A Fluorescently-labeled Tumor-specific Peptide for Intraoperative Visualization of Non-muscle Invasive Bladder Cancer: A Clinical Study

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Abstract

Bladder cancer is the fifth most common malignancy in humans. Cystoscopy under white light imaging is the gold standard for bladder cancer diagnosis, but these tumors are difficult to visualize and can be overlooked, resulting in high recurrence rates. We previously developed a phage display-derived peptide-based near-infrared imaging probe, PLSWT7-DMI, which binds specifically to bladder cancer cells and is nontoxic to animals. Here, we report the first-in-human application of this probe for near-infrared fluorescence endoscopic detection of bladder cancer. The purity, efficacy, safety, and nontoxicity of the probe were confirmed prior to its clinical application. Twenty-two patients diagnosed with suspected non-muscle invasive bladder cancer were enrolled in the present study. Following intravesical administration of the probe, the entire mucosa was imaged under white and near-infrared imaging using an in-house developed endoscope that could switch between these two modes. The illuminated lesions under near-infrared light were biopsied and sent for histopathological examination. We observed a 5.1-fold increase in the fluorescence intensity in the tumor samples compared to normal tissue, and the probe demonstrated a sensitivity and specificity of 91.2% and 90%, respectively. Common diagnostic challenges, such as small satellite tumors, carcinoma *in situ*, and benign suspicious mucosa, were visualized and could be distinguished from cancer. Further, no adverse effects were observed in humans. These first-in-human results indicate that PLSWT7-DMI-based near-infrared fluorescence endoscopy is a safe and effective approach for the improved detection of bladder cancer, and may enable thorough resection to prevent recurrence.

Introduction

Bladder cancer (BC) is the fifth most common human malignancy, with an incidence of 81.2 per 100,000 individuals[1]. At initial presentation, approximately 70% of patients present with non-muscle invasive bladder cancer (NMIBC), in which the tumor is superficial (stage carcinoma *in situ* [CIS]), or limited to the mucosa (Ta) and submucosa (T1), and up to 90% of NMIBC cases are urothelial neoplasms[2, 3]. Urothelial neoplasms are polymorphic in nature and can appear as solitary or multiple, flat, or exophytic, at any site on the surface of the bladder urothelium[4]. Bladder cancer usually has a good prognosis, particularly with early detection at stage Ta or T1. Although white light endoscopy, which is recommended by cancer prevention guidelines, is an easily accessible and non-invasive method to detect BC, up to 30% of tumors can be missed, particularly small satellite tumors and flat CISs[5]. These missed residuals lead to a high recurrence rate of 70%, and relapsed tumors may progress to infiltrative cancer[6], which has a significant impact on patient morbidity and quality of life. In addition, BC can incur high treatment expenses and is a burden on medical care systems. Therefore, improving the detection of BC, particularly those tumors that are often overlooked by current methods, would improve the prognosis of patients with NMIBC.

There are several barriers to overcome in the accurate diagnosis of NMIBC, including patient factors, physician factors, and the limitations of currently available diagnostic tests. Cancer prevention guidelines recommend imaging with white light (WL) endoscopy, which is the most commonly used diagnostic

method for NMIBC detection, but this method can still lead to misdiagnosis and missed diagnosis[7-9]. The main cause of missed diagnosis is that the lesions cannot be seen under WL, particularly in the case of multifocal flat cancers[10, 11]. A missed diagnosis can also affect the accurate detection and complete resection of tumors, resulting in recurrent or advanced carcinoma[10, 12]. The presence of inflammatory diseases can lead to misdiagnosis[13-15]. Under WL cystoscopy, it is difficult to distinguish between inflammatory lesions and cancer, as well as benign and malignant lesions, which often leads to misdiagnosis[16]. Although clinicians can make a reliable diagnosis by biopsy, the need for invasive biopsy is a barrier to quick detection of bladder cancer. Additionally, it is impractical to perform a biopsy assessment of every lesion. Therefore, it is necessary to develop new detection methods to improve the diagnosis of NMIBC.

