

PD-L1-targeted dual-modal MR/NIR-II probe for imaging and image-guided surgery of non-small cell lung cancer

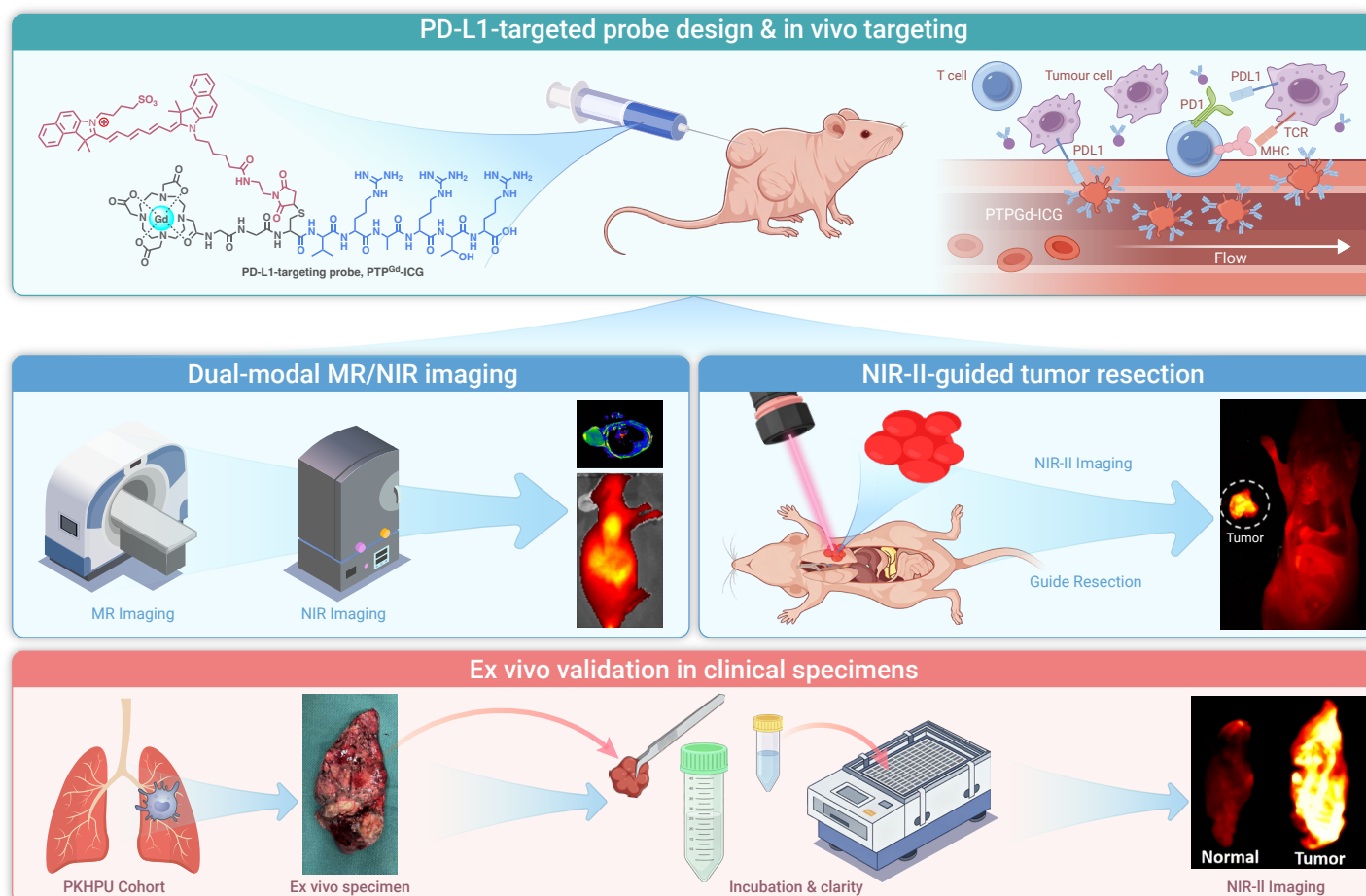
Qingyun Liu,^{1,2,3,8} Xiaohua Jia,^{4,5,8} Yutao Li,^{1,2,3} Lei Liang,⁴ Jun Wang,^{1,2,3} Defan Yao,^{7,*} Zhenhua Hu,^{5,6,*} and Kezhong Chen^{1,2,3,*}

*Correspondence: yaodefan@xinhumed.com.cn (D.Y.); zhenhua.hu@ia.ac.cn (Z.H.); mdkzchen@163.com (K.C.)

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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- A PD-L1-targeted MR/NIR-II dual-modal probe integrates preoperative imaging and intraoperative navigation.
- The probe shows high tumor-to-background ratios in xenograft, orthotopic, and metastatic tumor models.
- Rapid ex vivo NIR-II imaging accurately maps surgical margins and correlates with PD-L1 IHC in patients.

PD-L1-targeted dual-modal MR/NIR-II probe for imaging and image-guided surgery of non-small cell lung cancer

Qingyun Liu,^{1,2,3,8} Xiaohua Jia,^{4,5,8} Yutao Li,^{1,2,3} Lei Liang,⁴ Jun Wang,^{1,2,3} Defan Yao,^{7,*} Zhenhua Hu,^{5,6,*} and Kezhong Chen^{1,2,3,*}

¹Department of Thoracic Surgery, Peking University People's Hospital, Beijing 100044, China

²Thoracic Oncology Institute, Peking University People's Hospital, Beijing 100044, China

³Research Unit of Intelligence Diagnosis and Treatment in Early Non-small Cell Lung Cancer, Chinese Academy of Medical Sciences, 2021RU002, Peking University People's Hospital, Beijing 100044, China

⁴Department of Ultrasound, Aerospace Center Hospital, Peking University Aerospace School of Clinical Medicine, Beijing 100049, China

⁵CAS Key Laboratory of Molecular Imaging, Institute of Automation, Chinese Academy of Sciences, Beijing 100190, China

⁶School of Artificial Intelligence, University of Chinese Academy of Sciences, Beijing 100049, China

⁷Department of Radiology, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

⁸These authors contributed equally

*Correspondence: yaodefan@xinhumed.com.cn (D.Y.); zhenhua.hu@ia.ac.cn (Z.H.); mdkzchen@163.com (K.C.)

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Recurrence of non-small cell lung cancer remains a critical clinical challenge. Primarily due to undetectable micrometastatic lesions and incomplete tumor resection. Current preoperative imaging modalities lack sufficient resolution to identify small lesions, while intraoperative guidance relies on visual inspection or conventional fluorescence. Accurate preoperative diagnosis and precise excision of malignant tissue remain critical factors for improving patient prognosis. Considering the differential expression of PD-L1 in NSCLC and normal lung tissue, we developed a novel imaging probe (PTP^{Gd}-ICG). This probe enabled successful MRI-based tumor targeting with high contrast in tumor-bearing mice, with the experimental group showing a 2.39-fold higher signal-to-noise ratio (SNR) than the blocking group at 0.5 h post-injection. NIR-II fluorescence imaging guided tumor removal, with the probe exhibiting sustained tumor enrichment from 4-96 h post-injection and reaching a peak tumor-to-background ratio (TBR) of 8.77 at 72 h, enabling precise tumor resection in animal models. In addition, the probe exhibited significantly higher fluorescence intensity in human NSCLC tissues than in adjacent normal tissues, facilitating the assessment of positive surgical margins *ex vivo*. These findings demonstrate that PD-L1 is a promising imaging target for NSCLC. This strategy may enable preoperative identification of patients with high PD-L1 expression while facilitating rapid pathological assessment and intraoperative margin delineation.

INTRODUCTION

Lung cancer remains the leading cause of cancer-related mortality globally, with an estimated 2.2 million new cases and 1.8 million deaths in 2022 alone. Despite continuous advances in cancer diagnosis and treatment, establishing precise diagnostic and therapeutic strategies for lung cancer remains a major challenge in oncology.¹⁻³ Surgical resection remains the cornerstone of curative treatment for early-stage non-small cell lung cancer (NSCLC). However, postoperative recurrence and metastasis remain common, largely owing to incomplete tumor resection and the presence of occult micrometastatic lesions that are difficult to detect using current imaging modalities. In recent years, programmed death ligand 1 (PD-L1) has emerged as a pivotal regulator of tumor immune escape.⁴⁻⁶ Beyond its established role as a predictive biomarker for immune checkpoint inhibitor therapy,^{7,8} PD-L1 has become an attractive molecular target for visualizing the tumor immune microenvironment and evaluating intratumoral heterogeneity.^{9,10} Nevertheless, the clinical efficacy of immune checkpoint inhibitors is highly dependent on PD-L1 expression, with objective response rates varying substantially across different PD-L1 tumor proportion score (TPS) categories. Therefore, accurate, non-invasive, and dynamic assessment of PD-L1 expression is essential for optimizing patient selection, guiding immunotherapy, and facilitating precision surgical management.

Currently, PD-L1 evaluation primarily relies on immunohistochemical analysis of biopsy or surgical specimens. Although widely adopted in clinical

practice, this approach has several inherent limitations. First, tissue acquisition is invasive and pathological assessment is time-consuming, delaying therapeutic decision-making. Second, PD-L1 expression exhibits marked spatial and temporal heterogeneity, making single-site biopsies prone to sampling bias and inaccurate representation of the overall tumor immune landscape. Third, conventional pathological assessment cannot provide real-time intraoperative information for tumor localization or surgical margin evaluation, limiting its value in image-guided precision surgery. These limitations underscore the urgent need for non-invasive imaging approaches capable of dynamically monitoring PD-L1 expression *in vivo*.

