Editor's Summary

Lighting Up Bladder Cancer Lesions

Molecular imaging of bladder cancer could greatly improve on current methods of diagnosis, which rely on white light–based imaging that looks for superficial tissue changes, such as color and texture. To this end, Pan et al. targeted the cancer-specific marker CD47 that is present on the surface of solid tumors, including in the bladder. The authors attached a brightly fluorescent tag, called a quantum dot, to a CD47 antibody. The fluorescent antibody was instilled into human bladders with muscle and nonmuscle invasive cancer that had been recently removed from patients. Overall, 119 bladder regions were analyzed using the fluorescent antibody and blue light cystoscopy. The authors reported a sensitivity of 82.9% and a specificity of 90.5%. They further correctly identified five of six carcinoma in situ lesions—a diagnostic challenge for white light imaging. Because this approach only requires topical administration of a fluorescent antibody and the use of already available clinical tools, it is hoped that this molecular imaging approach to diagnosing various bladder cancers will translate after further optimization.

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CANCER IMAGING

Endoscopic molecular imaging of human bladder cancer using a CD47 antibody

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A combination of optical imaging technologies with cancer-specific molecular imaging agents is a potentially powerful strategy to improve cancer detection and enable image-guided surgery. Bladder cancer is primarily managed endoscopically by white light cystoscopy with suboptimal diagnostic accuracy. Emerging optical imaging technologies hold great potential for improved diagnostic accuracy but lack imaging agents for molecular specificity. Using fluorescently labeled CD47 antibody (anti-CD47) as molecular imaging agent, we demonstrated consistent identification of bladder cancer with clinical grade fluorescence imaging systems, confocal endomicroscopy, and blue light cystoscopy in fresh surgically removed human bladders. With blue light cystoscopy, the sensitivity and specificity for CD47-targeted imaging were 82.9 and 90.5%, respectively. We detected variants of bladder cancers, which are diagnostic challenges, including carcinoma in situ, residual carcinoma in tumor resection bed, recurrent carcinoma following prior intravesical immunotherapy with Bacillus Calmette-Guérin (BCG), and excluded cancer from benign but suspicious-appearing mucosa. CD47-targeted molecular imaging could improve diagnosis and resection thoroughness for bladder cancer.

INTRODUCTION

Combining optical imaging technologies with cancer-specific molecular imaging agents represents a potentially powerful means to facilitate endoscopic cancer detection and resection (1, 2). White light endoscopy is the primary approach to visualize, biopsy, and locally resect cancers of the gastrointestinal and urinary tract. However, white light endoscopy has several well-known shortcomings, including incomplete detection of multifocal and flat tumors, as well as inadequate visualization of tumor boundaries. These shortcomings can affect the thoroughness of resection and completeness of local staging, thereby contributing to cancer recurrence and progression. Fluorescently labeled cancer-specific molecular imaging agents may provide enhanced differentiation between tumor and adjacent normal or benign tissues. Therefore, there is significant interest in developing molecular imaging agents that can augment cancer visualization.

Bladder cancer, the fifth most common malignancy (3), is identified through white light cystoscopy followed by endoscopic resection for pathological diagnosis and local staging. About 80% of patients present with nonmuscle invasive cancer [stages Ta and CIS (carcinoma in situ), confined to superficial layer; stage T1, invading to the lamina propria] and are primarily managed through endoscopic resection (4). The recurrence rate for bladder cancer is among the highest of all cancers, reaching up to 78% at 5 years (5, 6), thus requiring lifelong endoscopic surveillance. For patients who present or recur with muscle invasive cancer (stage ≥T2), radical cystectomy is the standard treatment. In patients with recurring nonmuscle invasive cancer with high-risk features [high-grade T1 and/or CIS; early recurrence after intravesical Bacillus Calmette-Guérin (BCG) immunotherapy], radical cystectomy is also recommended for definitive treatment (7).

