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Intraoperative Sentinel Lymph Node Mapping of the Lung Using Near-Infrared Fluorescent Quantum Dots

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Abstract

Background—The presence of lymph node metastases is an important prognostic marker with regard to non-small-cell lung cancer (NSCLC). Assessment of the sentinel lymph node (SLN) for the presence of tumor may improve staging. Our objective was to develop an optical noninvasive imaging tool that would permit intraoperative SLN mapping and provide real-time visual feedback for image-guided localization and resection.

Methods—Invisible near-infrared (NIR) light penetrates relatively deeply into tissue and background autofluorescence is low. We have developed a NIR fluorescence imaging system that simultaneously displays color video and NIR images of the surgical field. We recently engineered 15 nm nonradioactive NIR fluorescent quantum dots (QDs) as optimal lymphotropic optical probes. The introduction of these QDs into lung tissue allows real-time visualization of draining lymphatic channels and nodes.

Results—In 12 Yorkshire pigs (mean weight 35 kg) we demonstrated that 200 pmol of NIR QDs injected into lobar parenchyma accurately maps lymphatic drainage and the SLN. All SLNs were strongly fluorescent and easily visualized within 5 minutes of injection. In 14 separate injections QDs localized to a mediastinal node, whereas in 2 injections QDs localized to a hilar intraparenchymal node. Histologic analysis in all cases confirmed the presence of nodal tissue.

Conclusions—We report a highly sensitive rapid technique for SLN mapping of the lung. This technique permits precise real-time imaging and therefore overcomes many limitations of currently available techniques.

The presence of lymph node metastases is an important prognostic marker with regard to non-small-cell lung cancer (NSCLC) [1]. Clinical staging of mediastinal lymph nodes using computed tomography (CT) is unreliable taking into account that up to 40% of enlarged nodes are actually benign and 25% of normal sized nodes are malignant [2]. Current surgical practice involves complete mediastinal node dissection as part of a formal anatomic tumor resection. Aside from the increased morbidity associated with this procedure, recurrent disease approaching 40% will develop in node-negative patients and they will die within 2 years [3]. This suggests that current staging methods often underestimate micrometastases with regard to regional lymph nodes and that focusing histopathologic examination on highly suspicious

nodes may improve staging. Thus a method to accurately assess nodal status and tailor selection of patients for adjuvant therapy in NSCLC is required.

The introduction of sentinel lymph node (SLN) mapping and biopsy revolutionized the assessment of nodal status regarding the spread of neoplasms, particularly melanoma and breast cancer [4]. The underlying hypothesis of SLN mapping is that the first lymph node to receive lymphatic drainage from a tumor site will contain tumor cells if there has been direct lymphatic spread [5]. Current techniques with regard to the identification of the SLN involve radioguided lymphatic mapping and/or visualization of the nodes with vital blue dyes [6].

Recent studies of SLN mapping have confirmed its feasibility in patients with NSCLC [7]. Several studies have indicated that the SLN accurately reflects the tumor status of regional mediastinal nodes exhibiting greater than 80% accuracy [8–11]. Present methods of SLN mapping for other organs are, however, not readily amenable for use with respect to staging NSCLC. The ideal method should be accurate, rapid, noninvasive, and potentially usable in a thoracoscopic setting. None of the current methods fulfill all of these criteria [12]. In the lung intraoperative mapping with blue dye has been determined to identify the SLN in fewer than 50% of patients because tissue penetration of the dye is poor and mediastinal lymph nodes are often anthracotic [13]. The use of radiolabeled tracers has improved the detection rate and accuracy of pulmonary SLN mapping, but the high radioactivity of the primary injection site may interfere with intraoperative in vivo detection of nearby nodes. If radioisotopes are injected intraoperatively the time period required for the tracer to migrate to the SLN may delay the operative procedure. Conversely preoperative injection of the radiocolloid tracer necessitates CT-guided injection anywhere from 6–24 hours before surgery.

We recently developed a completely portable near-infrared (NIR) fluorescence imaging system that permits real-time intraoperative SLN mapping using optimized quantum dots (QDs) [14–16]. QDs are bright fluorescent semiconductor nanocrystals that contain an inorganic core of metal and an outer soluble organic coating. These fluorescent biological labels are exceptional probes for SLN mapping because they are highly fluorescent, non-radioactive, and easily visible deep within tissue [17]. In this present study we investigated the feasibility of using QDs for mapping pulmonary lymphatic drainage and guiding excision of the SLN.

