CD47-targeted optical molecular imaging and near-infrared photoimmunotherapy in the detection and treatment of bladder cancer

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Transurethral resection of bladder tumor (TURBT) followed by intravesical therapy remains the most effective strategy for the management of non-muscle-invasive bladder cancer worldwide. TURBT has two purposes: to remove all visible tumors and to obtain tumor specimens for histopathological analysis. However, the detection of flat and small malignant lesions under white-light cystoscopy is extremely challenging, and residual lesions are still the main reason for the high recurrence rate of bladder cancer. We hypothesized that visual enhancement of malignant lesions using targeted optical molecular imaging could potentially highlight residual tumors in the bladder during surgery, and near-infrared photodynamic therapy (NIR-PIT) could kill exfoliated cancer cells and residual tumors. A mouse model of complete or partial bladder tumor resection was established under the guidance of optical molecular imaging mediated by indocyanine green and anti-CD47-Alexa Fluor 790, respectively. Once the tumor recurred, mouse model received repeated CD47-targeted NIR-PIT. After complete resection, there was no tumor recurrence. Furthermore, the growth rate of recurrent tumor decreased significantly after repeated NIR-PIT. Therefore, CD47-targeted optical molecular imaging can potentially assist urologists to detect and remove all tumors, and repeated NIR-PIT shows the potential to reduce tumor recurrence rates and inhibit the growth of recurrent tumors.

INTRODUCTION
About 75% of newly diagnosed bladder cancer (BC) manifests as early-stage (Tis, Ta, and T1) lesions confined to the mucosa or submucosa, known as non-muscle-invasive bladder cancer (NMIBC).1 Transurethral resection of bladder tumor (TURBT) is pivotal in the diagnosis and treatment of NMIBC. Unfortunately, during repeated transurethral resection (reTUR), residual tumors were detected in 17%–67% of Ta patients and 20%–71% of T1 patients, and most of the residual tumors (36%–86%) were located at the initial resection site.2 The high incidence of residual tumors reflected incomplete bladder tumor resection at the first operation. During the follow-up period, the 5-year recurrence-free survival rates of low-, intermediate-, and high-risk NMIBC were 43%, 33%, and 23%, respectively, and 21% of high-risk NMIBC patients progressed to muscle-invasive bladder cancer (MIBC).3,4 For NMIBC patients who progressed to MIBC, the 4-year cancer-specific survival rate was only 35%, and the prognosis was worse than that of patients with newly diagnosed MIBC who received radical cystectomy (RC) immediately.5 Tumor recurrence and progression not only endanger the life span of patients but also have double adverse effects on medical expenses and quality of life due to repeated surgical intervention, which has attracted more and more attention among urologists in recent years.6,7 Thus, optimizing detection and complete resection of early-stage BC are critical for prolonging overall survival and reducing medical costs.

During white-light cystoscopy (WLC)-assisted transurethral resection, urologists mainly rely on their own clinical experience and indirect visual feedback to determine the location and number of tumor lesions, and the boundary and depth of tumor invasion. Flat and small malignant lesions, particularly carcinoma in situ (CIS), are not easily displayed and diagnosed in the bladder wall, based on the feedback of visible images. CIS is a type of high-risk NMIBC that is confined to the mucosa, which can be easily confused with inflammatory lesions due to their similar structural appearance under WLC. Other imaging technologies commonly used in the diagnosis of BC, such as ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI), are mainly used for preoperative diagnosis and postoperative follow-up because they can not conveniently and accurately provide real-time dynamic-imaging information during transurethral resection. In recent years, urologists have begun to use enhanced-imaging technology for surgical navigation.
In this study, we hypothesized that CD47-targeted optical molecular imaging could be used to guide real-time bladder tumor resection and that near-infrared photodynamic (NIR-PIT) could inhibit the growth rate of recurrent tumor. On the basis of the specific binding of CD47 antibody to bladder tumor tissue, we established a novel partial or complete tumor resection model guided by optical molecular imaging, and then we recorded the tumor recurrence rate to test the clinical value of CD47-targeted optical molecular imaging in surgical navigation. Then, we explored the safety and efficacy of CD47-targeted NIR-PIT in xenograft mouse models with residual disease or tumor recurrence in vivo. Finally, we tested the diagnostic accuracy of CD47-targeted optical molecular imaging in detecting malignant lesions in vivo in an orthotopic rat model of BC.