Although central to the diagnosis, resection, and surveillance of bladder cancer, white light cystoscopy has recognized shortcomings, including difficulty in detecting flat neoplasms and differentiating malignancy from a spectrum of benign and inflammatory lesions. Recently, fluorescence imaging technologies have emerged as promising adjuncts to white light cystoscopy to enhance cancer identification and guide endoscopic resection (8). Blue light cystoscopy is a wide-field fluorescence technology based on blue light (375 to 440 nm) illumination of neoplastic cells that appear pink through selective accumulation of intravesically instilled photosensitive protoporphyrins (9). Confocal endomicroscopy enables in vivo microscopy using fluorescein as the imaging agent and a 488-nm laser as the light source (10–12). Despite the improved optical imaging provided by these technologies, limitations remain in their current application. For confocal endomicroscopy, fluorescein stains all cell types (normal and cancer), and real-time image interpretation of cellular morphology and tissue microarchitecture may be challenging. For blue light cystoscopy, increased uptake of protoporphyrins by inflammatory lesions is observed, thereby contributing to false positive rates of up to 30% (2, 8).

Here, we endeavored to develop a targeted imaging strategy to improve diagnostic sensitivity and specificity of the available optical imaging technologies by using a cancer-specific molecular imaging agent. As an easily accessible hollow organ, the bladder provides a well-established route for topical (that is, intravesical) administration of pharmacological agents. Intravesical administration minimizes potential systemic toxicity of the imaging agent, making bladder an attractive target organ for endoscopic molecular imaging and image-guided surgery. Previous studies identified CD47 as a surface marker of human solid tumors including bladder cancer (13). CD47 is expressed on more than 80% of bladder cancer cells (14). CD47 binds to signal regulatory protein α, a protein expressed on macrophages.
and dendritic cells, transmitting a signal to prevent phagocytosis. Blockade of CD47 signaling using anti-CD47 monoclonal antibodies enabled phagocytosis of bladder cancer cells in vitro, inhibited tumor growth, and prevented tumor metastases in xenotransplantation models (13, 14). Considering the expression and function of CD47 in bladder cancer, we aimed to evaluate fluorescently labeled anti-CD47 as an intravesical imaging agent for bladder cancer detection using optical imaging.

RESULTS

CD47 expression and distribution

We first analyzed CD47 mRNA expression using a published data set of bladder tumor specimens (n = 105) and matching normal urothelium obtained from sites distant to tumors (n = 52) (15, 16) with our previously established Web-based platform HEGEMON (Hierarchical Exploration of Gene Expression Microarrays Online) (17). With this platform, microarray samples were grouped on the basis of their annotations and analyzed to compare gene expression patterns. The overall CD47 expression level in both nonmuscle invasive (n = 33) and muscle invasive bladder cancers (n = 72) was significantly higher compared to that in normal urothelium (n = 52) (Fig. 1A). CD47 protein was detected in frozen human tissue sections of both normal urothelium and bladder tumor by immunofluorescence, but with differing distribution (Fig. 1B). The single layer of umbrella cells, which forms the luminal barrier between urine and the underlying tissues in normal urothelium [cytokeratin 18 (CK18)–positive cells (18)], did not express detectable levels of CD47, whereas the cancer tissue had widely distributed CD47 expression including on the luminal surface (Fig. 1B). This distribution led us to theorize that CD47 would be a viable target for molecular imaging of bladder cancer via topical administration of an imaging agent.

Ex vivo endoscopic molecular imaging

We next devised an ex vivo endoscopic molecular imaging protocol for human bladders (Fig. 2A). Fresh, intact bladders were obtained from human subjects who underwent radical cystectomy for muscle invasive or high-risk nonmuscle invasive bladder cancer (n = 26). Patient characteristics and final pathological diagnoses are given in table S1. A monoclonal CD47 antibody labeled with a fluorescent tag, either fluorescein isothiocyanate (FITC) or Qdot625, was instilled intravesically via a urinary catheter. After allowing sufficient time for antibody binding, excess antibody was removed by bladder irrigation. Bladders incubated with anti-CD47–FITC were imaged with confocal endomicroscopy (n = 5), and those incubated with anti-CD47–Qdot625 were imaged by blue light cystoscopy (n = 21).