Recently, new imaging technologies have been developed to enhance tumor identification. Endoscopic methods, such as narrow-band imaging[17] and blue light cystoscopy[18], were developed to enhance cancer identification and guide endoscopic resection[19]. However,, these methods are limited by high false-positive rates due to their low specificity[20]. Microscopic imaging techniques, including confocal laser endomicroscopy[21] and optical coherence tomography[22], can provide information on tissue microarchitecture and cellular morphology but are not practical for surveying the entire bladder due to their narrow field of view. Near-infrared (NIR) fluorescence imaging with targeted probes has emerged as a promising technique for precise imaging[23]. NIR fluorescent probes consist of NIR fluorescein and a targeting agent[24]. NIR fluorescein emits light beyond the visible light spectrum and is not interfered with by endogenous substances, thus improving the accuracy of NIR imaging. We previously developed a NIR-based cancer-specific agent[25] that had promising efficacy in preliminary tests. The targeting agent was developed based on molecular changes that are specific to cancer, thus providing high specificity and sensitivity. In the present study, we demonstrate the potential of a NIR-based imaging strategy for improving NMIBC detection using targeted contrast agents.

CD44 is a multifunctional transmembrane glycoprotein that is highly expressed in BC and is involved in cell adhesion, cell migration, tumor progression, and metastasis[26]. CD44v6, a CD44 isoform containing the CD44v6 exon, is also highly expressed in BC[27]. CD44v6 is a co-receptor of HGF in collaboration with the tyrosine kinase c-Met, and together they promote the migration and invasion of BC cells[28]. CD44v6 is a marker of poor prognosis in patients with BC[29]. In addition, CD44v6 is closely related to the degree of malignancy and the stage of BC. Therefore, targeting CD44v6 has tremendous potential for early BC diagnosis[30]. In a previous study, we identified a CD44v6-specific peptide, PLSWT (CSDRIMRGC), and labeled it with a NIR fluorescein, IRDye800CW, to synthesize the BC-specific NIR imaging probe PLSWT7-DMI[25]. As a tool for non-invasive *in vivo* identification of BC, the specificity of the probe has been confirmed in BC cells, tumor-bearing mice, and *ex vivo* human bladders. The *ex vivo* results revealed that CD44v6-targeted NIR imaging can pinpoint multifocal lesions. PLSWT7-DMI has a high sensitivity (up to 91.2%) and specificity (up to 90%). Specific peptides and NIR fluorescence provide a biological basis for enhanced contrast and can achieve desirable tumor-to-background ratios (TBR) for *in vivo* detection.

Preclinical evaluation of PLSWT7-DMI has strongly supported its feasibility for clinical translation. In the present study, we report our initial findings with NIR fluorescence-guided endoscopy using the imaging probe PLSWT7-DMI (Chinese Clinical Trials Database registration number: ChiCTR-DDD-17012616). The purity, efficacy, safety, and nontoxicity of the agent were confirmed before clinical application. We performed a first-in-human clinical study using this probe, and demonstrated that it can bind specifically to BC tissue for visualization by NIR fluorescence endoscopy, thus improving the detection of easily overlooked BC lesions.

Materials And Methods

Study design

In a previous study[25], we synthesized a phage display-derived peptide-based probe (PLSWT7-DMI) and demonstrated its specificity for BC cells *in vitro* and in a mouse model. In the present study, we first applied the probe to *ex vivo* bladder specimens from eight patients to confirm its binding specificity. The PLSWT7-DMI probe was then intravesically administered to patients diagnosed with NMIBC (n = 22). This study was approved by the ethics committee of the Fourth Hospital of Harbin Medical University, China (YXLLSC-2017-09), and was registered in the Chinese Clinical Trials Database (ChiCTR-DDD-17012616). Informed consent was obtained from each patient prior to participation. The study was performed under the guidelines of the Declaration of Helsinki.

Imaging of the entire bladder was performed using our in-house developed dual WL and NIR endoscopy imaging system. The number of lesions detected by WL or NIR imaging was compared. Illuminated lesions under NIR imaging and suspected lesions under WL imaging were biopsied and sent for pathological examination to explore the correlation between the appearance of fluorescent signals and cancer lesions. In total, 108 biopsies were analyzed and used for sensitivity and specificity calculations. All biopsies were reviewed by a single pathologist who was blinded to the imaging results.