RESULTS

The ability of different molecular tracers to identify human BC cells

To investigate the ability of different molecular tracers to identify human BC cells, cells were incubated with molecular tracers and then analyzed using flow cytometry. The ability of isotype control antibodies or negative control peptides to identify cancer cell lines is weak, whereas the molecular tracers (antibodies and peptides) have a significantly high ability to identify cancer cell lines (Figure 1A). For 5637 cells and UM-UC-3 cells compared with CA9 antibody NYZL1 and PLZ4 molecular tracers, the ability of CD47 antibody to identify BC cell lines is increased. And for RT4 cells compared with CD47 antibody NYZL1 and PLZ4 molecular tracers, the ability of CA9 antibody to identify BC cell lines is reduced.

Human BC cell lines express higher levels of CD47 than SV-HUC-1 cells

To determine whether the expression of CD47 in BC cells is higher than that in human normal urothelial cell line, cancer cells or SV-HUC-1 cells were incubated with anti-CD47-AF790 and its isotype control antibody IgG1-FITC and then detected using flow cytometry (Figure 1B). As controls, the ability of IgG1 to identify BC cells is non-specific. Therefore, although the CD47 mRNA expression in cancer cell lines and normal urothelial cell line is different, the curves look very similar (Figures 1B and 1C). The expression of CD47 mRNA in human BC cell lines or normal urothelial cell line was also analyzed. The results showed that, compared with SV-HUC-1 cells, the expression of CD47 mRNA in BC cells was significantly higher (5637 versus SV-HUC-1, p < 0.0001; UM-UC-3 versus SV-HUC-1, p = 0.0004; RT4 versus SV-HUC-1, p = 0.0065), which supported the results of flow cytometry (Figure 1C). Among the three types of BC cell lines, 5637 cells had the highest expression level of CD47 mRNA (5637 versus UM-UC-3, p < 0.0001; 5637 versus RT4, p < 0.0001).

In vitro NIR-PIT of human BC cell lines

We first evaluated CD47-targeted NIR-PIT in three human BC cell lines that express different levels of CD47: 5637, UM-UC-3, and RT4. Cells were incubated with anti-CD47-Alexa Fluor 790 (anti-CD47-AF790) and then exposed to increasing NIR light doses (0–32 J/cm²) to induce PIT. To measure the direct toxicity of NIR-PIT on cancer cells, propidium iodide staining and flow cytometry were used to detect the fraction of death cancer cells. Cells incubated with PBS and cells without irradiation were used as controls. In cancer cell lines, CD47-targeted NIR-PIT resulted in increased cell death in a light-dose-dependent manner. Significant cancer cells death started at 4 J/cm² in 5637 cells, 8 J/cm² in UM-UC-3 cells, and 8 J/cm² in RT4 cells, and the fraction of cell death increased with the increase of NIR light energy. At the maximum NIR irradiation level of 32 J/cm², over 80% of 5637 and UM-UC-3 cells and over 50% of RT4 cells died (Figure 2A).
To determine whether blocking CD47-SIRPα with CD47 antibody would activate the "eat me" signal, a phagocytosis assay was performed on human BC cell lines. Compared with cancer cell lines treated with isotype control antibody IgG1, CD47 antibody intervention significantly increased the phagocytosis of cancer cells by macrophages (Figure 2B).

In three BC cell lines, since 5637 cells have the highest expression of CD47 mRNA, we then evaluated the phagocytosis of 5637 cells by macrophages under CD47-targeted NIR-PIT (Figure 2C). The 5637 cells were incubated with PBS or anti-CD47 or anti-CD47-AF790 and then seeded onto 96-well plates pre-cultured with RAW 264.7 macrophages. The mixed cells were exposed to a light dose of 4 J/cm² to induce NIR-PIT. Non-irradiated 5637 cells were used as controls. Compared with the control group, the absorbance of cells incubated with anti-CD47-AF790 was significantly decreased under the light irradiation of 4 J/cm² (p < 0.0001), but this phenomenon was not observed in the PBS or anti-CD47 groups (p = 0.08 and 0.59, respectively). The results showed that CD47-targeted NIR-PIT has the dual function of inducing direct cancer cell death and enhancing phagocytosis.
Local recurrence occurred in the mouse model with residual tumor after surgery