The breakthrough of molecular imaging technology provides new ideas for solving this dilemma; molecular imaging has emerged as a transformative solution. Magnetic resonance imaging (MRI) provides excellent soft-tissue contrast and high-resolution anatomical information, making it an indispensable modality for preoperative tumor localization.^{11,12} Meanwhile, near infrared fluorescence (NIR) imaging technology, particularly within the second near infrared (NIR-II, 1000-1700 nm) window, enables highly sensitive, real-time visualization with superior tissue penetration and reduced background autofluorescence, offering unique advantages for tumor margin delineation and intraoperative navigation.¹³⁻¹⁶ Recent studies have further emphasized the growing value of non-invasive molecular imaging for dynamic biological characterization and highlighted multimodal imaging as a promising strategy to facilitate the clinical translation of precision medicine.^{17,18} However, most currently available imaging probes remain limited to single imaging modalities,^{8,19} restricting their ability to integrate accurate preoperative lesion localization with real-time intraoperative guidance. Consequently, an effective molecular imaging strategy capable of dynamically visualizing PD-L1 expression throughout the perioperative process remains unavailable.

To address this unmet need, we developed a PD-L1-targeted magnetic resonance/near-infrared dual-modal molecular probe, PTP^{Gd}-ICG, by integrating a PD-L1-specific peptide, a gadolinium-based MRI contrast moiety, and an NIR-II fluorescent dye into a single molecular platform. This probe enables a complementary imaging workflow that combines high-resolution preoperative MRI for accurate tumor localization with intraoperative NIR-II fluorescence imaging for real-time surgical navigation. We systematically evaluated its targeting specificity and imaging performance in cultured cells, murine lung cancer models, and human NSCLC specimens. Our findings demonstrate that PTP^{Gd}-ICG enables dynamic visualization of PD-L1 expression, accurate tumor localization, and fluorescence-guided tumor resection, providing a clinically translatable molecular imaging strategy for precision surgery and personalized management of NSCLC.

MATERIALS AND METHODS

Synthesis and characterization of MR/NIR imaging probe

The peptide PTP (CVRARTR-GG(DOTA)) was synthesized on rink amide resin (loading: 0.81 mmol/g) with the assistance of a CEM Liberty One microwave-assisted peptide synthesizer. After the attachment of all amino

acids, the product was transferred to a glass peptide synthesis vessel. Following Fmoc deprotection, the compound was conjugated with three tert-butoxycarbonyl (Boc)-protected 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). After DOTA conjugation, the product was cleaved from the solid support using a cleavage cocktail composed of trifluoroacetic acid-triisopropylsilane-H₂O (95:2.5:2.5). The solution was then removed, and the resulting white solids were precipitated by adding dry diethyl ether, followed by centrifugation at approximately 6,500 rpm for 15 minutes. Finally, the peptide was purified and isolated using preparative high-performance liquid chromatography (HPLC) with a C-18 reverse-phase column. The compound PTP^{Gd}-ICG was synthesized by chelation of gadolinium ions (Gd³⁺) with the DOTA chelator on PTP, followed by conjugation with the NIR fluorescent dye ICG via a thiol-maleimide reaction.

Theoretical binding interaction between PD-L1-targeting peptide and PD-L1

The initial binding pose of the PD-L1-targeting peptide PTP with the target protein PD-L1 was predicted using the AlphaFold3 (AF3) web server. Among the generated models, the structure with the highest ranking was selected for further structural analysis. Illustrations were generated using PyMOL.

Binding affinity of PD-L1-targeting probe to PD-L1

Probe-PD-L1 binding affinity was measured by surface plasmon resonance (SPR). A PD-L1-coated chip was prepared: First, an EDC/NHS mixture activated Fc1/Fc2 channels (10 μ L/min, 800 s). Then, 50 μ g/mL PD-L1 in NaAc (pH 4.0) was immobilized on Fc2 (10 μ L/min, 800 s; ~7893.3 RU). Channels were deactivated with ethanolamine HCl (10 μ L/min, 800 s). Probe, diluted in Running Buffer to concentrations (4.8–312.0 μ M), was injected over Fc1-Fc2 at 30 μ L/min. Association (60 s) and dissociation (90 s) phases were monitored using Running Buffer.

Physical characterization of PD-L1-targeting probe

The purified peptide was characterized by HPLC and high-resolution mass spectrometry (HRMS) analysis of the PD-L1-targeting peptide probe was conducted on a Waters Micromass® Q-TOF spectrometer equipped with an electrospray ionization (ESI) source. Ultraviolet-visible (UV-Vis) absorption spectra were recorded at room temperature across a range of concentrations (2.5 μ M to 40 μ M) using a Varian Cary 100 Conc UV-Visible spectrophotometer. Fluorescence emission spectra were acquired utilizing an F900 spectrometer (Edinburgh Instruments Ltd.), with excitation at the probe's characteristic absorption maximum wavelength.

Cell lines and cell culture

NSCLC lines HCC827 (RRID: CVCL_2063), A549 (RRID: CVCL_0023) and H520 (RRID: CVCL_1565), MLE (RRID: CVCL_0454), BEAS-2B (RRID: CVCL_0168) were purchased through the American type culture collection (ATCC). The HCC827-Luc stable cell line was generated by lentiviral transduction of parental HCC827 cells with a lentivirus encoding both firefly luciferase and a puromycin resistance gene, followed by puromycin selection to isolate a clonally stable population expressing both reporters. All cells were confirmed to be mycoplasma negative before use. Please see the in vitro targeting evaluation for the specific culture methods of the cell lines.

In vitro targeting evaluation

Five cell lines HCC827, A549, H520, MLE and BEAS-2B were cultured. HCC827, MLE and BEAS-2B were cultured in RPMI-1640 medium containing 10% FBS and 1% P/S at 37°C and 5% CO₂. A549 and H520 were cultured in DMEM containing 10% FBS and 1% P/S at 37°C in 5% CO₂. Approximately 1 $\times$ 10⁵/ml cells were seeded in glass bottom culture dishes (NEST, San Diego, CA; catalog no. 801002). After the cells grew to the required density (70%–90%), PTP-ICG 200 μ L of 0.01 mg/ml was added, and cells were incubated for 2 h in the dark, washed with PBS 3 times, fixed with 4% PFA for 10 min, and stained with DAPI for 10 min. Observations were made with a laser confocal microscope (LSM780, Carl Zeiss, Jena, Germany). For the binding site competition blocking experiment, cells were incubated with PTP unlabeled with ICG at 100 times the probe concentration for 2 h, washed three times with PBS, and then incubated with 200 μ L of PTP-ICG at 0.01 mg/ml.

Nucleus was stained with DAPI and observed by laser confocal microscopy.

Western blot, immunohistochemical staining and HE staining

The Cells were grown to 80–90% confluence, washed three times with cold PBS, and lysed in RIPA buffer with protease and protein phosphatase inhibitors. Lysates were collected by centrifugation at 12,000 $\times$ g for 30 min at 4°C. Protein concentrations were determined using the BCA protein assay kit. Protein samples were then boiled for 10 min, and 20 μ g of total protein were resolved by SDS PAGE and transferred to PVDF membranes. Blocking of the membrane with a 5% BSA was done for 1 h at room temperature. Immunoblotting was performed overnight using a 1:5000 dilution of anti-PD-L1 at 4°C, and GAPDH was used as an endogenous control. The membranes were washed three times with TBST and incubated with goat polyclonal anti-mouse IgG-HRP secondary antibody for approximately 1 h at room temperature. Finally, PD-L1 expression was detected by using enhanced chemiluminescence and calculated by quantifying western blot band intensities using the ImageJ.

HCC827 tumors were isolated from euthanized tumor bearing mice, fixed in 4% formalin, dehydrated with gradient ethanol, and then embedded in paraffin. Cut 4 μ m thick sections from formalin fixed paraffin embedded blocks and deparaffinize. The sample was subjected to antigen repair using high-pressure thermal repair method (pH 6.0 sodium citrate buffer, 121 °C), followed by blocking with endogenous peroxidase and blocking with 5% normal goat serum. Perform PD-L1 staining on the sample and detect it using the IHC kit. The Aperio AT2 panoramic scanning system (Leica Biosystems) was used to obtain full slice digital images under a 20 $\times$ objective lens. Clinical tumor tissue specimens are collected from clinical surgeries and stored in 4% paraformaldehyde after in vitro imaging. Tissue sections were embedded with embedding agents, frozen, fixed after sectioning, and subjected to hematoxylin and eosin staining (H&E) for pathological analysis.

Subcutaneous and orthotopic NSCLC xenograft models

6-week-old female BALB/c nude mice weighing around 20g was subcutaneously injected with HCC827 cell suspension at a concentration of 3 $\times$ 10⁶/100 μ L to establish a nude mouse subcutaneous xenograft model. The injection site is the subcutaneous area on the outer back of the right scapula. In addition, HCC827-Luc cell suspension with a concentration of 2 $\times$ 10⁶/100 μ L was injected into one lung of mice to establish an orthotopic NSCLC xenograft model.