Confocal endomicroscopy. Tissue-bound anti-CD47–FITC was detected with a clinical confocal endomicroscopy system. This system, used with a 2.6-mm fiber optic probe, provided a microscopic field of view (240 μm) and acquired video sequences at 12 frames/s (10–12). Image acquisition with the fiber optic probe was done either by opening the bladder after antibody incubation (n = 4) or through the working channel of a standard white light cystoscope (n = 1). Normal, cancer, and suspicious-appearing regions in each bladder were imaged and biopsied. The biopsies were histopathologically analyzed by a pathologist blinded to the imaging results. All five bladders were confirmed to have high-grade cancer by histology (table S1). Representative frames from confocal videos taken of normal urothelium and cancer lesions in each bladder with corresponding histopathology are shown in Fig. 2B. For quantitative analysis of anti-CD47–FITC signal in confocal videos, mean fluorescence intensity was determined as an average of all frames in a confocal video (fig. S1 and table S2). For each bladder, one video from a normal region and one video from cancer were used to calculate the fluorescence intensity ratio between anti-CD47–FITC binding to cancer and normal tissue (Fig. 2C).

To verify that the fluorescence detected on cancer was due to specific binding of anti-CD47–FITC, one bladder (Bl-1) was incubated with immunoglobulin G (IgG)–FITC isotype control and imaged with confocal endomicroscopy, followed by anti-CD47–FITC incubation and repeat imaging. Less than a twofold difference in fluorescence intensity was observed between normal urothelium and cancer lesion with IgG–FITC (Fig. 2, B and C). Upon subsequent incubation with anti-CD47–FITC, fluorescence intensity detected on the cancer lesion in Bl-1 was 95-fold greater than that on normal urothelium (Fig. 2, B and C). In all bladders (Bl-1 to Bl-5), anti-CD47–FITC binding to cancer lesions was between 95- and 1100-fold greater than that to normal urothelium in the same bladder. For six lesions in three bladders, duplicate confocal videos were captured to assess the reproducibility of image quantitation. Although

![Image](https://www.sciencetranslationalmedicine.org/content/6/260/ra148/F1.large.jpg)
Cup biopsy

Control before anti-CD47

Cancer-to-normal fluorescence signal ratio of each bladder (of normal and one of cancer lesion were used for calculation of the ratio (Table S2).

The mean pixel intensity of the second video collected was slightly lower, likely owing to photobleaching of FITC, there was good overall correlation for repeated imaging with a coefficient of determination \( R^2 \) of 0.934 (fig. S2).

Blue light cystoscopy. The confocal endomicroscopy results supported CD47 as an imaging target for bladder cancer. However, given the small field of view of confocal endomicroscopy and the requirement for en face contact between the probe and the tissue of interest, imaging the entire bladder mucosa is not practical. Blue light cystoscopy provides a wide-field view and can switch dynamically between white and blue light, allowing efficient imaging of the entire bladder and identification of lesions that might be overlooked with white light alone. Therefore, we further validated CD47 as a bladder cancer imaging target using blue light cystoscopy for ex vivo imaging of intact bladders as described in Fig. 2A. For these experiments, anti-CD47 was labeled with Qdot625, an inorganic fluorescent particle that has emission spectra compatible with the blue light cystoscopy system. Endoscopic molecular imaging using anti-CD47–Qdot625 with blue light cystoscopy was performed in 21 intact bladders (table S1).

Bladder cancer specificity by targeting CD47

To verify that the pink fluorescence observed under blue light on cancer lesions was due to cancer-specific binding of anti-CD47–Qdot625, one bladder was first incubated with Qdot625-labeled isotype control (IgG-Qdot625) and imaged with blue light cystoscopy. After being washed, the bladder was reincubated with anti-CD47–Qdot625 and imaged again. With the isotype control, no pink fluorescence was detected on either normal or tumor-appearing mucosa. After incubation with anti-CD47–Qdot625, pink fluorescence was observed on the tumor-appearing mucosa, but not the normal mucosa, indicating specific binding confirmed normal urothelium (Fig. 3A). The corresponding histopathology of a biopsy from the lesion with anti-CD47–Qdot625 binding confirmed CIS, whereas the biopsy of the region without anti-CD47–Qdot625 binding confirmed normal urothelium (Fig. 3A).

