction of observed lymph flow was used to guide further dissection to then identify the fluorescing LN.

3.3. Lymphatic mapping with isosulfan blue dye

In five animals, after SLN mapping with NIR fluorescent lymphatic tracer, isosulfan blue dye was injected at the same site. Unlike the large-HD NIR fluorescent tracers, the blue dye spread diffusely over the bladder wall and entered multiple lymphatic vessels (Fig. 2C). Multiple LNs including those with fluorescence from the NIR tracer and those without fluorescence acquired blue color following one injection of blue dye.

3.4. Optimization of the SLN mapping protocol

Lymphatic tracer type, injection volume and location, and intraluminal bladder pressure were systemically studied (Table 1). Consistent with previous studies [18], HSA800 and NIRQDs worked nearly equivalently. Optimal injection volume was less than 0.3 ml per injection site. Larger volumes resulted in overly bright signals in the bladder wall, which could “wash out” the view of the lymphatics. Covering the injection site or moving it out of the field of view also improved visualization contrast. Superficial injection into the serosa was superior to superficial injection into the mucosa or deep injection into the serosa, which is consistent with

the anatomical location of lymphatic channels. It should be noted that no attempt was made or required to cannulate lymphatic channels.

Bladder distension, and hence, intraluminal bladder pressure had an important effect on SLN mapping (Fig. 4). At intraluminal pressures below 20 cm H₂O, the bladder was difficult to inject, and fluorescence was obscured by bladder folds. Lymphatic flow was optimal at 20–40 cm H₂O. Lymphatic flow slowed as intraluminal pressure increased above 40 cm H₂O and ceased at 50–55 cm H₂O. Systemic blood pressure remained constant through this time.

3.5. Patterns of lymphatic drainage

Intraoperative NIRFI revealed considerable variability in the pattern of LN drainage. Although lymphatics tended to follow blood vessels, in some cases the lymphatic would cross midline and lead to a contralateral node. In two animals, lymphatics followed the urethra for a considerable distance before traversing to sacral LNs. In one animal, lymphatics traveled cranially along a persistent urachal ligament.

Lymphatic drainage did not proceed as expected anatomically from the four quadrants of the bladder. For example, fluorophore injected into the left caudal quadrant of the bladder could flow to the right medial iliac or hypogastric node. The LNs that fluoresced in one or more animals included medial iliac, hypogastric (formerly referred to as internal iliac), lumbar aortic, and sacral nodes. As expected, each tracer injection generally resulted in fluorescence in only one LN. In two animals, very slight fluorescence was noted in a second node in addition to the brighter fluorescence in the first node.

The pattern of fluorescence in the LNs varied between and within animals, and included bright fluorescence of the entire node (25% of samples), bright fluorescence of part of the node (45% of nodes), and positive rim of tissue along the surface of the node with speckled fluorescence inside the node (30% of samples). In some LNs in which only part of the node fluoresced, it was easier to detect the fluorescence *ex vivo* once the node had been removed.

3.6. Lymphatic mapping of naturally occurring InvTCC in pet dogs

NIR fluorescent SLN mapping with HSA800 (0.2–0.3 ml per injection site) was performed in five pet dogs with InvTCC (Table 2). When injected directly into tumor tissue, the HSA800 remained localized at the injection site with no migration into lymphatics (data not shown). When the fluorophore was injected into peritumoral tissues, migration into lymphatics was observed, and SLNs could be resected under image guidance. Injection of the fluorophore at multiple sites around the tumor resulted in fluorescence in more than one LN in some cases.

Cystoscopic injection of HSA800 was performed in one dog with InvTCC. Although the cystoscope used did not have NIRFI capabilities, it was not difficult to inject the fluorophore and to see a bleb of material under the mucosa at the injection site. The dog that received the fluorophore injection by this route had InvTCC predominantly in the urethra, and the injection was made into the urothelium lining the urethra caudally to the mass. Fluorescence was observed in lymphatics when the laparotomy was performed.

Four of the five pet dogs had LN metastases, which were identified at necropsy (Fig. 3B), and all four of these dogs had SNLs identified by NIRFI. No fluorescing nodes were found in one dog; this dog had lung metastases, but no LN metastases at necropsy. Fluorescence was observed in lymphatic vessels in and near the bladder in this dog, but lymphatic channels ended abruptly at thick adipose tissue in the abdomen. It is not known whether this was caused by fat obscuring lymphatics, blind lymphatics, or another cause.