In vivo MR imaging

HCC827 tumor bearing mice and blocking group were injected via tail vein. To inhibit activity, we injected PTP via tail vein into the blocking group of tumor bearing mice 12 hours before injection probe. HCC827 xenograft nude mice were intravenously injected with probe (100 μ L, 5 mM). MR imaging was performed before injection and at 2.5, 4.5, 6.5, and 24 h post-injection. T1 RARE and T1 mapping RARE axial images were obtained using a 9.4 T Bruker BioSpec system with the following parameters: FOV, 3.5 $\times$ 3.0 cm; slice thickness, 1 mm; interslice distance, 1 mm; TR, 500 ms; TE, 4.85 ms; number of averages, 2; image size, 200 $\times$ 172.

In vivo fluorescence imaging

Before fluorescence imaging, evaluate the in vitro cytotoxicity and in vivo safety of the probe. Select the HCC827 cell line with high expression of PD-L1 from the WB results to construct tumor bearing mice, and use the Blocking experiment as the control group. PTP-ICG was injected into tumor bearing mice via the tail vein at a dose of 3 μ g/g, and IVIS spectroscopy was used to capture images in vivo at specified time intervals (0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96 hours). The observation time was adjusted according to the changes in tumor imaging time in each group. NIR-II fluorescence imaging uses an InGaAs SWIR camera (Xenics Cheetah 640CL TE3) paired with a lens capable of transmitting NIR-II light (Spacecom VF50M SWIR) to capture NIR-II fluorescence. The blocking group was injected with unlabeled ICG PTP at a dose of 30 μ g/g, and after 12 hours, PTP-ICG was administered at a dose of 3 μ g/g body weight for fluorescence imaging. The fluorescence intensity is measured by ImageJ software, and the tumor background ratio (TBR) refers to the ratio of the fluorescence intensity between the tumor and the

surrounding tissue.

PTP-ICG NIR-I and NIR-II fluorescence imaging of blood vessels

The CMOS PCO camera is used for NIR-I fluorescence imaging, with an excitation wavelength of 792 nm and an acquisition device using a bandpass filter of 850 ± 40 nm. The exposure time for this process is 1–30 ms. NIR-II fluorescence imaging is performed using a high-tech NIR-II camera, which uses an excitation wavelength of 808 nm and an excitation power of approximately 20 mW/cm^2 . Multiple bandpass filters with wavelengths of 1100 nm, 1200 nm, and 1300 nm were used for acquisition channels, with exposure times ranging from 30 ms to 1 s. The spatial resolution of NIR-II imaging equipment is $62.5 \mu\text{m}$. Select 6-week-old BALB/c nude mice and inject PTP-ICG (50 μg per mouse) through the tail vein. Perform NIR-I and NIR-II fluorescence imaging on the vascular system of the hind limbs of mice, and measure the contrast of interest by drawing a cross-sectional fluorescence intensity distribution map.

In vitro cytotoxicity evaluation and in vivo safety of PTP^{Gd}-ICG

Evaluate the biocompatibility of the probe using the Cell Counting Kit-8 (CCK-8) assay method. Place HCC827 in a 96 well culture dish with 5000 cells per well. After adhesion, replace the culture medium containing different amounts (0, 5, 10, 20, 40, and 80 $\mu\text{g/ml}$) of PTP^{Gd}-ICG probes with fresh liquid. Subsequently, the cells were cultured for 0, 24, and 48 hours. The CCK-8 method is used to evaluate cell survival rate.

To evaluate the safety of PTP^{Gd}-ICG probe in vivo, a study was conducted using healthy BALB/c nude mice. Randomly divide the mice into four groups. PTP^{Gd}-ICG probe was administered via tail vein injection, while the control group received PBS injection. During the experiment, blood samples were collected from all mice 24 hours, 7 days, and 14 days before euthanasia. To determine the presence of acute or chronic toxicity, liver function of collected blood samples was evaluated by assessing alanine aminotransferase (ALT), albumin (ALB), alkaline phosphatase (ALP), and kidney function was evaluated by examining blood urea nitrogen (BUN), creatinine (CR), and uric acid (UA). To further investigate any pathological changes, important organs such as the heart, liver, spleen, lungs, kidneys, intestines, and prostate were washed with cold saline, then fixed and stained with H&E.

Bioluminescence imaging

To identify tumors, bioluminescence imaging (BLI) was performed using an in vivo imaging system (IVIS, PerkinElmer, USA). Anesthetize mice with tumors using 1.5–2% isoflurane, followed by intraperitoneal injection of D-fluorescein (150 $\mu\text{g/ml}$, 200 μl). Take BLI images 10 minutes after injection.

NIR-II fluorescence imaging guided NSCLC resection

Preoperative CT-BLI fusion imaging of HCC827-Luc orthotopic NSCLC mice was performed to determine the location and size of the tumors. Each mouse was injected with PTP-ICG 24 hours before surgery. Under isoflurane anesthesia, mice underwent surgery under white light and NIR-II fluorescence guidance. Tumor tissue was separated from surrounding tissue post-operatively to simulate the identification of tumor margins during radical lung cancer surgery. Perform NIR-II fluorescence imaging and BLI examination on separated tissues to confirm the presence of hidden NSCLC lesions. For HCC827-Luc lung cancer liver metastasis bearing mice, surgery was performed under white light and NIR-II fluorescence guidance 72 hours after injection of the probe. Tumor tissue was identified on the liver and excised to simulate the identification of lung cancer liver metastases. Finally, H&E and IHC were used for pathological analysis of the tissue.

Ex vivo cultivation and imaging of fresh human NSCLC specimen

Collect tumor specimens and adjacent normal tissues after lung cancer resection ($n=5$), and refer to supplementary data (Table S1) for patient and tumor characteristics. Store fresh specimens in tissue preservation solution, and then wash the bloodstains on the surface of the specimens with PBS. Incubate specimens with PTP-ICG at a concentration of 10 $\mu\text{g/ml}$ for 15 minutes, and use normal tissue specimens as controls. After incubation, the tissue specimens were washed three times with PBS and imaged using NIR-II camera. Further, freeze the specimen, slice it to a thickness of 4 μm , seal it

with 5% fetal bovine serum RT for 30 minutes, then incubate it with PTP-ICG (10 $\mu\text{g/ml}$) RT for 20 minutes, wash it three times with PBS, and seal it with anti fluorescence quenching tablets (including DAPI). Finally, three consecutive sections were cut for HE and PD-L1 immunohistochemical staining.

Statistical analyses

Quantitatively analyze near-infrared fluorescence images using ImageJ software and convert them to pseudocolor. The experimental data is expressed as mean $\pm$ standard deviation (SD), and the comparison between the two groups is conducted using Student's t-test (GraphPad Prism 9.5, * $P<0.05$ is considered significant, *, **, ***, **** represent $P<0.05$, $P<0.01$, $P<0.001$, $P<0.0001$ respectively).

RESULTS

Synthesis and characterization of dual-modal MR/NIR imaging probe

The PD-L1-targeting peptide (PTP), modified with DOTA, was synthesized using the conventional Fmoc-based solid-phase peptide synthesis (SPPS) methodology, as depicted in Figure 1A. The isolated peptide was characterized by HPLC and mass spectrometry, as shown in Figures S1–S2. The predicted binding conformation of the PD-L1-targeting peptide with PD-L1 was obtained from the AF3 web server, with the top-ranked model subjected to detailed analysis. As illustrated in Figure 1B, examination of the binding interface revealed that the positively charged residues within peptide established favorable electrostatic interactions with acidic residues in the PD-L1 binding pocket. Additionally, the threonine (T) residue was observed to form a hydrogen bond with PD-L1. Collectively, these electrostatic and hydrogen-bonding interactions are likely critical for stabilizing the peptide-protein complex. Notably, the C-terminal region of the peptide was found to be solvent-exposed, presenting an attractive site for chemical modification. This structural feature allows for the conjugation of functional groups at this position without disrupting the essential interactions anchoring the peptide to PD-L1. In alignment with this hypothesis, the synthesized derivatives L1–L3, which incorporate C-terminal modifications, exhibited no significant variations in binding affinity relative to each other. These results indicate that the C-terminal region can be strategically utilized for further functionalization, such as the incorporation of fluorescent probes or chelating agents like DOTA, thereby broadening the potential applications of this peptide scaffold without compromising its binding efficacy.

For MR and fluorescence imaging purposes, the PTP^{Gd}-ICG complex was synthesized by labeling the peptide with gadolinium (Gd) ions via coordination with the DOTA chelator, followed by conjugation with the fluorescent dye ICG using a thiol-maleimide reaction (Figure 1A). The dissociation constants (K_d) of PTP^{Gd}-ICG for PD-L1 were determined using SPR assays, yielding values of 1.0 mM (Figure 1C). This result confirmed that PTP^{Gd}-ICG maintains a strong affinity for PD-L1. Furthermore, the incorporation of MR/NIRF imaging groups into the peptide did not result in a significant decrease in the probe's targeting efficiency. The binding affinity is conducive to reversible interactions, thereby improving PD-L1 detection in MR/NIRF imaging with a high signal-to-noise ratio and minimal side effects. The absorbance and fluorescence spectra of PTP^{Gd}-ICG were analyzed in PBS at pH 7.4. As illustrated in Figure 1D, the absorption maxima of PTP^{Gd}-ICG were approximately located at 780 nm and increased with rising concentration. Moreover, PTP^{Gd}-ICG demonstrated a strong fluorescence signal at 825 nm, underscoring its NIRF properties, which enhance photon penetration through tissue and mitigate tissue autofluorescence effects (Figure 1E). Collectively, these results validate the rational design of the PD-L1-targeting peptide platform. Our findings establish a versatile peptide scaffold for developing advanced PD-L1 theranostics, where the peptide serves as a universal functionalization site for diagnostic or therapeutic payloads.