Additionally, biopsies from a bladder incubated with labeled anti-CD47 were analyzed by immunofluorescence to detect tissue-bound antibody using a secondary antibody against anti-CD47. Anti-CD47 was only found on the surface of biopsies from tumor tissue but not on normal urothelium (fig. S4), supporting the cancer-specific binding of anti-CD47 via topical administration.

Diagnostic accuracy of CD47-targeted imaging

Overall, 119 bladder regions were analyzed for co-registration of blue light cystoscopy diagnosis with histopathology (Table 1). True-positive anti-CD47 binding was found in 29 of 35 biopsies with histologically confirmed cancer (sensitivity, 82.9%), whereas 76 of 84 benign
biopsies were true negatives, with no evidence of anti-CD47–Qdot625 binding (specificity, 90.5%).

Most of normal urothelium and papillary urothelial carcinoma are easily distinguished by white light. From tissues that appeared to be benign urothelium under white light and had no detectable anti-CD47–Qdot625 binding with blue light cystoscopy, 54 of 57 biopsies were true negatives (Fig. 3B). For standard papillary urothelial carcinoma not otherwise specified (NOS), the most common type of bladder cancer, 15 of 19 biopsies were true positives with anti-CD47–Qdot625 binding (Fig. 3C, movie S1, and Table 1). In addition to papillary urothelial carcinoma NOS, aggressive variants were also positively identified with anti-CD47–Qdot625 binding, including three of three papillary urothelial carcinomas with squamous differentiation and two of two carcinomas with micropapillary features (Fig. 3C and Table 1). In addition, four of five biopsies of adenocarcinoma, a nonurothelial type of bladder cancer, were true positives with anti-CD47–Qdot625 binding (Fig. 3D and Table 1).

CIS is a flat, high-grade noninvasive urothelial carcinoma that poses a diagnostic challenge under white light and a significant risk of recurrence and progression. It typically appears as an erythematous patch with indistinct borders or may appear indistinguishable from normal urothelium. Differentiation from normal mucosa, benign lesions,
Inflammation, or BCG cystitis may be difficult. In our series, five of six instances of CIS were identified as true positives for anti-CD47–Qdot625 binding (Fig. 3C and Table 1). Strikingly, one biopsy with CIS appeared completely normal by white light, yet anti-CD47–Qdot625 binding was detected by blue light (movie S2). In this bladder, the region of CIS identified by CD47-targeted imaging was the only pathologically confirmed malignancy remaining in the cystectomy specimen. In another bladder with multifocal urothelial carcinoma of micropapillary variant, a CIS with micropapillary features was identified by anti-CD47–Qdot625 under blue light (Fig. 3C and Table 1). For the various benign lesions that appeared suspicious by white light, all squamous metaplasia (n = 6), 15 of 19 inflammatory lesions, and one of two ulcers (Fig. 3B) were diagnosed as true negatives by CD47-targeted imaging (movie S3). Although inflammation and ulceration potentially could provide anti-CD47 binding sites owing to denudation of urothelium or lymphocytic infiltration, 77% of these benign lesions were appropriately negative for anti-CD47–Qdot625 binding (Table 1).

Recurrent and residual carcinomas

Intravesical immunotherapy with BCG is the standard treatment for patients with nonmuscle invasive bladder cancer (Ta, T1, and CIS) with high-risk features (19). Although highly effective, prior BCG treatment can cause mucosal changes that confound both white light and standard protoporphyrin-based blue light cystoscopy (8). Common features of bladders with BCG exposure include granulomatous inflammation and denuded urothelium, both of which are potential sources of noncarcinoma-related binding of anti-CD47 that could lead to a high false positive rate. However, in bladders with a history of BCG exposure, diffuse nonspecific binding of anti-CD47–Qdot625 was not observed (n = 6) (Fig. 3E). Further, in a bladder with histologically confirmed granulomatous cystitis, anti-CD47–Qdot625 with blue light cystoscopy correctly identified recurrent carcinoma (Fig. 3E and movie S4).