In order to evaluate the real-time intraoperative guidance value of optical molecular imaging, partial or complete tumor resection was performed on BC xenograft models under CD47-targeted optical molecular imaging. Just as with the procedure of TURBT, two urologists independently determined whether there was residual disease in the wound on the basis of their own clinical experience and visual feedback. The urologists could only accurately point out that 2 of 32 mice with partial tumor resection (groups A, B, D and E) but mistakenly point out that 1 of 16 mice with complete tumor resection (groups C and F) had residual tumors. Thus, visual inspection for residual lesions in the wound had a sensitivity of 6.25% (2/32), a specificity of 93.75% (15/16), a positive predictive value of 66.67% (2/3), and a negative predictive value of 33.33% (15/45).

In the tumor volume measurement of the xenograft mouse model, microCT scanning has been proved to be more accurate than external caliper.

In groups C and F, the bladder tumor was completely removed under the guidance of optical molecular imaging, and there was no tumor recurrence. In groups A and D, the bladder tumor was partially removed under the guidance of optical molecular imaging, and the tumor recurrence rates were 87.5% (7/8) and 75% (6/8), respectively. In group E, mice received CD47-targeted NIR-PIT immediately and 24 h after surgery, and 50% (4/8) of those mice had tumor recurrence (Figure 3A), while the remaining 50% (4/8) of mice had no tumor recurrence (Figure 3B). In groups A, E, and E, the tumor recurrence rate of group E was lower than that of groups A and D, but the difference was not statistically significant (E versus A, p = 0.28; E versus D, p = 0.61).
Evaluation of the therapeutic effect of repeated CD47-targeted NIR-PIT in the xenograft mouse model with tumor recurrence

To evaluate the therapeutic effect of NIR-PIT, mice with tumor recurrence in group B received repeated CD47-targeted NIR-PIT. In groups A and B, tumor recurrence occurred in seven and eight mice, respectively. Seven mice in group B were randomly selected to receive repeated NIR-PIT, and seven mice with tumor recurrence in group A did not receive any intervention. Compared with the control group with no intervention, the tumor growth rate of mice in group B was significantly lower ($p < 0.0001$; Figure 3C).

Ex vivo optical molecular imaging of partially resected tumor specimens

In groups D and E, mice underwent partial tumor resection under the guidance of CD47-targeted optical molecular imaging, and then the tumor specimens and adjacent normal tissues (skin, fat, and muscle) were collected for in ex vivo optical molecular imaging (Figure 4A). Under optical molecular imaging, the mean fluorescence intensity (MFI) of tumor specimens and adjacent normal tissues (skin, fat, and muscle) were recorded. The fluorescence signal of tumor specimens was significantly higher than that of adjacent normal tissues ($p < 0.0001$). On average, the MFI of tumor specimens was about 2.5 times that of adjacent normal tissues (Figure 4B).

In vivo optical molecular imaging in an orthotopic rat model of BC

Two Wistar rats died of other causes before space-occupying lesions were found in the bladder. When space-occupying lesions were confirmed by microMRI scanning, anti-CD47-AF790 molecular tracer was infused into the bladder through the urethra, and then the entire bladder mucosa was imaged using hand-held NIR imaging equipment (Figure 5). During the examination, the bladder mucosa with fluorescence signal was marked, while the space-occupying lesions without fluorescence signal were considered as suspicious malignant lesions. For each rat, when the sum of the fluorescent-labeled sites and the suspicious malignant lesions is less than 5, a random biopsy of bladder mucosa shall be performed to make the total number of specimens not less than 5. Through this procedure, a total of 146...
tissue specimens were collected, including 87 fluorescent-labeled sites, 12 suspicious malignant lesions, and 47 random biopsies. Histopathological analysis confirmed that 80 of 87 fluorescent-labeled sites, 8 of 12 suspicious malignant lesions, and 3 of 47 random biopsies contained tumor tissue. In other words, among 146 specimens, 91 tumor tissues and 55 normal tissues were confirmed by histopathological analysis. Under CD47-targeted optical molecular imaging, fluorescence signals were detected in 80 tumor tissues, but not in 48 normal tissues. Therefore, the sensitivity and specificity of CD47-targeted optical molecular imaging in the detection of bladder tumor were 87.9% (80/91) and 87.3% (48/55), respectively.