PD-L1 expression in cell lines and cellular binding of probe

We detected the expression of PD-L1 in three human NSCLC cell lines (HCC827, H520 and A549), as well as in MLE and BEAS-2B cells, using Western blot analysis. The results showed that all tested cell lines expressed PD-L1, with HCC827 showing the highest expression level and MLE and BEAS-2B showing lower expression (Figure 2A). Quantitative analysis of the Western blot results was performed using BEAS-2B cells as the reference control

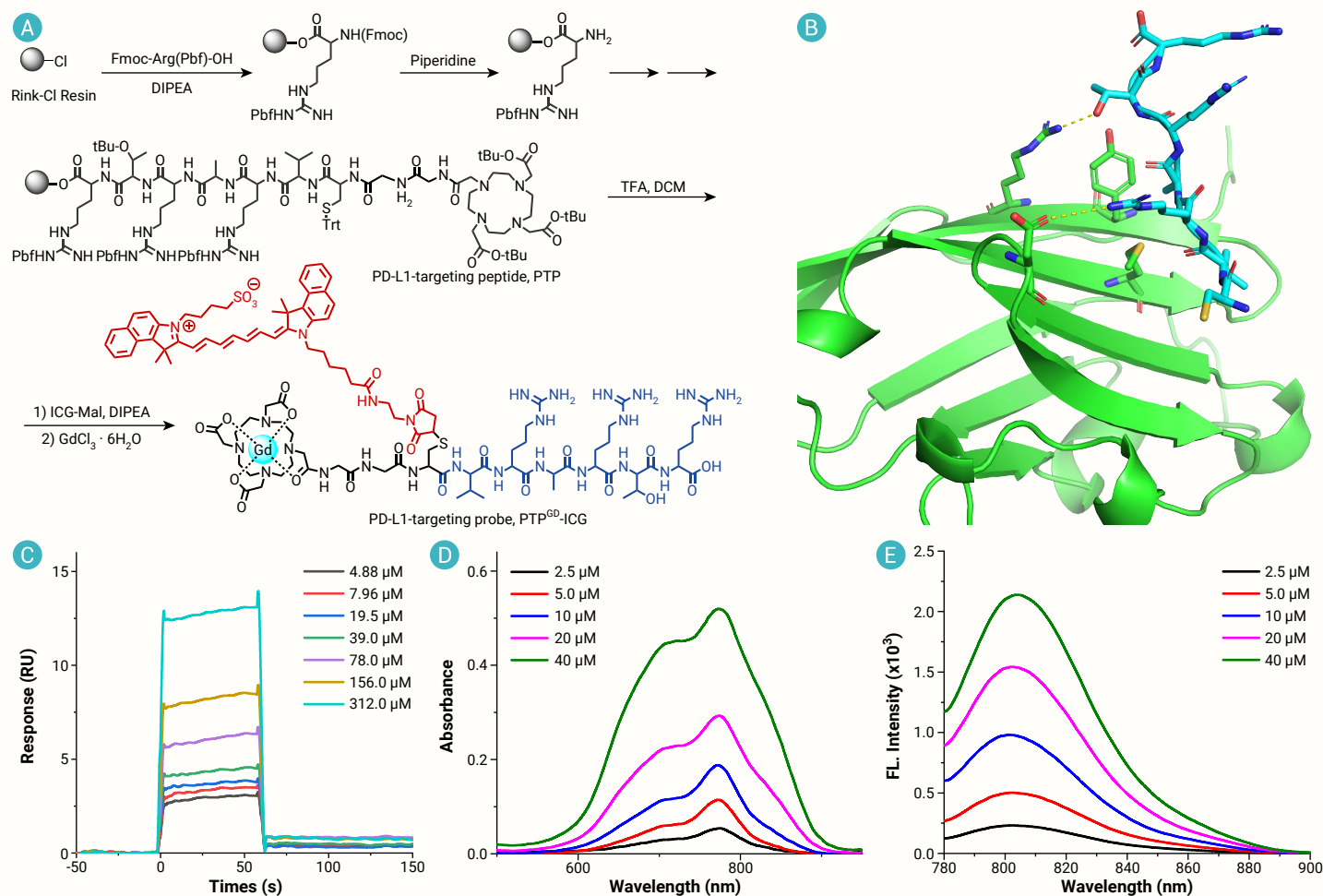


Figure 1. Preparation and characterization of PTP and PTP^{Gd}-ICG (A) Schematic synthesis route of PTP and PTP^{Gd}-ICG. (B) Predicted binding pose of PTP in complex with PD-L1. The peptide is shown as cyan sticks, while PD-L1 is displayed as a green cartoon. Yellow dashed lines indicate key interactions, including electrostatic contacts and hydrogen bonds, between the peptide and PD-L1. (C) Binding of PTP^{Gd}-ICG to PD-L1 at the indicated concentrations by SPR assay. (D, E) The absorption and fluorescence spectra of PTP^{Gd}-ICG in PBS at various concentrations.

(normalized to 1, PD-L1/GAPDH). The results showed that HCC827 cells exhibited significantly higher PD-L1 expression compared with MLE and BEAS-2B cells. Statistical analysis indicated that PD-L1 expression in HCC827 was significantly higher than that in H520 (** $P < 0.01$), MLE (** $P < 0.001$), and BEAS-2B (**** $P < 0.0001$) (Figure 2C). PD-L1 is mainly distributed on the cell membrane, and we investigated the cellular distribution of the probe. We incubated HCC827 cells with PTP-ICG. The results showed that the cells bound to the probe (Figure 2B, red fluorescence). To further verify the targeting specificity, a competitive blocking assay was performed using unlabeled PTP peptide. Pre-incubation with excess unlabeled peptide markedly inhibited probe binding, resulting in almost no detectable fluorescence signal in the blocking group, thereby confirming PD-L1-mediated targeting specificity. In addition, to evaluate expression-dependent uptake, fluorescence incubation experiments were further extended to H520, MLE, and BEAS-2B cells, which exhibit medium-to-low PD-L1 expression levels. The results demonstrated significantly reduced fluorescence signals in these low-expression cell lines compared with HCC827 cells, further supporting the correlation between probe uptake and PD-L1 expression levels. Other fields of view are shown in Figure S3. Quantitative analysis further confirmed that the fluorescence intensity of the blocking group and the low-expression cell lines was significantly lower than that of the HCC827 group (Figure 2D), which is consistent with the receptor-specific binding behavior.

In vivo MR imaging using PTP^{Gd}-ICG

To investigate the in vivo activity and PD-L1-targeted specificity of PTP^{Gd}-ICG, MR imaging was performed in mice bearing subcutaneous HCC827 tumor xenografts and blocking group (Figure 3A). At 0.5 h after tail vein injection

of PTP^{Gd}-ICG, the T₁WI signal of the HCC827 tumor significantly increased and reached its peak. At 24 h post-injection, the signal returned to baseline. In contrast, the blocking tumors showed no significant signal enhancement at the corresponding time point, and the overall signal was lower than that of the HCC827 group. There was a significant difference in tumor signal intensity between the two groups (Figure 3C). Additionally, as depicted in Figure S4, at 0.5h after injection of PTP^{Gd}-ICG, the SNR (signal-to-noise ratio) and CNR (contrast-to-noise ratio) of the experimental group were 2.39 times and 2.11 times higher, respectively, than those of the blocking group. These results demonstrated the in vivo PD-L1-targeting specificity of PTP^{Gd}-ICG, which could be effectively blocked by pre-injection of excess PTP peptide. We further investigated the expression of PD-L1 in the extracted tumors. The expression of PD-L1 in HCC827 tumors was confirmed at the tissue level using anti-PD-L1 antibodies (Figures 3B & D). The above results indicate that tumor uptake of PTP^{Gd}-ICG is attributed to the targeting ability of the PD-L1 ligand.