Prior tumor resection can also confound white light identification of residual carcinoma in the resection bed. Therefore, an important application of molecular imaging would be to identify residual carcinoma. The biopsies described in Table 1 include six biopsies taken from recent tumor resection beds in five different bladders. Of these biopsies, four were positive for anti-CD47–Qdot625 binding (Fig. 3F) and two were negative. Two of the four anti-CD47–Qdot625–positive resection bed biopsies represented true-positive binding (one CIS and one adenocarcinoma), and two represented false-positive binding (one ulcer and one inflammation). The two biopsies without detectable anti-CD47–Qdot625 were found to be true negatives (squamous metaplasia and ulcer).

DISCUSSION

Enhanced optical detection of bladder cancer has the potential to improve effectiveness of tumor resection and reduce cancer recurrence and progression. Here, we demonstrate endoscopic molecular imaging of human bladder cancer by targeting the cell surface protein CD47. CD47 is highly expressed in human bladder cancer but undetectable in the superficial layer of normal urothelium. We found CD47 to be a viable molecular imaging target with topically administered antibody-based optical imaging agents. The whole-organ imaging protocol developed here (Fig. 2A) mimics future in vivo endoscopic applications and facilitates clinical translation of CD47-targeted imaging for bladder cancer detection and resection.

Confocal endomicroscopy with anti-CD47 was investigated initially because the digital nature of the confocal images allowed for semiquantitative analysis in contrast to the analog video images collected from blue light cystoscopy. In vivo endoscopic molecular imaging with topical agents and confocal endomicroscopy has also been described for colonic dysplasia (20) and esophageal neoplasia (21). In the colon application, a FITC-labeled peptide was found to bind more strongly to dysplastic colonocytes than to adjacent normal cells, with 81% sensitivity and 82% specificity (20). In the esophageal study, another FITC-labeled peptide demonstrated 3.8-fold greater fluorescence intensity for esophageal neoplasia compared with Barrett esophagus and squamous epithelium, with 75% sensitivity and 97% specificity (21). In these studies, quantitation was determined by first selecting representative frames from video sequences, followed by comparing pixel intensities from different areas within a frame (20, 21). In our study, data from entire confocal video sequences of normal and cancer regions (12 frames/s, 12- to 50-s videos) were included for analysis. Using this unbiased image analysis strategy, we measured the increased fluorescence intensity in human cancer compared to normal tissue with anti-CD47 binding (Fig. 2C).

Although the confocal endomicroscopy allowed for quantitation, the small field of view precluded efficient scanning of the entire bladder

<table>
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<th>Histopathological diagnosis</th>
<th>Number of biopsies</th>
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<tr>
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<td>True positive</td>
<td>False positive</td>
<td>True negative</td>
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<td>6</td>
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<tr>
<td>Inflammation</td>
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<td>4</td>
<td>15</td>
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<td>1</td>
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<td>8</td>
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to detect lesions that might not be detected by white light. Blue light cystoscopy, which offers a wide-field fluorescence cystoscopy, allowed us to survey the entire bladder mucosa and investigate the sensitivity and specificity of CD47-targeted imaging. Anti-CD47 binds to a variety of bladder cancers, including papillary urothelial carcinoma and its variants (squamous and micropapillary), as well as adenocarcinoma, which accounts for <5% of bladder cancer (22). CD47 imaging identified known white light diagnostic challenges including CIS, positive residual cancer in resection bed and surgical margins, and recurrent disease in the context of BCG cystitis. Presence of CIS is a well-established risk factor for bladder cancer progression, and BCG is the standard treatment for patients with high-risk nonmuscle invasive bladder cancer, including CIS. In this high-risk patient cohort, CD47 imaging may improve CIS detection and better stratify patients who may benefit from BCG treatment as well as assess BCG treatment response. In addition, CD47 imaging identified residual cancer in the base and margin of prior resection beds, which may help improve the thoroughness of tumor resection.