**DISCUSSION**

According to their molecular size, molecular tracers can be subdivided into the following five classes: monoclonal antibodies, antibody fragments, protein scaffolds, peptides, and small molecules. So far, there is no study to investigate the difference of the ability of different molecular tracers to identify BC cells. By consulting the relevant literature on PubMed, Web of Science, and Scopus, the molecular tracers used in the diagnosis and treatment of BC were summarized and analyzed. The results showed that the main molecular tracers for bladder tumors were monoclonal antibodies and peptides (Table 1). Epidermal growth factor receptor (EGFR) was overexpressed in 74% of bladder tumor tissues, and was low-expressed in normal transitional epithelium. However, the results of The Cancer Genome Atlas (TCGA) project demonstrated that the amplification rate of EGFR in MIBC was only 11%, which limited it being an effective target for the management of BC. Human epidermal growth factor receptor 2 (HER-2) was overexpressed in about 40% of bladder tumor tissues but not in normal bladder epithelial cells. Immuno-histochemical analysis showed that the expression of CA9 in normal urothelial cells was negative but that more than 70% of malignant lesions were positive, and that the expression level of CA9 in Ta stage and low-grade NMIBC was significantly increased. Meanwhile, as a urine marker, the sensitivity and specificity of CA9 in the diagnosis of BC were 86.2% and 95.1%, respectively, and the prediction accuracy of BC was significantly higher than that of urine exfoliated cytology. CD47 was overexpressed in more than 80% of BC cells, and it was overexpressed in both NMIBC and MIBC but not in normal urothelial cells. The sensitivity and specificity of anti-CD47-Qdot625-mediated optical molecular imaging in the detection of bladder malignant lesions were 82.9% and 90.5%, respectively. Therefore, in this study, we chose CD47 antibody and CA9 antibody as the research objects. Unlike monoclonal antibodies, the binding site of peptides to BC cells is not clear, so it is impossible to quantitatively analyze the expression level of this site on the cell membrane. Thus, NYZL1 and PLZ4 peptides screened by different techniques were selected. NYZL1 peptide was screened by phage display technology, while PLZ4 peptide was screened by the “one bead, one compound” combinatorial chemistry method.

After BC cells were incubated with molecular tracers, the results of flow cytometry showed that, compared with the other three molecular tracers (NYZL1, PLZ4, and CA9 antibodies), the ability of CD47 antibody to identify cancer cells was increased. Meanwhile, compared with normal urothelial cells, the ability of CD47 antibody to identify BC cells was increased, and the expression level of CD47 mRNA in BC cells was significantly higher. And then, we found that in CD47-expressing BC cell lines CD47-targeted NIR-PIT has a direct killing effect on cancer cells in a light-dose-dependent manner. At the maximum light-energy level, the specific cell death rate of 5637 cells with the highest expression of CD47 mRNA induced by NIR-PIT was up to 89.66%.

Urologists mainly rely on clinical experience, endoscopy training, and indirect visual feedback when delineating and resecting bladder malignant lesions. However, determining the boundary and number of
bladder tumors based on these subjective assessments is challenging and may lead to residual disease, which is the main cause of early tumor recurrence and ultimately affects the oncological prognosis. Complete resection with negative tumor margins must also be balanced with preserving the surrounding structures to minimize the occurrence of bladder perforation. Although the EAU guidelines recommend selective biopsy of the wound after transurethral resection, this procedure can examine only a small part of the tumor base and is prone to sampling errors. A systematic review of the clinical data of 8409 NMIBC patients showed that 17%–67% of patients with stage Ta and 20%–71% of patients with stage T1 had residual tumors at the time of reTUR, and most (36%–86%) of them were located at the initial resection site. Meanwhile, the rates of tumor upstaging in Ta and T1 patients were 0%–8% and 0%–32%, respectively. As a result, the recurrence and progression rates of NMIBC patients were 15%–61% and 0.2%–17% at 1 year, and 31%–78% and 0.8%–45% at 5 years, respectively. Compared with the newly diagnosed stage T2 BC patients who underwent RC immediately, patients with NMIBC upstaged to stage T2, confirmed by histopathological analysis after RC had a worse oncological prognosis.