Material and Methods

Intraoperative NIR Fluorescence Imaging System

The large animal intraoperative NIR fluorescence imaging system used in this study has been described in detail previously [16]. Briefly the system simultaneously displays color video and NIR fluorescence images of the surgical field allowing instantaneous imaging of lymphatic flow and providing real-time visual feedback for image-guided localization and dissection [18]. Screen images are updated 15 times per second. In the present configuration NIR fluorescent excitation light was supplied by two 150 W halogen light sources, in which custom 725–775 nm band pass filters (Chroma Technology Corp, Brattleboro, VT) are installed. White light excitation was conducted via a 150 W halogen lamp (Model PL-900; Dolan-Jenner Industries, Lawrence, MA) depleted of wavelengths greater than 700 nm. Fluency rates for white light (400–700 nm) and NIR excitation light were 2 mW/cm² and 5 mW/cm², respectively. The Orca-ER (Hamamatsu Corp, Bridgewater, NJ) NIR camera settings included maximum gain, 2 × 2 binning, 640 × 480 pixel resolution, and an exposure time of 67 ms. Data was acquired and quantified on a Dell computer using Labview (National Instruments Corp., Austin, TX).

NIR Fluorescent Lymphatic Tracer Preparation

The complete synthesis of quantum dots used in this study has been previously described [14]. Briefly we synthesized type-II NIR fluorescent semiconductor nanocrystals (QDs) with a hydrodynamic diameter of 15–20 nm, a maximal absorption cross-section, a 840–860 nm fluorescence emission, an aqueous quantum yield greater than or equal to 13%, and a stable oligomeric phosphine coating. The hydrodynamic parameters of these QDs were engineered to maximize both rapid uptake into lymphatics and retention in the first draining lymph node.

Surgical Preparation

Adult Yorkshire pigs ($n = 12$, mean weight 35 kg) of either sex were used in this study. Animals were obtained from EM Parsons (EM Parsons & Sons, Inc., Hadley, MA). All animals received humane care in compliance with institutional protocols and the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (National Institutes of Health publication 85 to 23, revised 1985). Investigators complied with the 1996 NRC Guide for the Care and Use of Laboratory Animals.

General anesthesia was induced with 5 mg of intramuscular tamsulosin (Telazol) and 5% isoflurane/6L O₂. Animals were endotracheally intubated and ventilated with 100% oxygen and anesthesia was maintained with 1.5%–2% isoflurane. Continuous oxygen saturation and heart rate were monitored throughout the experiment. An intravenous catheter was placed in the left marginal ear vein and maintenance intravenous fluid was infused. The left internal jugular vein was cannulated for central venous access as necessary. Either a median sternotomy or a wide clam-shell thoracotomy was used to gain access to the thoracic cavity for lymphatic mapping.

Lymphatic Mapping and SLN Identification

Pulmonary lymphatic mapping was performed by injecting 100 μ L of either 2 μ mol/L NIR QDs in phosphate buffered saline (PBS), pH 7.4, or 4 ml of 1% (17.6 mmol/L) isosulfan blue (Lymphazurin; BenVenue Laboratories Inc., Bedford, OH) intraparenchymally using a tuberculin syringe. Injections were positioned approximately 1.5 cm deep into the medial surface of the right and left upper and lower lobes. Completely reproducible parenchymal injections were performed by one surgeon after refinement of the technique. For colocalization studies QDs were injected 1 minute before isosulfan blue because the latter quenches the QD-fluorescence of the lymphatic vessels and SLN. Preinjection images were obtained to document tissue autofluorescence. Lymphatic flow was visualized in real-time using the imaging system. The first lymph node encountered on the path delineated by the fluorescent lymphatic vessels was defined as the SLN. Time from injection to first appearance in the SLN was recorded.

NIR Fluorescence-Guided Nodal Dissection

After the identification of lymphatic vessels and the SLN, real-time imaging was used to guide careful dissection of the SLN. Adjacent nodes, which did not exhibit fluorescence, were also excised as negative controls. Postresection NIR fluorescence imaging of the surgical field was performed to confirm that all sentinel node tissue had been excised.

Nodal Migration

In a subset of injections the identified SLN was not immediately dissected, but allowed to remain in situ for 4 hours to determine if QDs migrated beyond the single SLN as well as to determine migratory time course. Lymphatic drainage of the porcine lung is known to advance superiorly, traversing subsequent mediastinal nodes [19].

Histologic Analysis

Nodal tissue was embedded in optimal cutting temperature compound (OCT) (Miles Laboratories, Elkhart, IN), snap-frozen, and cryosectioned at 6 μm onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were stained with hematoxylin and eosin (H + E) and then examined and photographed to confirm nodal tissue. Consecutive unstained sections were photographed on a NIR fluorescence microscope [20].