The study design and procedures are summarized in Fig. 1. We imaged small satellite tumors and carcinomas *in situ* and compared these with ulcers and inflammation. A biopsy was taken from the tumor and from the disease-free margin for fluorescence microscopy and histopathological correlation analysis.

Procedures

All patients underwent clinical examination and collection of vital signs (heart and respiratory rate, body temperature, and blood pressure) before and after PLSWT7-DMI administration. Intravenous blood samples (3 mL) were collected for hematology and biochemistry prior to PLSWT7-DMI exposure, and at 2–4 days after the administration of PLSWT7-DMI (“swish”) an 18-French urinary catheter was inserted into each patient. The front part of the catheter was cut off to make the catheter a working channel, and 50 ml of PLSWT7-DMI solution (180 pmol/ml) was intravesically instilled. The catheter was clamped,

and the bladder was incubated for 30 min, followed by washing with 200 ml of normal saline three times. Details on the assessment of drug toxicity are provided in Supplementary Material and Scheme 1. All biopsies were sent for H&E staining and were reviewed by a pathologist who was blinded to the imaging results.

Cell culture

The human BC cell lines EJ and RT112, and the human normal urothelial cell line SV-HUC-1, were obtained from the Cell Culture Center at the Institute of Basic Medical Science (Chinese Academy of Medical Sciences) in 2016. The cell lines were checked and authenticated within 6 months using STR analysis (Biowing, Shanghai, China). The cells were grown at 37°C and 5% CO₂ in a humidified atmosphere. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technology, Carlsbad, CA, USA) supplemented with 10% FBS.

Synthesis of fluorescently labeled peptides

BC-specific peptide selection and probe synthesis were performed as previously described ([17](#)). The C-terminus of CSDRIMRGC was attached by a GGGK linker to IRDye800CW (PLSWT7-DMI) or FITC (PLSWT7-FITC) to prevent steric hindrance. The peptide CQRSPIHDC was synthesized as a control for non-specific binding. The probe was purified to >95% by HPLC using a C18 column (Waters Inc.) with a water (0.1% TFA)-acetonitrile (0.1% TFA) gradient. Further characterization was performed using either ESI (Waters Inc.) or MALDI-TOF mass spectrometry (Hitachi America, Ltd.). The excitation and emission spectra of the fluorescently labeled peptides were measured using a spectrophotometer (Ocean Optics). The peptide-binding assays and toxicology analysis are described in the Supplementary Materials and Methods.

Ex vivo imaging of human bladders

Fresh bladder specimens were collected from patients (n = 8) who underwent radical cystectomy. These patients were in the subgroup of high-risk NMIBC patients who underwent radical cystectomy according to cancer prevention guidelines. After radical cystectomy, whole bladder specimens were immediately removed and washed three times with 0.9% saline solution. The bladder specimens were first sprayed with the control (cPLSWT7-DMI) probe, and images were obtained after 30 min using our in-house endoscopy system. After washing to remove the control probe, the bladders were resprayed with PLSWT7-DMI, and images were obtained. The mean fluorescent density was quantified using Image Pro Plus 6.0 (Media Cybernetics, Inc.).

Clinical evaluation of PLSWT7-DMI fluorescence endoscopy

Patients

From September 2017 to August 2018, 22 patients diagnosed with NMIBC were enrolled in the present study. Patient characteristics are provided in Table 1. All patients enrolled were female because the length of the in-house developed endoscope was only 19 cm, limiting access into the bladder via the urethra. The criteria for patient inclusion were the presence of a suspected bladder tumor on prior cystoscopy, ultrasound, or cytology, and no history of surgical or medical therapy for BC during the preceding 6 months. Patients with gross hematuria or a history of allergic reactions to proteins (such as milk, eggs, or beef) and patients who were pregnant, breastfeeding, or did not utilize adequate contraceptive measures were excluded.