Several optical-imaging technologies have been developed as an auxiliary mode of WLC to assist urologists in improving intraoperative decision-making. According to the imaging field of view, optical-imaging technology can be subdivided into two modes, namely macroscopic imaging and microscopic imaging. PDD and NBI belong to the macroscopic-imaging mode and examine a wide area of bladder mucosa in a way similar to WLC. Through additional contrast enhancement, suspicious malignant lesions can be highlighted, and then the patient’s oncological prognosis is improved. Due to the lack of tumor specificity, although PDD and NBI can improve the detection rate of flat lesions and CIS, the incidence of false-positive results will increase if there are inflammatory lesions, acute bleeding, and previous intravesical therapy in the bladder. Confocal laser endoscopy (CLE) and optical coherence tomography (OCT) are real-time microscopic-imaging modes. Through high-resolution imaging of suspicious malignant lesions, the pathological information about the changes in tissue microstructure and cell morphology can be obtained. Unfortunately, only a limited area of bladder mucosa can be examined under the visual field of CLE and OCT. Therefore, the application of microscopic-imaging mode needs to be combined with an additional, macroscopic-imaging technology (such as WLC, PDD, or NBI) to determine the location of suspicious malignant tissues first.

In this study, we first established a mouse model of complete or partial bladder tumor resection under the real-time guidance of optical molecular imaging. Indocyanine green (ICG) is a non-toxic fluorescent dye that can be administered intravenously and then used for optical molecular imaging, which has been approved by the U.S. Food and Drug Administration. Although ICG lacks tumor specificity, it can accumulate in tumor tissues via the enhanced permeability and retention effect. Thus, under optical molecular imaging mediated by ICG, residual lesions in the surgical wound can be detected after tumor resection. Theoretically, compared with ICG, fluorescent dye-labeled CD47 antibody is a more valuable molecular tracer in the detection of BC due to its tumor specificity. There was no tumor recurrence in the mouse model of complete tumor resection under the real-time guidance of CD47-targeted optical molecular imaging, indicating that CD47-targeted optical molecular imaging has potential navigation value in bladder tumor resection. Meanwhile, CD47-targeted optical molecular imaging has high diagnosis accuracy in the detection of BC in an orthotopic rat model. Then, we evaluated the therapeutic effect of CD47-targeted NIR-PIT in a mouse model with residual bladder tumors. Compared with the mice in group A (no intervention) and group D (immunotherapy alone), the tumor recurrence rate of the mice in group E decreased after NIR-PIT intervention, but the difference was not statistically significant. Similarly to intravesical therapy after TURBT, we will explore whether CD47-targeted NIR-PIT maintenance therapy can significantly reduce the recurrence rate in mice with residual tumor in the future. Finally, the mice that underwent partial tumor resection (group B) received CD47-targeted NIR-PIT maintenance therapy after tumor recurrence, and the tumor growth rate was significantly lower than that of mice without intervention (group A).

Due to its easy access, the urinary tract is very suitable for endoscopic molecular imaging and NIR-PIT. Human bladder is a hollow organ with high compliance, which provides an ideal closed darkroom for endoscopic molecular imaging without the interference of external light sources. In addition to systemic intravenous administration, intravesical instillation provides an alternative route to increase the...
local concentration of molecular tracers and reduce systemic side effects. Using a NIR cystoscopic camera, the fluorescence signal from fluorescent-dye-labeled molecular tracer would allow for intraoperative optical molecular imaging, thereby helping the urologist to improve the detection rate of flat and small lesions and evaluate the resection margin to reduce recurrence. After transurethral resection, a light-guide bundle is inserted into the bladder through a catheter, and PIT can be induced by direct NIR light irradiation, thereby specifically killing the exfoliated tumor cells and residual diseases. The local concentration of molecular tracers and reduce systemic side effects. Using a NIR cystoscopic camera, the fluorescence signal from fluorescent-dye-labeled molecular tracer would allow for intraoperative optical molecular imaging, thereby helping the urologist to improve the detection rate of flat and small lesions and evaluate the resection margin to reduce recurrence. After transurethral resection, a light-guide bundle is inserted into the bladder through a catheter, and PIT can be induced by direct NIR light irradiation, thereby specifically killing the exfoliated tumor cells and residual diseases. The fresh intact tumor specimens were collected after en bloc resection and imaged using a fluorescence camera and a fluorescence microscope, respectively. Through fluorescence images, pathologists can evaluate the surgical margins and the depth of tumor invasion and then provide urologists with useful information about which patients need to receive reTUR or RC (Figure 6).