In terms of safety, analysis of the CCK-8 cell viability assay demonstrated that different concentrations of the PTP-ICG probe had no statistically significant effect on the viability of HCC827 cells, indicating favorable cytocompatibility across all tested concentrations (Figure S5). To further investigate the in vivo clearance behavior of PTP^{Gd}-ICG, a time-course pharmacokinetic study was performed by measuring gadolinium concentrations in blood samples collected from 5 min to 2 h post-injection using ICP-MS after microwave digestion. As shown in Figure S6A, blood gadolinium levels decreased rapidly within 120 min, indicating efficient systemic clearance of the probe. In addition, clearance pathways were evaluated by quantifying gadolinium content in urine and feces collected over 24h. Quantitative ICP-MS analysis revealed

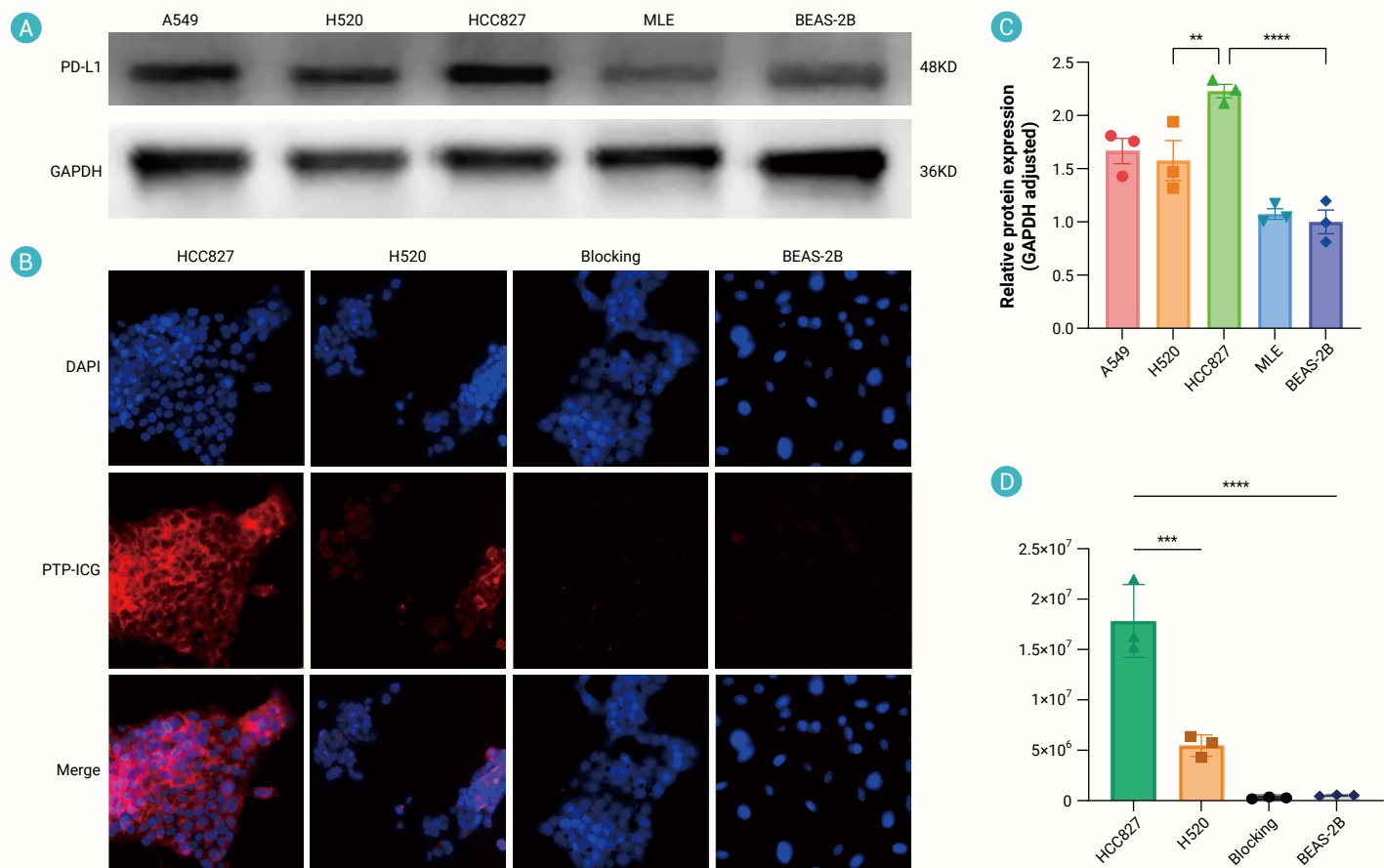


Figure 2. Cell lines PD-L1 expression and cellular binding of PTP-ICG (A) PD-L1 protein expression in HCC827, A549, H520, MLE and BEAS-2B cell lines. (B) In HCC827 cell lines, PTP-ICG (green) is localized to the tumor cell membrane, and the nuclear DAPI channel is blue. (C) Quantitative analysis of WB results. (D) Fluorescence intensity histogram of incubated HCC827, H520, BEAS-2B and Blocking. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

that urinary and fecal excretion accounted for $42.53 \pm 3.62\%$ and $31.48 \pm 2.79\%$ ID/g, respectively, at 24h post-injection, suggesting combined renal and hepatobiliary elimination of the probe (Figure S6B). Residual gadolinium analysis further showed that accumulation was mainly detected in the kidneys and liver, with minimal retention in other major organs. The in vivo safety profile of PTP^{gd}-ICG was further assessed at 1, 7, and 14 days following tail vein administration. Serum biochemical examinations, including albumin (ALB), alkaline phosphatase (ALP), creatinine (CREA), and urea (UREA), were conducted to evaluate hepatic and renal function. No abnormal fluctuations were observed in any measured indicators, and all values were comparable to those of the control group (Figures 3E-H). Additionally, histopathological examination of major organs using H&E staining was performed to further assess systemic toxicity. No obvious morphological abnormalities, inflammatory infiltration, or pathological damage were observed in the major organs of tumor-bearing mice at 1, 7, or 14 days after injection (Figure 3I). These findings collectively indicate that PTP^{gd}-ICG exhibits favorable biosafety, efficient clearance behavior, and does not induce significant toxicity in normal tissues.

In vivo specificity and biodistribution of probe

Based on the WB analysis results, we selected the PD-L1-overexpressing cell line HCC827 to construct subcutaneous xenograft mouse models, divided into an experimental group and a blocking group. PTP-ICG ($3\mu\text{g/g}$ mouse body weight) was injected into the tail vein of tumor-bearing mice, and NIR-I and NIR-II fluorescence images were collected at multiple time points to evaluate the specificity of the probe and its in vivo distribution (Figures 4A-B). NIR-I fluorescence imaging showed that the liver displayed strong fluorescence signals that gradually weakened over time. After 4 h, fluorescence signals were detected in the tumor area. The tumor to background ratio (TBR) gradually increased over time, enabling clear differentia-

tion of the tumor from surrounding normal tissues within 24 h. The highest TBR (4.67 ± 0.32) was reached at 72 hours. In contrast, no obvious fluorescence signal was observed in the tumor area of the blocking group ($\text{TBR} = 1.8 \pm 0.12$). The NIR-II fluorescence images showed the same trend as the NIR-I images. The TBR of the experimental group (8.77 ± 1.45) and the TBR of the blocking group (2.89 ± 0.82) showed significant differences ($P < 0.001$, student t-test) (Figures 4C-D). PD-L1 IHC and HE staining were also performed on the tumor (Figure 4G).

To verify the NIR-II imaging advantage of PTP-ICG, we performed non-invasive vascular imaging of the hind limbs and abdomen of intact mice. The signal-to-noise ratio (S/N) was defined as the ratio of the fluorescence signal to the background signal of the surrounding tissue. The S/N values for hind limb vascular imaging were 1.16 at 840 nm, 1.47 at 1100 nm, 1.89 at 1200 nm, and 1.98 at 1300 nm. As the wavelength extended from 840 nm in NIR-I to 1300 nm in NIR-II, the imaging resolution and signal-to-noise ratio of small blood vessels improved (Figures 4H-O). The fluorescence imaging between the two groups demonstrated the targeting specificity of the probe, and compared with NIR-I, NIR-II provided higher tissue penetration, lower background, and higher imaging contrast. (Figures 4E-F).

NIR-II fluorescence imaging-guided NSCLC resection

HCC827-Luc cells were used to construct orthotopic lung cancer mouse models. By utilizing preoperative BLI images collected from small animal live imaging systems (Figure 5Ai) and 3D CT-BLI co-registered images (Figure 5Aii), the precise location of the tumor was determined. 24 hours after injection of PTP-ICG, the thoracic and abdominal cavities of the mice were opened using tissue scissors to fully expose the lungs and tumor tissue. Under NIR-II guidance, tumor resection was performed (Figure 5B i-iii), and the tumor tissue was successfully separated from the lung. After euthanasia, the major organs were harvested and white light, NIR-II images, and BLI images (Figure