PLSWT7-DMI-based NIR imaging of NMIBC in humans

An 18-French urinary catheter was inserted into each patient. The front part of the catheter was cut off to make the catheter a working channel, and 50 ml of PLSWT7-DMI solution (180 pmol/ml) was intravesically instilled. The catheter was clamped, and the bladder was incubated for 30 min, followed by washing with 200 ml of normal saline three times. Imaging was performed using an in-house developed NIR imaging endoscopy system, which is capable of both WL and NIR imaging. The endoscope was inserted into the bladder to obtain images of the entire mucosa under WL, followed by NIR imaging. The number of tumors detected under WL and NIR conditions was recorded. A biopsy instrument was inserted into the bladder via the catheter, and the illuminated lesions were biopsied. All biopsies were sent for H&E staining and were reviewed by a pathologist who was blinded to the imaging results.

Immunofluorescence analysis of PLSWT7 distribution

To assess the distribution and expression of PLSWT7 target sites, frozen NMIBC tissue and normal mucosa were sectioned into 5- μ m thick slices, acetone fixed, and air dried for 30 min. The sections were then blocked with 1% BSA in PBS for 30 min, and incubated with PLSWT7-DMI and rabbit monoclonal anti-CK20 antibody overnight at 4°C. The slides were washed three times with PBS, incubated with goat anti-rabbit antibody conjugated with rhodamine red-X for 30 min, and counterstained with DAPI for 5 min. Images were obtained using fluorescence microscopy (Olympus FV3000).

Statistical analysis

Data were analyzed using SPSS version 20 (IBM Corp., Armonk, NY, USA). Results are presented as the mean $\pm$ standard deviation (SD) for experiments performed in triplicate. Two-tailed independent two-sample *t*-tests were used to assess the differences in fluorescence intensities and tumor-to-background ratios between the groups. The detection rate was calculated as the number of tumors detected in one mode divided by the total number of detected tumors. Differences were considered to be statistically

significant at $p < 0.05$. To determine the diagnostic accuracy of PLSWT7-DMI fluorescence endoscopy, sensitivity was calculated as $TP/(TP + FN)$ and specificity was calculated as $TN/(TN + FP)$, where TP indicates true positives, FN indicates false negatives, TN indicates true negatives, and FP indicates false positives.

Results

Peptide discovery and characterization

In vivo phage display technology was used to identify BC-specific peptides in our previous study (17). After three rounds of biopanning, phage clone P7 was selected based on its high affinity for RT112 (a BC cell line). P7 was isolated, sequenced, and translated into the corresponding peptide, CSDRIMRGC (PLSWT7), and labeled with IRDye800CW (PLSWT7-DMI) or fluorescein isothiocyanate (PLSWT7-FITC). The chemical structure is shown in Fig. 1A. Mass spectrometry was used to determine the mass/charge ratio (m/z) of PLSWT7-DMI, and its monoisotopic mass based on this was determined to be 2514.8 Da (Fig. 1B). The fluorescence spectra for PLSWT7-DMI and IRDye800CW in phosphate-buffered saline (PBS) at an excitation wavelength of 774 nm revealed an emission peak at 789 nm (Fig. 1C). Thus, no shift was observed after IRDye800CW labeling. The apparent dissociation constant (K_d) and association rate constant (K) of PLSWT7-FITC were 42 nM (Fig. 1D) and 0.14 min^{-1} (Fig. 1E), respectively. The results were determined using the least-squares fit of the data. To evaluate the specificity of the peptide *in vitro*, RT112, EJ, and SV-HUC-1 (a normal urothelium cell line) were incubated with the probes, and higher fluorescence intensity was observed in tumor cells incubated with PLSWT7-DMI, indicating efficient binding to RT112 and EJ cells. However, no fluorescence was observed following incubation with cPLSWT7-DMI (scrambled peptide CQRSPIHDC, labeled with IRDye800CW) or PBS. Additionally, pretreatment with PLSWT7 prevented PLSWT7-DMI from binding (Fig. 1F), and minimal fluorescent signals were observed in SV-HUC-1 cells incubated with PLSWT7-DMI, suggesting that the binding of PLSWT7-DMI occurred via the targeting of specific sites on tumor cells. The binding of CD44v6 to RT112 cells also occurred in a dose-dependent manner. The level of PLSWT7-DMI binding was higher in non-transfected cells than in silenced cells (Fig. 1G). These peptides bind to RT112 mouse bladder tumor cells, which express murine CD44v6 of RT112 cells, and an anti-CD44v6 blocking antibody inhibited the cellular binding of CD44v6. These findings indicate that the binding of CD44v6 to CD44v6-overexpressing cells is specifically mediated by CD44v6. In addition, CD44v6 preferentially binds to plates coated with either human or mouse recombinant CD44v6 compared to control plates coated with albumin (Fig. 1H).