In summary, the ability of CD47 antibody to identify BC cell lines is increased compared with the other three molecular tracers (NYZL1, PLZ4, and CAIX antibodies). CD47-targeted optical molecular imaging has high diagnosis accuracy in the detection of BC and has potential navigation value in the resection of BC. CD47-targeted NIR-PIT has a direct killing effect on cancer cells in a light-dose-dependent manner and can inhibit the growth rate of recurrent tumors. The combination of targeted optical molecular imaging and NIR-PIT may meet the needs of special imaging for the detection of all malignant lesions and precise targeted therapy, thus providing a complementary option in the management of BC.

**MATERIALS AND METHODS**

**Reagents**

ICG for injection was purchased from Dandong Yichuang Pharmaceutical (Liaoning, China). Anti-CD47, anti-CD47-fluorescein isothiocyanate (anti-CD47-FITC) and its isotype control antibody normal mouse IgG1-FITC, anti-CA9-FITC and its isotype control antibody normal mouse IgG2a-FITC, and anti-CD47-AF790 were purchased from Santa Cruz Biotechnology (Oregon, USA). CSSPIGRHC (NYZL1) peptide and its negative control CRIGSPHSC (cNYZL1) peptide CQAGRMGFC (cPLZ4) peptide and its negative control CQDGRMGFC (PLZ4) peptide and its negative control CQAGRMGFC (cPLZ4) peptide were synthesized by Chinese Peptide (Hangzhou, China) using the standard solid-phase fluorenylmethyloxycarbonyl chloride chemistry. All peptides were purified by high-performance liquid chromatography with a minimum purity of 99.0%. The sequence and structure of each peptide were characterized and verified by mass spectrometry. FITC-labeled NYZL1 (NYZL1-FITC), FITC-labeled cNYZL1 (cNYZL1-FITC), FITC-labeled PLZ4 (PLZ4-FITC), and FITC-labeled cPLZ4 (cPLZ4-FITC) were also synthesized in the same manner as above.

**Cell lines**

RAW 264.7 macrophages, human urothelial cell line SV-HUC-1, and human BC cell lines 5637, UM-UC-3, and RT4 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All three BC cell lines are derived from male patients, but have different grades and genome instability groups. According to the urothelial BC cell lines index,13 RT4, 5637, and UM-UC-3 have a low, intermediate, and high genome instability group, respectively. Apart from TERT and
TP53 mutations in all three cancer cell lines, additional gene mutations are FGFR3 in RT4 cells and KRAS2 in UM-UC-3 cells. All cell lines were cultured under specific conditions recommended by the official website of the Cell Bank of the Chinese Academy of Sciences.

Flow cytometry
Three BC cell lines were trypsinized from a culture flask and then incubated with molecular tracers and its isotype control antibodies or negative control peptides, respectively. The molecular tracers used in this assay were as follows: anti-CD47-FITC, anti-CA9-FITC, NYZL1-FITC, and PLZ4-FITC. Flow cytometry was performed on the FACSCanto II flow cytometry system (BD Biosciences, USA), and the data were analyzed using FlowJo software. Then, SV-HUC-1 cells were incubated with anti-CD47-FITC, and the expression of CD47 on cell membrane was detected by flow cytometry.

Figure 6. Schematic diagram of targeted optical molecular imaging and NIR-PIT for the management of BC
Using a NIR cystoscopic camera, the fluorescence signal from fluorescent dye would allow for intraoperative optical molecular imaging, thereby helping the urologist to improve the detection of flat and small lesions and evaluate the resection margin to reduce recurrence. After transurethral resection, a light-guide bundle is inserted into the bladder through a catheter, and PIT can be induced by direct NIR light irradiation, thereby specifically killing the exfoliated tumor cells and residual diseases. The fresh intact tumor specimens were collected after en bloc resection and imaged using a fluorescence camera and a fluorescence microscope, respectively. Through the fluorescence images, pathologists can purposefully select tissue samples for histopathological analysis to evaluate the surgical margins and the depth of tumor invasion and then provide urologists with useful information about which patients need to undergo reTUR or RC.
Reverso transcription-quantitative polymerase chain reaction

According to the manufacturer’s protocol, TRIzol reagent (Thermo Fisher Scientific) was used to extract total RNA from SV-HUC-1 and three BC cell lines, respectively. The yield of RNA was determined using a spectrophotometer (Thermo Fisher Scientific), and the integrity was evaluated using agarose gel electrophoresis stained with ethidium bromide.