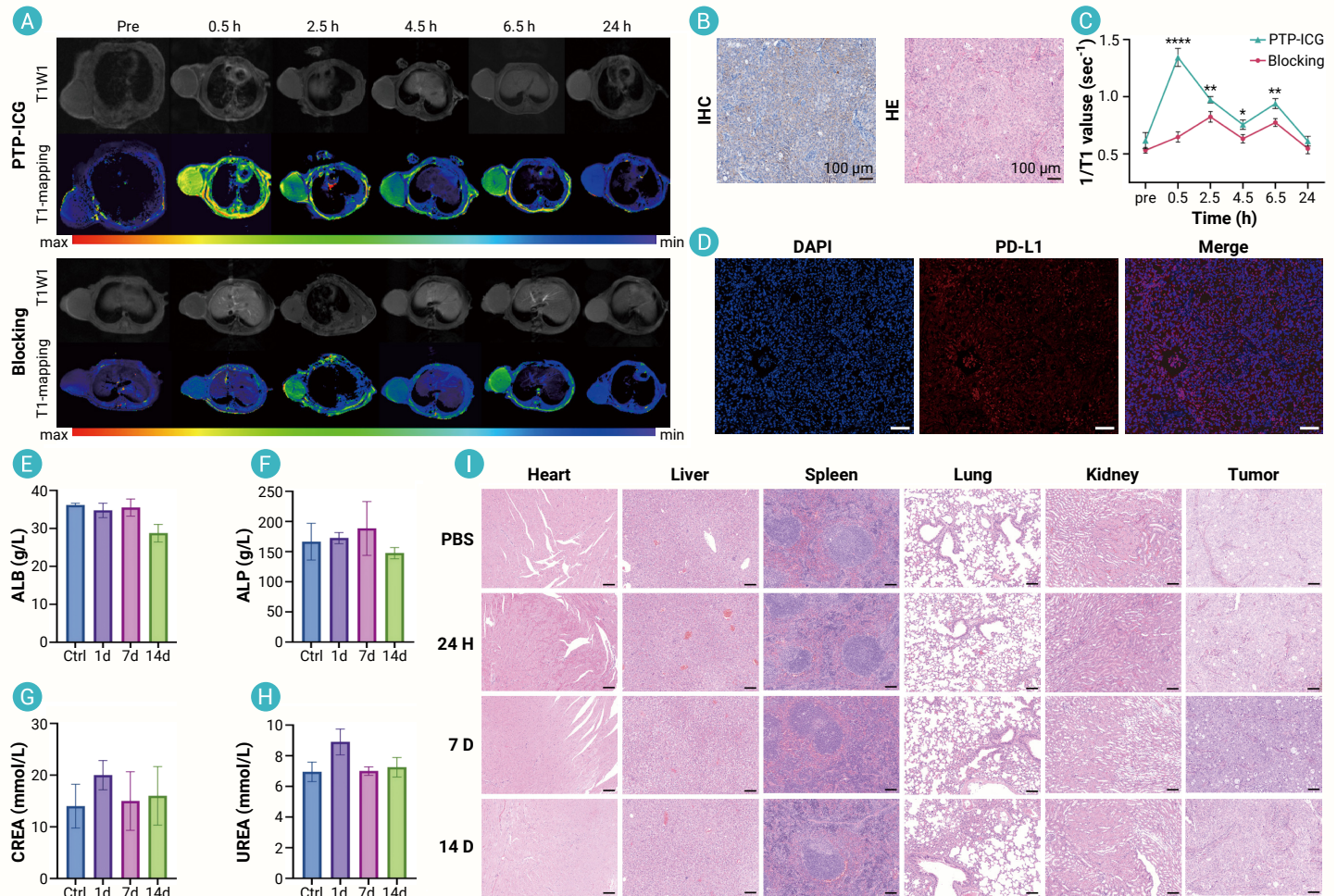


Figure 3. In vivo MR imaging recognition of subcutaneous tumor in mice (A) MR imaging of mice bearing HCC827 tumors at 0.5, 2.5, 4.5, 6.5 and 24h post-injection via tail vein with PTP^{Gd}-ICG. To inhibit PD-L1 activity, PTP were injected into HCC827 tumor-bearing mice before injection of PTP^{Gd}-ICG. (B) Immunohistochemical staining of PD-L1 and pathological HE staining results. Scale bar is 100 μ m. (C) 1/T1 values of tumors in the above MR images (* $p < 0.05$). (D) Fluorescence imaging of HCC827 tumor sections stained with DAPI and anti-PD-L1 antibody. (E-H) The liver and kidney function measurements of BALB/c nude mice were evaluated after intravenous injection of PBS or PTP^{Gd}-ICG at different time intervals (24 hours, 7 days, 14 days). (I) Each group has H&E stained sections of important organs (heart, liver, spleen, lungs, kidneys, intestines, tumors). ALP: alkaline phosphatase; ALB: albumin; CREA: creatinine; UREA: urea. Scale bar is 100 μ m.

5B iv-vi) were collected. The excised tissues were subsequently embedded for histological analysis to verify the nature of the lesions and PD-L1 expression, and vertical sections were prepared. Tumor tissue was confirmed by H&E and immunohistochemical staining (Figure 5H).

To further evaluate the detection ability of the probe for metastatic lesions, we constructed an HCC827-Luc liver metastasis mouse model, and tumor localization was performed using BLI images (Figure 5Ci). Considering the high hepatic fluorescence background caused by probe metabolism, NIR-II imaging was performed 72 h after injection of PTP-ICG (Figure 5C ii). Under NIR-II guidance, the abdominal cavity was opened to fully expose the liver, allowing clear visualization of metastatic tumors with strong fluorescence signals, whereas the liver and kidneys showed minimal fluorescence. The tumors were then resected. After euthanizing, the major organs were collected for white light, NIR-II, NIR-I and BLI imaging (Figure 5C iii-viii). Postoperative pathology confirmed that the excised tissues were all tumor tissues, and immunohistochemistry showed positive PD-L1 (Figure 5I). The experimental group of mice were euthanized at 24 hours and the highest TBR point (72 hours), and their organs and tumors were dissected for in vitro NIR-I and NIR-II imaging. PTP-ICG was found to be mainly distributed in the kidneys, liver, and tumor tissues at 24 h, while at 72 h, it was mainly distributed in tumor tissues (Figures 5D-G).

In vitro incubated and imaging of fresh human NSCLC specimens

Tumor and adjacent tissue samples were collected from five patients who underwent NSCLC surgery. Fresh specimens were incubated with PTP-ICG,

followed by white light and NIR-II fluorescence imaging. Enhanced fluorescence signals were observed in the tumor tissue regions, while the adjacent normal tissues exhibited weaker fluorescence signals (Figure 6A). Subsequently, standard H&E staining confirmed that areas with stronger fluorescence signals corresponded to tumor tissue. In addition, immunohistochemistry confirmed that PD-L1 was highly expressed in the tumor regions (Figure 6B). Measurement and quantitative analysis of NIR-II fluorescence signal intensity revealed that signal intensity in all tumor regions was significantly higher than in adjacent tissues, as shown in Figure 6C. Statistical analysis of the H-SCOREs and fluorescence intensity of 10 tissue specimens from five NSCLC patients revealed a positive correlation between the two ($R^2 = 0.951$) (Figure 6D).

DISCUSSION

This study successfully constructed a PD-L1-targeted MR/NIR II dual-modal peptide probe (PTP^{Gd}-ICG) and systematically validated its molecular specificity, imaging performance, and biosafety across cellular, animal, and human tissue models. Collectively, these findings demonstrate that PTP^{Gd}-ICG enables accurate visualization of PD-L1-positive tumors while exhibiting favorable biocompatibility, supporting its potential for clinical translation.

Our in vitro experiments showed that PTP-ICG specifically bound to PD-L1-expressing NSCLC cells, whereas minimal fluorescence signals were observed in the blocking group and in the low-PD-L1-expression cell line. These findings are consistent with our WB experimental results. To verify the safety of PTP-ICG, we conducted cytotoxicity experiments, evaluated animal