We assessed the cancer specificity of CD47 targeting by sequential incubation of bladders with fluorescently labeled IgG isotype control and anti-CD47. For both confocal endomicroscopy (Fig. 2B) and blue light cystoscopy (Fig. 3A), enhanced cancer-to-normal contrast was observed with anti-CD47 compared to the IgG isotype control, indicating that the fluorescence signal detected only after anti-CD47 incubation was the result of specific binding to CD47. Detection of anti-CD47 bound to the luminal surface of biopsy from tumor but not normal tissue further supported specific targeting of CD47 via topical administration of the antibody.

Wide-field fluorescence imaging of CD47 offers several potential advantages over currently approved blue light cystoscopy based on protoporphyrin (for example, hexaminolevulinate), which has been shown to improve bladder cancer detection and decrease recurrence (23, 24). Owing to the potential for false-positive fluorescence, protoporphyrin-based imaging is not recommended for recent tumor resection or BCG treatment. Hexaminolevulinate is also indicated for single use only owing to concerns of hypersensitivity. Because hexaminolevulinate-based imaging requires uptake by viable cells, a direct comparison was not feasible in our study based on ex vivo bladders. Future in vivo comparative studies are needed to confirm our ex vivo findings that molecular imaging of CD47 improves bladder cancer detection and may not be prone to false-positive signals with recent tumor resection or BCG.

To translate CD47-targeted imaging to clinical use, an imaging agent for in vivo human use will need to be established. Bladder is a storage organ with established intravesical route to receive topically administered pharmacological agents including live attenuated vaccines (for example, BCG) and high-dose chemotherapy (for example, mitomycin) without significant systemic absorption. The CD47 antibody used here is a mouse monoclonal antibody and unlikely to be suitable for human use; however, a humanized, good manufacturing practice–grade monoclonal CD47 antibody is currently being studied for therapeutic use in metastatic cancers in a phase 1 clinical trial (25). If this antibody proves safe in human, it could be conjugated with fluorophores for optical molecular imaging. Even if this CD47 antibody is not compatible for systemic use, intravesical administration may prove a safe alternative.

FITC-labeled imaging agents, similar to the anti-CD47–FITC used in this study, have been shown to be safe for topical use in the gastrointestinal tract in combination with confocal endomicroscopy (21) and are likely to be safe in the bladder as well. For clinical translation of a Qdot625-labeled antibody, additional animal testing will be required to determine the safety profile of intravesical anti-CD47–Qdot625 prior to human trials. Quantum dots are a class of inorganic fluorescent particles that is currently not approved for human use because the heavy metal core may pose a risk when administered systemically (26). However, given the advantages of inorganic fluorescent particles (for example, improved quantum yield and photostability) (27), efforts are under way to improve their biocompatibility and reduce the potential toxicity. A recent study using quantum dots covered with a nontoxic shell demonstrated no acute toxicity in rhesus monkeys 90 days after intravenous administration (28). Further, in vivo safety and favorable pharmacokinetics in humans were shown for an inorganic silica particle (C-dot) with near-infrared fluorescence as a cancer-diagnostic imaging agent (29, 30). This may prove a viable alternative with a corresponding near-infrared endoscopic camera if the use of the Qdot625-labeled anti-CD47 proves a limitation to translation. Another possibility includes the use of IRDye 800CW, an organic fluorophore in the near-infrared range approved for investigational use in humans (31).