Real-time PCR was performed using LightCycler 480 II Real-time PCR Instrument (Roche, Switzerland) with a PCR reaction mixture that included cDNA, 2× PerfectStart Green qPCR SuperMix, forward primer, reverse primer, and nuclelease-free water. The expression level of CD47 mRNA was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. The primer sequences were as follows: human CD47 (5'-GCTCTAAACAAGTCCACTGTC-3' and 5'-CATCTTCAAAGGCACTCTCC-3'), and human GAPDH (5'-CCTCACAGTTGCCATGTAGA-3' and 5'-TGATACATGACAAGGTGCG-3').

In vitro CD47-targeted NIR-PIT of BC cell lines

BC cell lines were removed from a culture flask using trypsin and incubated with phosphate-buffered saline (PBS), anti-CD47, or anti-CD47-AF790. Then, cancer cells were seeded onto 96-well low-adhesion plates and irradiated with NIR (760–800 nm) light-emitting diode (Shanghai WinWorld Trading, SM0760). The light source was placed 0.2–0.3 cm above the cells and produced a light-power density of 100 mW/cm², which was measured with an optical power meter (Thorlabs, PM 100D). Cancer cells incubated with anti-CD47-AF790 received increasing light-energy levels of 0, 1, 4, 8, 16, or 32 J/cm², whereas cells incubated with anti-CD47 received 32 J/cm² or no irradiation. After 30 min of cell intervention, 10% of the cell suspension volume of propidium iodide (10 μg/mL) was added to each well of the 96-well plate and incubated at room temperature for 20 min followed by flow cytometry to detect the percentage of dead cells.

In vitro phagocytosis assay

Phagocytosis analysis was performed to evaluate the phagocytosis of anti-CD47-induced macrophages on cancer cells. RAW 264.7 macrophages were pre-cultured in a 15-mm laser confocal culture dish overnight. Three BC cell lines were removed from the culture flask using trypsin and incubated with PBS, IgG1-FITC, or anti-CD47-FITC, respectively. The cancer cells were added to the laser confocal culture dish, co-cultured with RAW 264.7 macrophages, and then observed by laser-scanning confocal microscopy.

The 5637 cells were removed from the culture flask using trypsin and incubated with PBS, anti-CD47, or anti-CD47-AF790, respectively. Cells were then seeded onto 96-well plates, in which RAW 264.7 macrophages were pre-cultured overnight. Cancer cells and macrophages were co-cultured and treated with 4 J/cm² NIR light intervention or no irradiation. Cell viability was measured by the CCK-8 method. Absorbance was detected at 450 nm by a microplate reader, and the data were analyzed using Thermo Scientific SkanIt.

Animal studies

Female Wistar rats (6 weeks old) and athymic NU/NU nude mice (4 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). All animals were kept in pathogen-free and suitable temperature (25 ± 1°C) conditions with a light-dark cycle of 12/12 h. Water and standard diet were provided ad libitum, and the cages were changed twice weekly to maintain hygienic conditions. All procedures involving the animals were performed in accordance with the guidelines formulated by the Ethics Committee for Animal Experimentation of Shanxi Medical University (permission no. SYDL20200010).