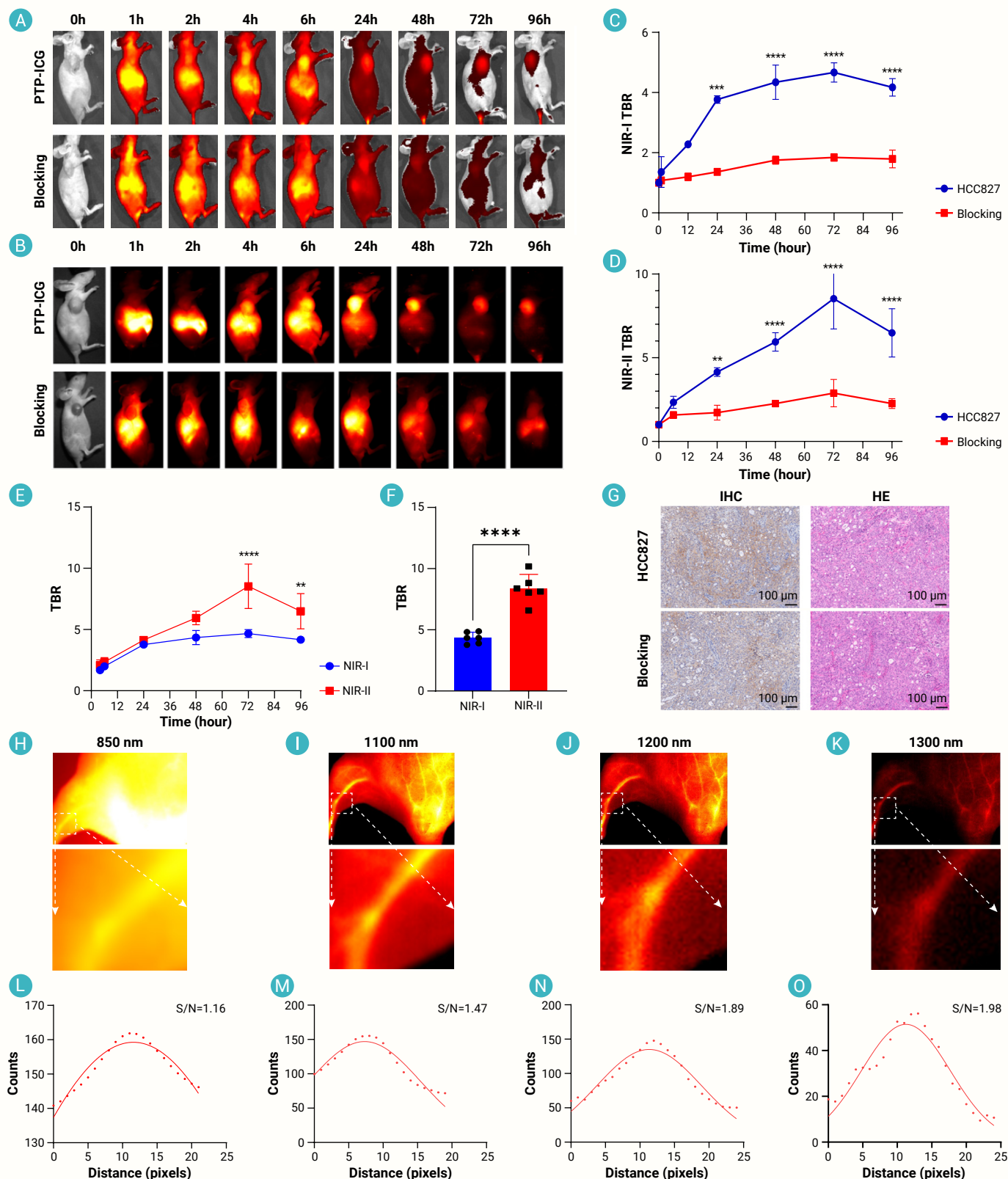


Figure 4. In vivo specificity and biodistribution of PTP-ICG (A) BLI imaging and NIR-I fluorescence imaging at different time points of intravenous injection PTP-ICG in HCC827 subcutaneous xenograft tumour models and in HCC827 subcutaneous tumour blocking experiments. (B) BLI imaging and NIR-II fluorescence imaging after injection of PTP-ICG from 1 h to 96 h. (C) Tumour-to-background (TBR) ratio of NIR-I fluorescence signal intensity, and the skin fluorescence signal intensity around the tumour was selected as the background. (D) BLI imaging and NIR-II fluorescence imaging after injection of PTP-ICG from 1 h to 96 h. (E) The TBR for HCC827 experimental group of NIR-I and NIR-II fluorescence signal intensity. (F) NIR-I and NIR-II fluorescence signal intensity at 72 hours. (G) IHC analysis of PD-L1 in the tumors and Pathological HE staining results. (H-K) PTP-ICG NIR-II imaging of blood vessels. (L-O) Fluorescence cross-sectional intensity distribution of hind limbs in NIR-I and NIR-II windows. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

blood biochemical parameters, and examined tissue safety. Our findings indicate that after 24 and 48 hours incubation within the concentration range of 0-

80 $\mu\text{g/ml}$, the probe did not significantly affect cell viability. In addition, animal safety experiments demonstrated that PTP-ICG has no acute or long-term

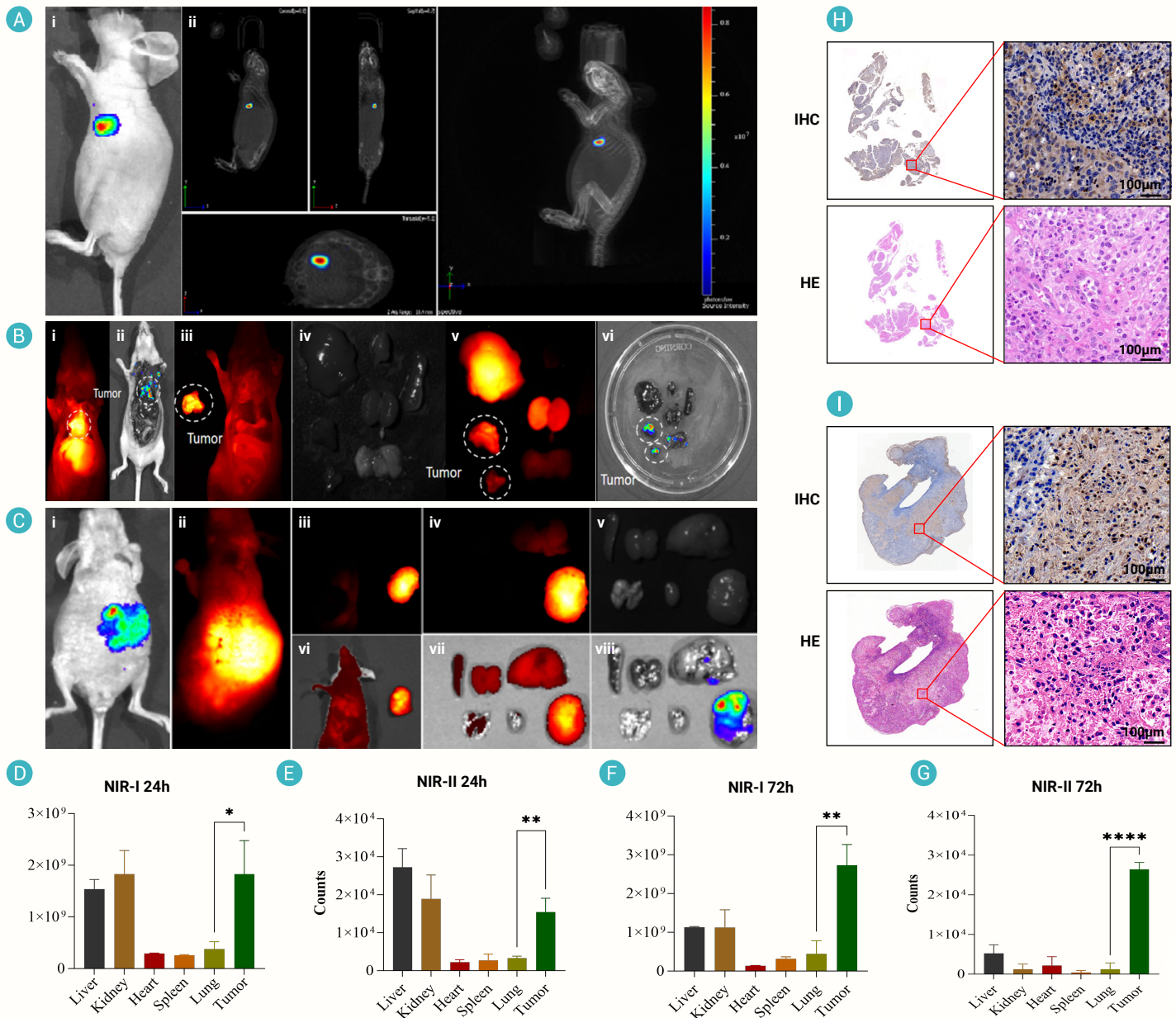


Figure 5. NIR-II fluorescence imaging-guided orthotopic / metastasis lung cancer resection (A) BLI (A i), 3D CT-BLI fusion image (A ii) of an in situ tumor-bearing mouse. (B) NIR-II fluorescence (B i), BLI (B ii), and NIR-II fluorescence (B iii) images of mice after thoracotomy and liver resection. White light (B iv), NIR-II fluorescence (B v), and BLI (B vi) images of important organs and tumors in vitro. (C) Preoperative BLI (C i) and NIR-II fluorescence (C ii) images of mice with lung cancer liver metastasis. Intraoperative resection of tumors using NIR-I fluorescence (C vi), NIR-II (C iii) fluorescence images, as well as white light (C v), NIR-II fluorescence (C iv), NIR-I (C vii) fluorescence, and BLI (C viii) images of important organs and tumors in vitro. (D) NIR-I fluorescence signal intensity in important organs and tumors of the HCC827 experimental group at the 24-hour time point. (E) NIR-II fluorescence signal intensity at the 24-hour time point. (F) NIR-I fluorescence signal intensity at the 72-hour time point. (G) NIR-II fluorescence signal intensity at the 72-hour time point. (H) PD-L1 IHC and HE staining results of lung in situ tumor. (I) PD-L1 IHC and HE staining results for liver metastasis from lung cancer.

biological toxicity. This confirms its safety profile, providing a foundation for future translational and clinical investigations.

Traditional preoperative lung cancer staging relies on CT/PET-CT, which often underestimates tumor burden due to limited soft-tissue contrast and false positives from inflammation.^{20,21} Our probe addresses this gap by leveraging MRI's high anatomical resolution ($\leq 100 \mu\text{m}$) for non-invasive PD-L1 mapping. In vivo imaging experiments demonstrated that PTP^{Gd}-ICG probes accurately localized mouse tumor lesions through MRI. During surgery, PTP-ICG probes enabled real-time identification of tumor tissue under NIR fluorescence guidance and facilitate targeted resection. This capability aligns with clinical needs for identifying occult metastases—critical for accurate TNM staging and surgical planning. Consistent with its specific PD-L1-targeting capability, PTP-ICG generated a high tumor-to-background ratio (TBR), enabling clear discrimination between tumor tissue and surrounding normal organs and supporting accurate tumor delineation in vivo. In addition,

our results demonstrated that NIR-II fluorescence imaging outperformed conventional NIR-I imaging in distinguishing PD-L1-positive tumors from adjacent normal tissues, consistent with the well-recognized advantages of reduced tissue scattering, lower autofluorescence, and improved tissue penetration in the NIR-II window. By utilizing this technology, surgeons can potentially non-invasively and dynamically monitor PD-L1 expression while achieving more accurate tumor localization before surgery. The orthotopic NSCLC mouse model accurately simulates the challenge of identifying tumor margins in real life. To further evaluate the translational potential of this strategy, fresh tissue specimens from five patients with NSCLC were subjected to ex vivo NIR-II fluorescence imaging using PTP-ICG. Based on tissue sample incubation, NIR-II fluorescence imaging demonstrated favorable tumor-to-normal tissue contrast, providing preliminary evidence supporting its clinical applicability. Guided by NIR-II fluorescence imaging during surgery, surgeons may more readily identify submillimeter occult tumor lesions, thereby