For pharmacological/toxicological analysis of PLSWT7-DMI, rats were administered four different doses of PLSWT7-DMI by oral gavage ($n = 12$ animals per dose). After 15 days, the rats showed no peptide-related acute adverse effects according to clinical signs, chemistries, or necropsy (Table S1).

Ex vivo endoscopic NIR imaging

Specific binding of PLSWT7-DMI to NMIBC was confirmed *ex vivo* using fresh human bladder specimens ($n = 8$) from patients who had undergone radical cystectomy. Multifocal tumors and CIS were present in the mucosa. After incubation with PLSWT7-DMI, tumors were clearly visible, but the normal mucosa was not, indicating specific targeting of the probe (Fig. 2A). Quantitative analysis showed that the fluorescence density was significantly higher in urothelial carcinoma and CIS tissue than in the normal mucosa after incubation with PLSWT7-DMI, with no difference observed with cPLSWT7-DMI incubation (Fig. 2B). These results suggest that PLSWT7-DMI binds specifically to human BC tissue, and thus can be translated into clinical applications.

Endoscopic NIR imaging with PLSWT7-DMI in NMIBC patients

To determine whether PLSWT7-DMI specifically binds to NMIBC *in vivo*, we enrolled 22 NMIBC patients and compared the absolute fluorescence of tumor tissue with that of the adjacent normal tissue following intravesical probe administration. The bladders were washed and emptied, and intravesically instilled with a control probe (cPLSWT7-DMI), and images were obtained using an in-house developed endoscopy system. After washing to remove the control probe, bladders were re-instilled with PLSWT7-DMI and imaged again. With the control probe, no contrast was detected between the normal and apparent tumor mucosa (Fig. S1). After instillation with PLSWT7-DMI, urothelial carcinomas at stage Ta and T1 were clearly stained (Fig. 3A), but the normal mucosa was not (Fig. 3B). Quantitative analysis revealed that the mean fluorescence density was significantly higher in the tumor tissue than in the normal mucosa after incubation with PLSWT7-DMI, while no difference was observed with the control probe cPLSWT7-DMI (Fig. 3C), indicating that PLSWT7-DMI specifically binds to tumors. Moreover, the TBR of PLSWT7-DMI was 4.58-fold higher than that of cPLSWT7-DMI (Fig. 3D). After treatment with PLSWT7-DMI, the signal ratio between the lesion and margin in stage Ta and T1 tumors was higher than that in the normal tissue (Fig. 3E). Furthermore, T1 urothelial carcinomas showed higher TBRs than the patient of Ta stage (Fig. 3F).

The illuminated lesions were biopsied using NIR imaging. Pathological examination confirmed that the probe-bound lesions corresponded to NMIBC tissue, while the lesions without probe binding were identified as normal mucosa.

Detection accuracy of PLSWT7-DMI fluorescence endoscopy for NMIBC

A total of 108 lesions (68 cancerous lesions and 40 benign lesions) were analyzed to compare PLSWT7-DMI fluorescence endoscopy and histopathological diagnosis (Table S2). For urothelial neoplasms, 20.6% ($n = 14$) were found only by NIR imaging, and 7.4% ($n = 5$) were found only by WL imaging. Small satellite tumors and CIS overlooked under WL imaging were identified using probe-based NIR imaging

(Fig. 4A). Under WL imaging, 54 NMIBC lesions were detected, while nine additional lesions were detected by NIR imaging (Table S2), including six small foci and three CIS that were overlooked by WL endoscopy. These results indicate that PLSWT7-DMI-based NIR fluorescence endoscopy may reduce the number of overlooked NMIBC lesions, thus facilitating complete resection and reducing the recurrence rate of BC.