Results

Real-Time NIR Imaging System

The NIR imaging system was able to unobtrusively provide real-time images to the surgeon during the entire procedure (Fig 1). While operating the surgeon can view color video, NIR fluorescence, and pseudocolored merged images of the operative field. This latter image is particularly useful to the surgeon because it permits visualization of lymphatic flow against a background of normal anatomy. By using a nonanatomic color such as lime green (i.e., a pseudocolor) for this overlay, lymphatic flow and the SLN can be clearly delineated. The imaging system's variable field of vision allows zooming capability for more precise localization and dissection as required. Finally the system is self-contained on a rolling cart allowing complete portability.

Lymphatic Mapping of the Lung

Tissue autofluorescence of the thoracic cavity and lung parenchyma were minimal. After intraparenchymal injection of 200 pmol of QDs on the medial surface of a lobe, fluorescence was immediately visible at the injection site whether viewed from the medial surface (approximately 1.5 cm deep) or through the lung from the lateral surface (approximately 5 cm deep). Lymphatic channels could be observed extending into the hilum and, within 5 minutes, a single lymph node was fluorescent (Fig 2). In most injections the lymphatic pathway between the injection site and the SLN was clearly visible. There was no visible leak of QDs from lymphatic vessels or nodal tissue; minimal leakage of QDs at the injection site did not affect visualization of the lymphatic vessels or SLN. A single SLN was identified after each of the 16 injections (Table 1). In 11 animals fluorescent lymphatic channels terminated in one mediastinal SLN (Fig 3). In one animal left upper and left lower lobe QD injections terminated in a hilar intraparenchymal lymph node (Fig 3). Image-guidance allowed all target SLNs to be removed easily with a high degree of precision. Adjacent nodal tissue was also resected and demonstrated no fluorescence, whereas postresection imaging of the surgical site confirmed no residual fluorescent nodal tissue. No noticeable inflammatory changes or edema was observed grossly or histologically in the injection site, intervening lung parenchyma, or nodal tissue. Oxygen saturation and heart rate remained stable throughout the case. Fluorescence microscopy demonstrated that the QDs localize to the subcapsular and intermediate sinuses of the SLNs.

Because isosulfan blue is considered the gold-standard visible lymphatic tracer, coinjection of QDs and isosulfan blue was performed to document migration to the same SLN. In four separate animals, after intraparenchymal injection of 200 pmol of QDs on the medial border of the right upper lobe and identification of the afferent lymphatic channels and one mediastinal SLN, 4 ml of 1% isosulfan blue (4000 μl) was injected into the same area. In all injections QDs identified the SLN within 1 minute, whereas isosulfan blue could be visualized in the same node within 4 minutes (Fig 4). Some quenching of the QD fluorescence was expected and observed. In all four injections, isosulfan blue identified the same SLN as did the fluorescent QDs. In contrast to QDs, isosulfan blue was difficult to recognize in the mediastinal nodes and dye extravasation reduced clarity. Once isosulfan was visualized in a mediastinal node, the node was immediately resected and sectioned. No adjacent nodes were blue or fluorescent.

Nodal Migration

After standard QD injection into the right upper and right and left lower lobes in three separate animals as described above, a single mediastinal SLN became fluorescent within 5 minutes. There was no observed migration of QDs beyond the identified SLN at 4 hours. Furthermore QDs remained localized to the subcapsular and intermediate sinuses of the SLNs and did not travel further.

Comment

We report the technique of SLN mapping of the lung using NIR fluorescent QDs in a large animal model approaching the size of humans. This technique permits precise, rapid, real-time, intraoperative SLN imaging and therefore overcomes many of the limitations of current lymphatic mapping techniques. NIR QDs can reliably identify lung lymphatics as well as the SLN and indicates promise for future application in human lymphatic mapping. Injection of just 200 pmol of NIR QDs into various lobes quickly identifies the afferent lymphatics and the SLN within 5 minutes of injection. Image-guided dissection is easily and accurately accomplished given the brilliant fluorescence and low-tissue scatter of the NIR QDs. Importantly the current mapping agent, isosulfan blue, colocalizes to the nodes identified by NIR QDs. These observations suggest that NIR QDs are optimal lymphotropic optical probes and can be used to provide highly sensitive and specific, real-time, intraoperative SLN mapping in the lungs in contrast with current mapping techniques which are inadequate and technically limited.