Although our data indicate that CD47-targeted imaging will allow detection of a wide variety of bladder cancer types, the comparable CD47 expression in nonmuscle invasive and muscle invasive disease suggests its limited value as a prognostic marker. Surface antigens such as epidermal growth factor receptor (EGFR) (32, 33) or prostate stem cell antigen (PSCA) (34–36) also have differential expression between normal urothelium and bladder cancer tissues. Evidence from small-animal studies indicates that targeting EGFR may be effective for identifying and treating a particularly aggressive form of bladder cancer (37), and PSCA may be especially useful in detecting CIS (36). However, EGFR expression in bladder tumors varies between 23 and 72% (38, 39), and PSCA is expressed at low levels in the umbrella cells of normal urothelium and absent from up to 36% of muscle invasive bladder cancer (36). The absence of EGFR and PSCA from a large number of bladder tumors makes them nonideal targets for endoscopic molecular imaging in general, but improved detection and diagnosis may be achieved in combination with a more ubiquitous target, such as CD47.

Our findings shed new light on identification and validation of molecular imaging targets for bladder cancer and potentially for other epithelial cancers. That CD47 can serve as a cancer-specific imaging agent illustrates that both expression and in situ distribution are important criteria for target selection. Our whole-organ imaging approach takes advantage of the ease of accessing the bladder lumen, the bladder’s well-established intravesical route for drug administration, and the availability of clinical grade fluorescence imaging technologies including confocal endomicroscopy and blue light cystoscopy (8). We demonstrate that this type of approach is feasible and may be adaptable for development and validation of imaging agents in other hollow organs. The intended endoscopic application of anti-CD47 imaging will primarily benefit patients with nonmuscle invasive disease. These patients represent most of bladder cancer patients whose cancer is managed endoscopically. Radical cystectomy is typically not recommended for low-risk nonmuscle invasive disease; thus, all bladders imaged for this study were from patients with a history of muscle invasive (stage ≥T2) or high-risk cancer with invasion into the lamina propria (stage T1). We believe that CD47 imaging will be applicable for low-risk
nonmuscle invasive disease, as well as high-risk and invasive, given the similar expression pattern of CD47 in nonmuscle invasive and muscle invasive human bladder cancer. Together, our study provides evidence for CD47 as a bladder cancer imaging target with potential for clinical translation.

**MATERIALS AND METHODS**

**Study design**

This study was designed to investigate CD47 as an endoscopic molecular imaging target for bladder cancer. The differential expression and distribution of CD47 on normal urothelium and bladder tumor specimens were first analyzed using a published gene array data set and snap-frozen tissue sections. With the approval of the local institutional review board, fluorescently labeled anti-CD47 was evaluated as a molecular imaging agent to detect bladder cancer in ex vivo intact human bladders \( n = 26 \). Antibody was administered intravesically, and bound antibody was detected using a clinical grade endoscopic imaging system, either confocal endomicroscopy or blue light cystoscopy. The whole-organ imaging approach was used to determine the feasibility of using topical CD47-targeting agents for endoscopic imaging and to assess the cancer specificity and diagnostic accuracy of CD47-targeted imaging for potential in vivo translation.

Imaged bladders were not selected on the basis of tumor stage, grade, or prior treatment, because patients with a history of bladder cancer undergoing radical cystectomy were eligible for participation in the study. For anti-CD47 imaging with blue light cystoscopy, analysis of biopsied regions was dependent on co-registration of blue light diagnosis and histopathology. Of 131 biopsies, 119 were analyzed and used for sensitivity and specificity calculation, and 12 were excluded because of indeterminate histopathology or blue light diagnosis. All biopsies were reviewed by a single pathologist (R.V.R.) who was blinded to the imaging results.

**CD47 molecular imaging with confocal endomicroscopy**

Fresh intact bladders were obtained from the operating room immediately after radical cystectomy. The bladders were catheterized with an 18-French (Fr) urinary catheter, and 50 ml of anti-CD47–FITC (1:100 diluted in saline) was instilled. The catheter was clamped, and bladder was incubated on a rotator at 37°C for 30 min, followed by irrigation with 100 to 200 ml of sterile saline three times. The bladders were opened longitudinally \( n = 4 \) for white light visual examination of the urothelium or examined intact \( n = 1 \) with a standard 21-Fr white light cystoscope (Karl Storz). The confocal probe was then placed under direct visualization or through the cystoscope on the normal and suspicious/tumor regions identified under white light for confocal endomicroscopy (Mauna Kea Technologies) \((11)\). Ex vivo cystoscopy was conducted under saline irrigation to provide distension of the bladder mucosa. After imaging, representative lesions of normal and cancer-appearing mucosa that were imaged were biopsied for histopathological correlation. The biopsies were processed and stored at −80°C as described above for analysis.