Partial or complete tumor resection in xenograft mouse model

A total of 2×10⁵ 5637 cells in 50% Matrigel (Corning) were injected subcutaneously at the left forelimb pit of NU/NU nude mice. The calculation formula of tumor volume is (l × w²)/2, where l = length and w = width. Tumor resection was performed when the volume reached ~500 mm³. For evaluation of CD47-targeted optical molecular imaging-assisted tumor resection and NIR-PIT in vivo, mice were randomly divided into six groups: A, B, C, D, E, and F, with eight mice in each group. Under the real-time guidance of optical molecular imaging mediated by ICG, 95% of the tumor in group A and group B was removed, and complete tumor resection was performed in group C. Under the real-time guidance of CD47-targeted optical molecular imaging, 95% of the tumor in groups D and E was removed, and complete tumor resection was performed in group F. Mice in group E received NIR light irradiation at an energy level of 100 J/cm² immediately and 24 h after surgery. ICG (2.5 mg/kg) or anti-CD47-AF790 (100 μg) was administered intravenously 24 h prior to tumor resection.

Before the wound was closed, two independent urologists were asked to distinguish which mice underwent complete tumor resection and which mice underwent partial tumor resection. Neither of them had prior knowledge about experimental design and surgical method. After sufficient hemostasis in the surgical area, the two urologists independently examined the wound and recorded the suspicious areas without any enhanced visual tools.

MicroCT scanning

In order to investigate the clinical value of routine imaging technology during surgery, microCT scanning (Bruker, Germany) was performed before wound closure to determine whether there was residual tumor in the wound and its location. Thirty representative animals (5 mice in each group) were imaged using the microCT before surgery, and then the mice were imaged again by utilizing the microCT after the tumor was partially or completely removed.

In vivo NIR-PIT of xenograft mouse model with tumor recurrence

Mice in groups A and B underwent partial tumor resection under the guidance of ICG-mediated optical molecular imaging, and the tumor recurrence was observed after surgery. When the tumor volume reached ~100 mm³, the therapeutic efficacy of repeated NIR-PIT in xenograft mouse model with tumor recurrence was evaluated. Mice
in group B received NIR light irradiation at day 1 (100 J/cm²) and day 2 (100 J/cm²) after intravenous injection of 100 μg anti-CD47-AF790, once a week for 3 weeks.

**Orthotopic rat model of BC**
To induce the occurrence of space-occupying lesion in bladder, 30 female Wistar rats were given drinking water containing 0.05% N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN; Tokyo Chemical Industry Japan). After 18 weeks, Wistar rats were imaged using a micro-MRI (Bruker, Germany) to detect whether a space-occupying lesion occurred in the bladder. Rats without a space-occupying lesion continued to be fed drinking water containing 0.05% BBN, and micro-MRI was performed once a week until a space-occupying lesion occurred.

**In vivo optical molecular imaging in orthotopic rat model of BC**
Intravesical instillation of 2 μg of anti-CD47-AF790 was performed in rats with a -occupying lesion using the urethra through an angiocatheter 24-G intravenous indwelling needle plastic sheath (BD Bioscience). After 30 min, a small incision was made in the lower abdomen of the rat to expose the bladder. The bladder was cut longitudinally, and the entire bladder mucosa was imaged using the NIR imaging device. Under the guidance of CD47-targeted optical molecular imaging, the fluorescent sites in the bladder mucosa were marked. Then, the marked site and the suspicious malignant lesions visible by naked eye were removed. For each rat, if fewer than five specimens were removed through the above-mentioned steps, a random biopsy of bladder mucosa was needed to make the total number of specimens not fewer than five. The specimens were fixed in 4% paraformaldehyde and then embedded in paraffin for hematoxylin-eosin (HE) staining.

**Statistical analysis**
Qualitative statistics were expressed as numbers and percentages. For tumor recurrence rate, Fisher’s exact test was used to compare the differences between two groups. Descriptive statistics were recorded as mean ± standard deviation. For CD47 mRNA expression level, unpaired t test was used to assess the differences between two groups. For phagocytosis of 5637 cells by macrophages and the tumor growth rate, two-way ANOVA was used to evaluate the differences between control and experimental groups. Statistical analysis was performed using the statistical software GraphPad Prism, version 7.0 for Windows. Significant differences were reached when p < 0.05.

**AVAILABILITY OF DATA AND MATERIALS**
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**AUTHOR CONTRIBUTIONS**
Y.J.Y. and X.F.Y. performed the conception and analyzed data. Y.J.Y., X.T.Y., J.W.L., and C.L. performed experiments and analyzed data. Y.J.Y., C.L., and X.F.Y. wrote, reviewed, and revised the manuscript.

**DECLARATION OF INTEREST**
The authors declare no competing interests.

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