4. Discussion

Assessment of SLN status to guide surgical planning is the standard of care in breast cancer and melanoma, and is being evaluated in other forms of human cancer [18–20]. There is also interest in SLN mapping in patients with urinary bladder cancer [11–13]. The ability to effectively identify SLNs at the time of cystectomy could impact several areas of InvTCC research and potentially patient management in the future such as (1) generating prognostic information, (2) individualizing the extent of surgery, (3) assessing need for adjuvant therapy, and (4) understating biological mechanisms of lymphatic metastasis [11].

The combination of radioactive lymphatic tracer and blue dye has been reported to allow SLN identification in patients with InvTCC, but poor sensitivity and specificity of blue dye, and the inability to identify an SLN in all patients still are concerns [8,11–13]. Optical imaging using NIR fluorescent lymphatic tracers provides real-time assessment of lymphatic flow from the point of injection to the SLN(s), relatively high sensitivity due to the penetrating ability of NIR photons, and elimination of the need for radioactivity. NIRFI has been validated in large animal models for SLN mapping of skin [16,17], lung [21], esophagus [22], pleural space [22], GI tract [16,21], and spontaneous melanoma [23]. Our study of invasive bladder cancer confirms that the anatomy of lymphatic flow is highly variable among individuals, and can involve structures such as the urachus and presacral nodes, and that SLN mapping must be performed on an individual basis.

In performance of LN mapping, it is important to avoid disruption of lymphatics during the surgical procedure or to deliver the lymphatic tracer prior to surgery. Cystoscopic injection in the urothelium caudally to urethral InvTCC in a dog resulted in fluorescence in lymphatics near the tumor. It is anticipated that similar cystoscopic injections made in the bladder wall would be equally successful. Other lymphatic imaging agents have been successfully delivered into the bladder wall of humans with InvTCC via cystoscopic injection, with injections made at four points around the tumor mass [11].

Large animal models of human cancer are rare. Our study utilized terminally ill pet dogs with naturally occurring InvTCC. Canine bladder cancer closely resembles InvTCC in humans with regard to histopathological characteristics, cellular and molecular features, and biological behavior including sites and frequency of metastasis [15]. Of particular importance for translation of our results into human studies, we have recently described a formulation of two clinically available agents that can be used for SLN mapping in humans [16].

In this study, injection volume, technique, and location, and intraluminal bladder pressure were optimized for NIRFI. Histologically, normal lymphatics have been localized to the lamina propria and connective tissue surrounding muscle [24], and tumor lymphatics in InvTCC to the peripheral zone surrounding the tumor [11,25]. It is not surprising that fluorophore injections had to be made around rather than in the tumor. Interestingly, moderate bladder distention was required for optimal lymphatic mapping of the bladder. Even with superficial, serosal injection of NIR fluorescent tracer, no migration was observed with maximal (> 50 cm H₂O) or minimal (< 10 cm H₂O) bladder distention.

In this study, fat obscured the view of the fluorescence in obese animals. There is still, however, reason to believe that NIRFI will be of value in obese humans. In the obese dogs, the direction of the lymphatic flow was used to guide further dissection along lymphatics to the SLN. In addition, a second-generation imaging system [23] with greatly improved optics has recently become available.

5. Conclusions

This study describes the use of invisible NIR light for successful SLN mapping of invasive urinary bladder cancer in animals. With recent improvements in NIR fluorophore formulation, the study results should be immediately translatable into testing in humans with InvTCC.

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Fig. 1.

Real-time intraoperative near-infrared (NIR) fluorescence imaging system. Light paths for color video (solid lines) and NIR fluorescence (dotted lines) are indicated. The system has two wavelength-isolated light sources of 400- to 700-nm “white” light and 725- to 775-nm NIR fluorescence excitation light, which generate fluence rates of 0.5 mW/cm² and 5 mW/cm², respectively. The 400- to 700-nm white light permits normal visualization of the surgical field without producing background fluorescence that would otherwise occur with full-spectrum room lights and surgical lights.

A.