We successfully identified a SLN in 100% of the injections, with most being solitary mediastinal nodes. This distribution is not unusual because pigs are reported to exhibit no pulmonary nodes located along the segmental and lobar bronchi. In studies of porcine cadavers it was determined that lymph from the lungs drains directly into mediastinal nodes and that there are no histologically identifiable lymph nodes proximal to these [19]. Our results concur with this report, except in one animal where both the left upper and lower lobe injections terminated in an intraparenchymal SLN in the left hilum. All other animals sustained direct drainage to a mediastinal node. In humans the lymphatic drainage of the lungs is primarily to the bronchopulmonary nodes. Lymph drains directly into mediastinal nodes approximately 25% of the time and might be important with regard to the development of “skip” metastases [2,21,22].

Several studies have documented the feasibility and usefulness of SLN mapping with regard to NSCLC. Current practice in thoracic oncology supports a complete anatomic mediastinal lymph node dissection for patients with NSCLC to accurately stage the cancer. Although usually straightforward mediastinal lymph node dissection can be associated with considerable morbidity [2]. By identifying a specific lymph node for histopathologic staging, pulmonary SLN biopsy could eliminate the need for a nontherapeutic mediastinal lymph node dissection and its associated complications. Intraoperative SLN biopsy may be especially useful in smaller sized tumors [23]. By allowing the pathologist to focus on one node, or even one area of one node, for analysis, SLN biopsy can potentially uncover more micrometastatic disease [24].

Despite the worldwide validation of the SLN hypothesis and its growing use in many forms of cancer the current technique exhibits considerable limitations when applied to NSCLC. The use of blue dye cannot adequately identify the SLN in thoracic malignancies because of poor tissue penetration and the presence of anthracosis. Recent reports of SLN mapping with regard to NSCLC have thus abandoned this tracer in favor of various radioactive lymphotropic probes. A radiocolloid is injected around the tumor either preoperatively or at the time of thoracotomy, and a hand-held gamma probe is used to detect a “hot” node. In contrast to lymphatic mapping with the blue dye method, where success is defined as the identification of

a blue lymphatic channel leading to a node, radiocolloid lymphatic mapping indicates no standard definition of success. Criteria defining a positive SLN have been largely arbitrary and empiric, usually based on ratios of background to SLN radioactivity. Detection of a true SLN with the radioisotope method can also be confounded by varying amounts of “shine through” radioactivity from the primary injection site essentially causing increased background radioactivity and decreasing ratio results.

Tracer particle size exhibits an important impact on migration time in SLN mapping. Particles less than 5 nm partition into blood and those between 5–10 nm can migrate through nodal tissue often resulting in false-positives, whereas those > 1000 nm largely remain at the injection site [25]. Blue dye contains a range of less than 5 nm particle size and thus can pass through the SLN and traverse multiple nodes in addition to becoming intravascular. Because radioisotopes exhibit a more uniform particle size, various preparations of technetium-99m have been used with regard to pulmonary SLN mapping. Liptay and colleagues intraoperatively injected technetium-99m sulfur colloid and used a hand-held gamma probe to identify highly radioactive nodes [8,9]. Migration time varied considerably, but on average radiocolloid migration was detectable after 10–15 minutes. In most instances only 1 SLN was identified. Technetium-99m sulfur colloid possesses a 40 nm particle diameter and thus requires anywhere from 10–60 minutes for migration to the SLN. Technetium-99m tin colloid, with a particle diameter of 1000 nm, has also been used [10]. Because migration time of this large tracer particle exceeds 6 hours, preoperative injection was necessary. The need for CT-guided tracer injection 12–24 hours before surgery, however, severely limits the usefulness of this agent. To avoid the use of a radioactive tracer, Nakagawa and associates used ferumoxides, a 100 nm diameter nonradioactive superparamagnetic tracer particle, to perform SLN mapping in NSCLC patients [26]. Because of technical limitations regarding their magnetometer, they could only perform ex vivo SLN identification after complete nodal dissection.

Pulmonary SLN mapping using NIR QDs overcomes these limitations. QDs can be engineered to precise sizes to optimize migration time and SLN retention. The optimal size of 15–20 nm allows for rapid transit to, and retention in, the first node. Additionally these optical biological probes are bright in the NIR region and can easily be viewed at depths of approximately 1 cm of skin, allowing visualization of a mediastinal SLN beneath thick pleura. The intraoperative use of NIR QDs to visually map lymphatic flow and identify SLNs obviates the necessity for preoperative radiocolloid injections and the use of blue dye. Instead surgeons can track lymphatic flow in real-time and guide their nodal dissection with the aid of on-line video feedback after one simple intraoperative injection. NIR fluorescence from the injection site does not interfere with visualization of the SLN on the video monitor and the actual surgical field remains unchanged because NIR QDs are invisible to the human eye. Finally the entire NIR-imaging system could be potentially translated to a thoracoscopic platform because it is optically based and completely portable.