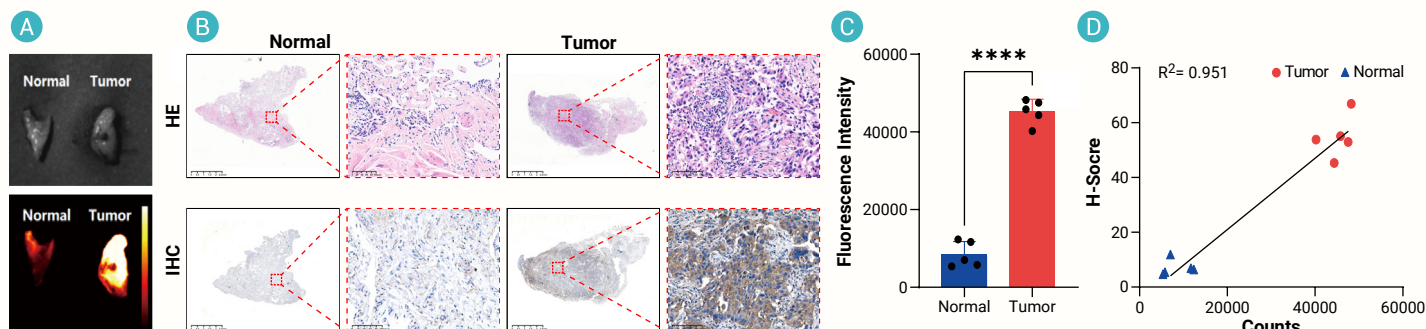


Figure 6. In vitro cultivation and imaging of fresh NSCLC specimens (A) White light images and NIR-II fluorescence images of NSCLC tissue in vitro and adjacent normal lung tissue. (B) The corresponding H&E staining and PD-L1 IHC staining of the above organizations. (C) Paired statistical analysis revealed a significant difference in NIR-II fluorescence intensity between NSCLC and normal lung tissue ($P < 0.0001$). (D) The fluorescence intensity is positively linearly correlated with the expression of H-SCORE in PD-L1 ($R^2 = 0.951$).

improving the completeness of tumor resection and potentially reducing postoperative recurrence. However, the sample size of human tissues remains limited, which may not fully capture the clinical heterogeneity of NSCLC. PD-L1 expression is highly heterogeneous across patients and tumor regions; therefore, this strategy is expected to be particularly beneficial for patients with PD-L1-high tumors and may be less suitable for PD-L1-low or PD-L1-negative tumors.

Beyond its value for intraoperative navigation, non-invasive assessment of PD-L1 expression is equally important for precision immunotherapy. PD-1/PD-L1 immune checkpoint inhibitors (as monotherapy or combination with chemotherapy) have become a core therapeutic strategy for advanced non-small cell lung cancer (NSCLC),²² and PD-L1 expression levels (especially tumor proportion scores $TPS \geq 1\%$ and $\geq 50\%$) are key biomarkers guiding clinical medication.^{23,24} At present, the evaluation of PD-L1 mainly relies on immunohistochemistry (IHC) staining. However, although IHC remains the current clinical gold standard, its limitations are evident. On one hand, IHC relies on invasive biopsy procedures. Due to factors such as tumor location and patient physical condition, obtaining sufficient tissue samples is challenging for some patients. On the other hand, NSCLC exhibits significant spatiotemporal heterogeneity. A biopsy from a single lesion or time point cannot fully reflect the overall PD-L1 expression status of the tumor. In recent years, PET/CT imaging of radioactive isotopes targeting PD-L1 has provided a new approach to address the limitations of IHC. The core advantage of this type of technology is that it can dynamically reflect the expression level of tumor PD-L1 through semi-quantitative parameters such as SUVmax, demonstrating significant correlation with clinical efficacy (such as progression-free survival).²⁵⁻²⁷ In addition, it can identify the differential PD-L1 expression among different lesions within the same patient, addressing the shortcomings of single-lesion biopsy. In terms of efficacy prediction, some studies have confirmed that the degree of uptake of PD-L1 probe by lesions is positively correlated with immune therapy response.^{25,28}

However, current PD-L1-targeted PET/CT imaging strategies still face several important limitations. Radiolabeled monoclonal antibodies are bulky and have poor penetration, requiring 4 to 7 days after injection to achieve optimal imaging results, thereby prolonging patient wait time.²⁵ Long-half-life radionuclides increase the radiation dose to normal tissues such as liver and bone marrow, limiting the feasibility of repeated testing. Although PD-L1 PET/CT of small molecules and peptide fragments has been studied, the spatial resolution of PET make it difficult to identify small metastases or deep lesions, while insufficient soft tissue contrast on CT may lead to the omission of low-signal lesions. Compared with antibody-based imaging probes, peptide probes possess several intrinsic advantages, including their smaller molecular size, superior tissue penetration, faster blood clearance, lower immunogenicity, and shorter imaging windows. These characteristics are particularly advantageous for perioperative molecular imaging, where rapid target accumulation and timely surgical guidance are essential. The favorable pharmacokinetic properties of PTP^{Gd}-ICG enabled high-resolution MR imaging within 0.5 h after administration, providing a practical imaging time window for subsequent NIR-II fluorescence-guided surgery.

In this context, the introduction of MR technology provides a key breakthrough in solving the above-mentioned problems. The MR/NIR II dual-mode peptide probe (PTP^{Gd}-ICG) used in this study significantly compensates for the limitations of PET/CT. Firstly, MR imaging has a natural high soft tissue contrast, which can clearly distinguish small lesions within the lung parenchyma, making up for the missed detection of low signal metastases by CT. Secondly, the relaxation effect improves the spatial resolution of MRI and solves PET's difficulty in visualizing fine details. Furthermore, MR does not require radioactive nuclide labeling, avoiding radiation exposure from long half-life isotopes. High-resolution imaging with PTP^{Gd}-ICG can be completed just 0.5 hours after injection, greatly reducing waiting time. In addition, the multi parameter imaging characteristics of MR can provide comprehensive information about the tumor microenvironment (such as edema, necrosis, and blood flow perfusion), which complements the single metabolic information of PET and more comprehensively reflects the biological characteristics of PD-L1 positive tumors. Meanwhile, NIR-II fluorescence imaging provides real-time intraoperative visualization that cannot be achieved by PET or MRI alone, allowing seamless integration of preoperative lesion localization and intraoperative surgical navigation within a single molecular imaging platform.

Overall, the synergistic integration of MRI and NIR-II fluorescence combines high-resolution anatomical imaging with real-time surgical navigation, offering a promising strategy for PD-L1-guided tumor imaging. However, several limitations should be acknowledged. First, PD-L1 heterogeneity limits the universal applicability of this approach, and it is more suitable for PD-L1-high NSCLC patients rather than all tumor types. Second, the current study lacks systematic evaluation in PD-L1-negative tumor models, which is necessary to define the biological performance boundary of the probe. Third, the current human tissue validation is limited in sample size, and larger clinical cohorts are needed to confirm diagnostic robustness. Future prospective studies involving larger patient cohorts, together with longitudinal evaluation of imaging-guided therapeutic decision-making, will be essential to validate the clinical utility and translational potential of this MR/NIR-II dual-modal molecular imaging strategy.

CONCLUSION

In conclusion, we developed a PD-L1-targeted MR/NIR-II dual-modal imaging probe (PTP^{Gd}-ICG) for integrated preoperative tumor localization and intraoperative fluorescence-guided surgery in NSCLC. The probe demonstrated excellent targeting ability and imaging performance in multiple preclinical models, while rapid imaging of fresh human specimens further supported its translational potential for tumor margin assessment. This study establishes an integrated imaging strategy combining MRI, NIR-II fluorescence, and pathological validation, providing a promising platform for precision surgery and future clinical translation for PD-L1-positive NSCLC.

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AUTHOR CONTRIBUTIONS

Each author's individual contributions to this work were depicted with CRediT Taxonomy. Qingyun Liu and Xiaohua Jia contributed equally to this work. Qingyun Liu and Xiaohua Jia: Investigation, Writing-original draft. Yutao Li and Lei Liang: Validation, Visualization. Zhenhua Hu and Defan Yao: Methodology. Jun Wang, Zhenhua Hu and Kezhong Chen: funding acquisition, Supervision, Writing-review & editing. The order of the two co-first authors was decided according to the workload and total contribution to this research. All authors contributed to and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

This study strictly adheres to all relevant ethical standards. The study involving patients has been approved by the Institutional Review Committee of Peking University People's Hospital (Approval No. 2023PHB124-001), and all patients have provided written informed consent. All animal procedures were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Peking University Health Science Center (certification number: DLASBE0357).

DATA AND CODE AVAILABILITY

The data supporting the findings of this study, including imaging data and analysed datasets, will be made available from the corresponding author upon reasonable request, beginning at the time of publication.

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-med.2026.100251>