Suspicious benign lesions that were difficult to discriminate under WL, such as inflammation and ulcers, could be easily differentiated by PLSWT7-DMI-based NIR fluorescence endoscopy (Fig. 4B). The mean fluorescent density did not differ among normal mucosa, ulcers, and inflammation (Fig. 4C), indicating negligible probe binding to benign lesions. Ten out of thirteen inflammatory lesions and three out of three ulcers were invisible under NIR (Table S2), suggesting that mistaken resection of benign lesions would be minimized if our technique was used during tumor resection.

Overall, PLSWT7-DMI-based NIR fluorescence endoscopy resulted in a more thorough detection of urothelial neoplasms at different tumor stages than WL endoscopy (Fig. 4D). The sensitivity of probe-based NIR imaging was 91.2% (62/68), specificity was 90% (36/40), and the false positive rate was 10% (4/40), as shown in Table 2.

Distribution of PLSWT7-DMI in bladder tissue

We used immunofluorescence to further examine the distribution of PLSWT7-DMI in the tumor and normal tissue. Frozen sections of biopsies from the detected lesions and the adjacent normal mucosa were obtained during the present study. In representative images of NMIBC and normal mucosa, we found that the PLSWT7-DMI signal was higher in NMIBC tissue than in normal tissue and overlapped with CK20 expression (Fig. S2), indicating that PLSWT7-DMI was localized in NMIBC tissue but not in the normal urothelium. The tumor-selective distribution of PLSWT7-DMI may serve to illuminate tumor lesions during intraoperative imaging.

Safety and adverse effects

PLSWT7-DMI was well-tolerated by the study subjects, and no adverse pharmacological activity or allergic reactions occurred during the study. Three patients experienced surgical fevers within the first postoperative day (36.8–38.5°C), which subsequently subsided. No damage to liver or renal function was observed. None of the patients complained of nausea, vomiting, or gross hematuria within one week after surgery. No symptoms of urinary tract infection were reported (Table S3).

Discussion

With a recurrence rate of up to 70% within 5 years, tumor recurrence is a major issue that affects the survival of patients that are treated for NMIBC. A substantial percentage of tumor recurrences are likely to be caused by persistent tumors that were overlooked at the initial resection[31], despite urologists aiming

to detect as many neoplastic lesions as possible to minimize the possibility of future relapse. In the present study, we present the first in-human assessment of the use of NIR fluorescence endoscopy with a specific probe, PLSWT7-DMI, to detect NMIBC lesions. We show that intravesical administration of PLSWT7-DMI is safe and effective in improving NMIBC detection, and successfully demonstrate its potential for detecting overlooked neoplastic lesions, which may minimize future relapse. Topical application of imaging probes is an effective method for tumor imaging[28]. The bladder is a storage organ, representing an established intravesical route for topical administration of pharmacological agents without significant systemic absorption. Compared with systemic delivery, topically administered probes are not diluted by the volume of distribution, thus requiring smaller quantities and decreasing the risk of toxicity and the cost of implementation[29, 30]. In the present study, no adverse effects were observed after intravesical administration of PLSWT-DMI, suggesting that the probe was well-tolerated in humans.

In a previous study, we used *in vivo* phage-display technology to identify the BC-specific peptide PLSWT7. Compared with antibodies, which are commonly used as targeting agents, peptides are easier to label and more cost-effective, and have lower immunogenicity[32]. The peptide PLSWT7 had a high binding affinity with rapid onset in urothelial neoplasm cells. As urothelial neoplasms account for the majority of NMIBC cases, PLSWT7 was labeled with IRDye800CW to detect NMIBC endoscopically under NIR imaging in the present study. Before translating PLSWT7-DMI targeted imaging for clinical use, a rigorous validation process and focused regulatory strategy were applied. Mass spectrometry and high-performance liquid chromatography (HPLC) were used to elucidate the structure and synthesis of PLSWT7-DMI. In addition, toxicological tests were performed to confirm the safety of the probe in rats. To further evaluate the feasibility of PLSWT7-DMI for clinical use, *ex vivo* bladder specimens from high-risk NMIBC patients were used to assess probe-based imaging. Multifocal tumors and CISs were clearly visualized. Similar results were also reported by Pan et al.[33]and Golijanin et al.[34]. However, endoscopically-managed low-risk NMIBC accounts for many BC cases. Furthermore, real-time intraoperative endoscopy involves many challenges that are not encountered in *ex vivo* imaging, such as the limited range of motion of the endoscope, the length of the procedure, and increased background signal as a result of non-thorough bladder washing. Therefore, low-risk NMIBC patients were enrolled in the present study, and NIR fluorescence endoscopy with PLSWT7-DMI was performed *in vivo*. As expected, PLSWT7-DMI showed positive binding to Ta and T1 urothelial neoplasms with 90% specificity and 91.2% sensitivity. However, these results need to be verified in a larger cohort with a wider range of pathological types of NMIBC. In particular, the sensitivity may be lower for other types of NMIBC (such as squamous cell carcinoma and adenocarcinoma), as target site expression may vary among tumor cells.