A limitation regarding the clinical use of NIR QDs for SLN mapping is their potential toxicity. These biological probes contain heavy metals at their cores with an amphiphilic organic coating. Cadmium, telluride, selenide, and alkyl phosphines exhibit known acute and chronic toxic profiles as isolated heavy metals, but their toxicities as precomplexed nanocrystals are unknown. In our nonsurvival animal studies we observed no evidence of acute toxicity; heart rate and rhythm, blood pressure, and oxygen saturation remained stable during prolonged operative procedures. As we reported elsewhere extrapolating known dose-effect relationships for inhalation and oral exposures of these heavy metals indicates that our dose of 200 pmol of NIR QDs corresponds to a dose fifty times lower than that defined as causing respiratory symptoms and three-hundred times lower than that required to produce renal insufficiency [14]. Of importance to this study, most of the injected dose is removed by resecting lung and nodal tissue, so toxicity may, in fact, be negligible.

In summary we report a highly sensitive technique for pulmonary SLN mapping using NIR fluorescent QDs that overcomes many of the formidable limitations of current techniques. The brilliant fluorescence and low tissue scatter of NIR QDs, along with their precisely engineered size, constitutes them as optimal lymphotropic optical probes. Thus in the future they may prove useful with regard to SLN mapping of human lung cancer.

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Fig 1.
Intraoperative invisible near-infrared (NIR) fluorescence imaging system. View of the NIR fluorescence imaging system deployed in the operating room.

Fig 2. Sentinel lymph node (SLN) mapping of the right upper lung lobe in a pig using invisible near-infrared (NIR) fluorescent quantum dots (QDs). (A) Depicted from top to bottom are NIR fluorescence images of the surgical field before QD injection (autofluorescence), during QD injection, 45 seconds after injection (lung retracted), 1 minute after injection, and after SLN resection. For each time point, color video (left), NIR fluorescence (middle), and color-NIR merge (right) images are presented. Fluorescence images exhibit identical exposure times and normalization. QDs rapidly localize to the SLN (white arrow). Lack of fluorescence in the nodal basin after resection confirms complete removal of the sentinel nodal tissue. (B) NIR QD retention in the resected SLN compared with regional negative controls. (C) Histologic

analysis of frozen sections of the SLN (B). Photomicrographs ($\times 20$ and $\times 40$) illustrating the same field with representative hematoxylin and eosin (H + E)-stained sections and consecutive unstained sections photographed on a NIR fluorescence microscope.

Fig 3.

Modified lung schematic. Mapping of sentinel lymph nodes (SLNs) in the pig lung. Invisible near-infrared quantum dots were injected into the right and left upper and lower lobes. A mediastinal SLN was identified after 14 injections (X), whereas an intraparenchymal hilar SLN was identified after 2 injections (Y). (Reprinted with modification from [1] by permission of The C. V. Mosby Co.)

Fig 4.

Isosulfan blue colocalization. (A) Depicted from top to bottom are invisible near-infrared (NIR) fluorescence images of the surgical field during quantum dot (QD) injection, 1 minute after QD injection (just before isosulfan blue injection), and 5 minutes after QD injection (i.e., 4 minutes after isosulfan blue injection). For each time point, color video (left), NIR fluorescence (middle), and color-NIR merge (right) images are presented. Fluorescence images exhibit identical exposure times and normalization. QDs rapidly localize to the sentinel lymph node (S) from their injection site (I). Coinjection of isosulfan blue (90 seconds after QD injection) quenches the QD fluorescence as evidenced by the loss of QD fluorescence at 5

minutes. Isosulfan blue colocalizes to the same lymph node as the QDs (S). (B) NIR QD and isosulfan blue retention (arrow) in the resected sentinel lymph node.

Table 1

Sentinel Lymph Node Location

Injection Site	Number of Injections	Intraparenchymal Alone	Mediastinal Alone	Both
RUL	7	0	7	0
RLL	3	0	3	0
LUL	3	1	2	0
LLL	3	1	2	0
Total	16	2	14	0

LLL = left lower lobe; LUL = left upper lobe; RML = right middle lobe; RUL = right upper lobe.