**CD47 molecular imaging with blue light cystoscopy**

For wide-field fluorescence imaging using blue light cystoscopy, anti-CD47 was labeled with Qdot625 (excitation, 405 nm; emission, 625 nm; Life Technologies) by amine-thiol cross-linking per manufacturer’s protocol. Anti-CD47–Qdot625 (50 ml, 10 nM in saline) was instilled into fresh intact bladder specimens, incubated, and rinsed as described for anti-CD47–FITC. For imaging, a clinical blue light cystoscopy system (Karl Storz), which is capable of both standard white light and fluorescence imaging using a blue light (375 to 440 nm) and detection of red light above 610 nm, was used \((7)\). The 21-Fr cystoscope was inserted into the intact bladder to survey the entire mucosa first under white light, then blue light. Under blue light, tissues with anti-CD47–Qdot625 bound showed pink fluorescence, and tissue without anti-CD47–Qdot625 binding demonstrated blue background. A tangential angle of incidence of blue light to bladder mucosa may cause false fluorescence. Positive anti-CD47–Qdot625 binding was defined as a discrete area with persistent pink fluorescence independent of distance and angle of observation. The entire cystoscopy was video-recorded to facilitate colocalization between image findings and histopathology.

Three strategies were used for colocalization of anti-CD47–Qdot625 binding with histology. The most stringent strategy was used for the majority of bladders \( n = 16 \) by direct cystoscopic biopsy of areas of interest using standard biopsy instrument under fluorescence guidance in the intact bladder. The second strategy was cystoscopic injection of toluidine blue or India ink into the bladder mucosa adjacent to the regions of interest using a 27-gauge endoscopic needle inserted through the working channel of the cystoscope followed by pathological sampling of the inked areas after formalin fixation \( n = 5 \) \((fig. S3)\). The third strategy was to correlate distinct regions of interest with the detailed pathological description and analysis. This approach was used for areas that were not amenable to biopsy or dye injection \( n = 4 \) (for example, bladder neck) and areas with obvious gross pathology \( n = 4 \) (for example, solitary papillary tumor). True positive was defined as a histologically confirmed bladder cancer that exhibited pink fluorescence under blue light. True negative was defined as histologically confirmed benign mucosa \( n = 5 \) (for example, normal, inflammation) with background blue fluorescence.

**Statistical analysis**

Statistical analysis was performed using R software. All data are presented as means ± 95% confidence interval. For CD47 gene expression analysis, the significance of CD47 expression levels in different tissue groups was analyzed by pairwise \( t \) tests. A threshold to identify high and low gene expression levels was computed on the basis of StepMiner as described previously in BooleanNet \((15, 17)\). For the reproducibility analysis of fluorescence intensity measurement using confocal endomicroscopy, linear regression analysis was used in R software. For the diagnostic accuracy of anti-CD47–Qdot using blue light cystoscopy, sensitivity was calculated as TP/(TP + FN), and specificity as TN/(TN + FP), where TP is true positive, FN is false negative, TN is true negative, and FP is false positive.

**SUPPLEMENTARY MATERIALS**

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Materials and Methods

Fig. S1. Software algorithm for fluorescence intensity analysis of confocal videos.

Fig. S2. Fluorescence signal reproducibility of CD47 imaging using confocal endomicroscopy.

Fig. S3. Colocalization of blue light cystoscope images and histology by intravesical dye injection.

Fig. S4. Immunofluorescence detection of tissue-bound anti-CD47 after imaging.

Table S1. Patient characteristics and histopathological diagnoses.

REFERENCES AND NOTES