Overlooking small satellite tumors at TUR is one of the major obstacles in the successful treatment of patients with NMIBC, as it results in incomplete tumor control and a high rate of recurrence. CIS is a flat, high-grade NMIBC that poses a diagnostic challenge under WL imaging, and has a significant risk of recurrence and progression[34]. To detect as many NMIBC lesions as possible, the detection of both small multifocal lesions and CISs must be optimized. PLSWT7-DMI-based NIR fluorescence endoscopy detected a greater number of tumors, including three CISs and six small tumors that were not visualized

under WL imaging. It is expected that an increase in tumor detection rates will reduce the early recurrence rate of NMIBC[35]. Accordingly, a reduction in the tumor recurrence rate is expected if PLSWT7-DMI fluorescence endoscopy is adopted as a standard procedure during NMIBC resection. Although further trials are needed to confirm these findings, we expect that its use will have a positive impact on the outcomes of patients with NMIBC in clinical practice.

During WL tumor resection, surgeons must remove all suspected lesions to ensure the complete removal of tumors, because the naked eye cannot differentiate malignant lesions from benign lesions[36]. Although the use of 5-aminolevulinic acid (5-ALA) provides enhanced contrast, the non-specific accumulation of 5-ALA in benign lesions, including inflammation and ulcers, leads to high false-positive rates[26]. This can lead to unnecessary mucosal injury, which provides sites for tumor implantation and recurrence[4]. In contrast, PLSWT7-DMI-based NIR imaging clearly demarcated the tumors via positive binding of the cancer-specific probe. In contrast, benign lesions exhibited negligible fluorescence due to negative probe binding. Therefore, the application of PLSWT7-DMI should enable surgeons to easily discriminate between malignant and benign lesions. The false positive rate of PLSWT7-DMI (10%) was much lower than that of 5-ALA reported in other studies[27]. Thus, the risk of unnecessary mucosal injury, a critical factor in NMIBC recurrence, could be reduced with PLSWT7-DMI.

Conclusion

In the present study, we demonstrated that PLSWT7-DMI-based NIR fluorescence endoscopy is a feasible and safe approach for detecting NMIBC lesions. The method showed good specificity and sensitivity for the detection of NMIBC. Small satellite tumors and flat CISs that evaded detection under WL imaging were identified using probe-based NIR imaging, and benign lesions could be differentiated from malignant lesions. However, some of the limitations of this study include the exclusion of male patients due to the size of the endoscope and the limited number of patients that were included in the cohort. As a commercial NIR cystoscope is not currently available, a customized NIR endoscope system was developed for the present study. However, the customized endoscope could only be used in female patients because of its limited length. With improved endoscopes, the validity of the method may be evaluated in male patients with NMIBC in future studies. Further, the present study was limited to only 22 patients with NMIBC, suggesting the need for these results to be verified in a larger cohort in future trials.

Declarations

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Conflicts of interests

There are no conflicts of interest to declare

Author contributions

Wenting Shang and Li Peng wrote the manuscript, in addition to designing, performing, and analyzing all experiments. Wenting Shang synthesized and characterized the probe. Kunshan He and Chongwei Chi designed the imaging system. Pengyu Guo, assisted with collecting the information on NMIBC patients. Han Deng assists with manuscript modification. Yu Liu assisted with data analysis. Jie Tian and Wanhai Xu designed, supervised, and analyzed all experiments, in addition to assisting with manuscript preparation.

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Tables

Tables 1 and 2 are not available with this version.