B.

C.

Fig. 2.

Near-infrared (NIR) fluorescent sentinel lymph node (SLN) mapping in the pig. (A) NIR fluorescence imaging immediately after injection (Inj.) into the bladder wall; lymphatic channels (arrowheads) are identified within 10 s, and fluorescence in nodes within 30 s to 3 min (bottom). Shown are the color video image, NIR fluorescence image, and a pseudocolored (lime green) merge of the two for each condition. (B) Postresection, ex vivo processing of the SLN. (C) Isosulfan blue injection into the bladder wall.

Fig. 3. Photomicrographs (hematoxylin-eosin stain; original magnification: $\times 200$) of resected sentinel lymph nodes (SLNs). Histology was used to confirm that fluorescing structures viewed intraoperatively were lymph nodes. (A) Histologic section of SLN removed from a normal laboratory dog. The lymphoid follicle (LF) and subcapsular sinus (S) are labeled. (B) Histological section of SLN removed from a dog with invasive transitional cell cancer (InvTCC). Neoplastic cells fill the subcapsular sinus (S). Lymphoid follicle (LF) is also labeled.

Fig. 4.

The effect of intraluminal bladder pressure on sentinel lymph node (SLN) mapping. Intraluminal bladder pressure was varied from 10 cm H₂O (top) to 45 cm H₂O (bottom), with only intermediate pressures (middle), resulting in free flow of the tracer from the injection site to the SLN. Shown are the color video image, near-infrared fluorescence image, and a pseudocolored (lime green) merge of the two, for each condition.

Table 1

Parameter optimization for NIR fluorescent SLN mapping of urinary bladder cancer

Parameter	Conditions tested	Optimal
Tracer injection volume	0.1–0.5 ml per injection	0.2–0.3 ml HSA800 0.1 ml NIRQD
Site of injection	Serosal or mucosal injection	Serosal injection
	Deep or superficial injection	Superficial
Intraluminal bladder pressure	0–55 cm H ₂ O	Moderate distention (20–40 cm H ₂ O)

NIR, near-infrared; SLN, sentinel lymph node; NIRQD, near-infrared fluorescent quantum dot.

Table 2

NIR fluorescence imaging of lymph node drainage in dogs with naturally occurring InvTCC of the urinary bladder

Dog no./ tumor location ^a	NIR fluorescence imaging findings	Necropsy findings
Dog 1/midbladder	Pale diffuse fluorescence in 2 nodes	InvTCC metastases in 2 fluorescing nodes; no other metastases found
Dog 2/trigone, midbladder	Bright fluorescence in 1 node; pale fluorescence in 1 node	InvTCC metastases in 2 fluorescing nodes, and in nonfluorescing nodes and lung
Dog 3/urethra	Bright fluorescence in 1 node	InvTCC metastases in fluorescing node, and in nonfluorescing nodes and lung
Dog 4/urethra, trigone	Bright fluorescence in lymphatics; no fluorescence in nodes found	InvTCC metastases in lung; no metastases in nodes found
Dog 5/urethra, trigone ^b	Very pale fluorescence in 1 node; bright area of fluorescence adjacent to urethra (possible node?) ^{b,c}	InvTCC metastases in fluorescing node, and in nonfluorescing nodes and lung

NIR, near-infrared; InvTCC, invasive transitional cell cancer.

^aTumor location was categorized as urethra, trigone, midbladder, or apex.

^bThe initial injection of the fluorophore in this dog was made via cystoscopy into the wall of the urethra caudal to the tumor. Owing to the tumor location and small urethral diameter, cystoscopic injection cranially to the tumor mass was not possible. An abdominal incision was made, and three additional fluorophore injections were made in the bladder wall cranially to the tumor mass. Movement of the fluorophore was observed in real time from these injection sites. Following further dissection of tissues, fluorescence in lymphatics between the cystoscopic injection site and tumor mass was observed, but this fluorescence ceased at the edge of the tumor mass.

^cAfter fluorophore injection cranially to the tumor mass, a very bright area of fluorescence was noted along the outer surface of the proximal urethra away from injection sites and away from the tumor mass. Histopathologically, this lesion comprised InvTCC cells. It could not be determined if this was nodal tissue that had been effaced with InvTCC.