Figures

Figure 1

Fluorescently labeled peptide that are specific to NMIBC. (A) Chemical structure of the CSDRIMRGC peptide with a GGGK linker and an IRDye800CW label, denoted PLSWT7-DMI. (B) Fluorescence emission

spectra for PLSWT7-DMI and IRDye800CW with an excitation wavelength of 471 nm resulting in an emission peak of 519 nm. (C) Mass spectrum of PLSWT7-DMI. (D) Binding affinity (Kd) of PLSWT7-DMI to RT112 cells. (E) Binding kinetics (K) of ASY*-FITC to RT112 cells. Solid line represents the least-squares fit. (F) Fluorescence intensity (mean sores) of RT112, EJ, and SV-HUC-1 cells incubated with phosphate buffer saline (PBS), cPLSWT7-DMI, PLSWT7-DMI with blockade, or PLSWT7-DMI. The PLSWT7-DMI-treated group showed higher binding affinity than that of the other groups. *p < 0.05 versus cPLSWT7-DMI-treated cells. (G) Flow cytometry analysis of the binding of FITC-labeled cPLSWT7 and FITC-labeled cPLSWT7 +blockade at the indicated concentrations in RT112 cells. **, p < 0.01; data represents the mean ± S.E. of three independent experiments. ***, p < 0.001. (H) Binding of PLSWT7-DMI to recombinant human CD44V6 and mouse CD44V6. Bovine serum albumin (BSA) was used as the control protein. NMIBC: non-muscle invasive bladder cancer, cPLSWT7-DMI: scrambled labeled peptide control.

Figure 2

Ex vivo endoscopic NIR imaging of human bladder specimens. (A) The colocalization of PLSWT7-DMI binding and histopathology as shown by representative white and blue light images with corresponding hematoxylin and eosin (H&E) staining. PLSWT7-DMI binding was detected under NIR lighting on multifocal urothelial carcinomas and flat CIS. Scale bars, 50 μ m. (B) Mean fluorescence density (mean $\pm$ SEM) in urothelial carcinoma, CIS, and normal tissues post administration of PLSWT7-DMI or the scrambled control cPLSWT7-DMI. * $p < 0.05$ vs. normal tissue treated with PLSWT7-DMI. WL: white light, NIR: near infrared, CIS: carcinoma in situ.

Figure 3

NIR fluorescence endoscopy of NIMBC in humans with the tumor-specific probe PLSWT7-DMI. (A) Representative images of WL and NIR imaging of stage Ta, T1 urothelial carcinoma and normal mucosa (B), with corresponding H&E staining to demonstrate the colocalization of PLSWT7-DMI binding and histopathology. Scale bars, 100 μ m. (C) Quantification of the mean fluorescent density and (D) tumor-to-background ratio (TBR) in cancer and normal mucosa tissue post-administration of PLSWT7-DMI and cPLSWT7-DMI. Values are presented as the mean $\pm$ SEM. * $p < 0.05$ vs. urothelial carcinoma treated with PLSWT7-DMI. WL: white light, NIR: near infrared, cPLSWT7-DMI: scrambled control peptide. (E) The signal ratio between the lesion and margin in stage Ta and T1. (F) TBR for all patients stage T1 and Ta.

Figure 4

PLSWT7-DMI-based NIR fluorescence endoscopy enables the detection of bladder cancer tumors and benign lesions. (A) Small satellite tumors and CIS were overlooked using WL endoscopy but were clearly visualized under NIR endoscopy after incubation with PLSWT7-DMI. H&E staining confirmed the presence of tumor cells. Scale bars, 100 μ m. (B) Ulcer and inflammatory lesions showed no probe binding. H&E staining confirmed benign lesions. (C) Mean fluorescent density (mean $\pm$ SEM) of normal mucosa and benign lesions post-administration of PLSWT7-DMI. (D) Number of tumors detected at various stages under NIR or WL imaging. *p < 0.05 vs. WL endoscopy. WL: white light, NIR: near infrared, CIS: carcinoma in situ.

Figure 5

Scheme 1: Schematic overview of the PLSWT7-DMI clinical trial. 22 patients with BC confirmed by biopsy consented to the clinical study.

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