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Near infrared photoimmunotherapy for lung metastases

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Abstract

Lung metastases are a leading cause of cancer related deaths; nonetheless current treatments are limited. Near infrared photoimmunotherapy (NIR-PIT) is a new cancer treatment that combines the specificity of intravenously injected antibodies that target tumors with the toxicity induced by photosensitizers activated by NIR-light. Herein, we demonstrate the efficacy of NIR-PIT in a mouse model of lung metastases.

Experiments were conducted with a HER2, luciferase and GFP expressing cell line (3T3/HER2-luc-GFP). An antibody-photosensitizer conjugate (APC) consisting of trastuzumab and a phthalocyanine dye, IRDye-700DX, was synthesized. *In vitro* NIR-PIT-induced cytotoxicity was light dose dependent. With 3D culture, repeated NIR-PIT could eradicate entire spheroids. *In vivo* anti-tumor effects of NIR-PIT included significant reductions in both tumor volume ($p = 0.0141$ vs. APC) and bioluminescence image (BLI) ($p = 0.0086$ vs. APC) in the flank model, and prolonged survival ($p < 0.0001$). BLI demonstrated a significant reduction in lung metastases volume ($p = 0.0117$ vs. APC). Multiple NIR-PIT doses significantly prolonged survival in the lung metastases model ($p < 0.0001$). These results suggested that NIR-PIT is a potential new therapy for the local control of lung metastases.

Keywords

near infrared photoimmunotherapy; bioluminescence; HER2 receptor; lung metastases

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5. Author Contributions

K.S. designed and conducted experiments, performed analysis and wrote the manuscript; K.S. is a Japanese pulmonologist and has performed many bronchoscopic and thoracoscopic investigations for patients. T.N. and M.M performed analysis; P.L.C. wrote the manuscript and supervised the project; and H.K. planned and initiated the project, designed and conducted experiments, wrote the manuscript, and supervised the entire project.

6. Conflict of interest

None declared

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1. Introduction

The lung is among the most common sites of metastatic disease and is the most common cause of cancer-related death [1–4]. Existing treatment often includes a sequential combination of surgery, chemotherapy and, less commonly radiotherapy [1,5–7]. Surgery results in acute damage to the chest wall and has a long recovery period. Chemotherapy is directed systemically and therefore causes both systemic and local toxicities to the lung. Radiotherapy cannot be delivered to the entire lung and can result in damage to the mediastinum, lung and heart. Therefore, there is an urgent need for a highly targeted therapy, which could locally control pulmonary metastases, yet avoids local and systemic side effects.

The lung is the ideal organ for light therapy because light is transmitted easily throughout the air-containing lung without significant attenuation. Of course, the chest wall is too thick to allow sufficient light to pass but modern minimally invasive thoracoscopy could enable the delivery of light to virtually all parts of the lungs.

The concept of using targeted light therapy with photosensitizers is over three decades old [8,9]. However, most photosensitizers do not target tumors with sufficient selectivity to be used alone. Attempts to target photosensitizers have often been limited by the hydrophobicity of traditional photodynamic therapy (PDT) sensitizers which dominate the pharmacokinetics of antibody-PDT conjugates. However, if the photosensitizer is highly hydrophilic, then the pharmacokinetics of an antibody-photosensitizer are dominated by the antibody and true targeting is possible. IRDye700DX, (IR700, a silica-phthalocyanine dye) is a highly hydrophilic photosensitizer that is excited with near infrared (NIR) light. When conjugated to a monoclonal antibody, this antibody photosensitizer conjugate (APC) is able to treat tumors, a process that has been called “near infrared photoimmunotherapy” (NIR-PIT) which differs from traditional PDT not only in the hydrophilicity of the photosensitizer, but also in its reliance on NIR-light that has better tissue penetration than lower wavelength light used with PDT. This NIR-PIT APCs demonstrate similar intravenous pharmacokinetics to naked antibodies, resulting in highly targeted tumor accumulation with minimal non-target binding. When bound to targeted cells and exposed to NIR, APCs induce rapid, selective and irreversible damage to the cell membrane. *In vitro* studies have demonstrated that NIR-PIT is highly target cell-specific, therefore, non-target expressing cells suffer no toxic effects even when they are immediately adjacent to treated cells [10,11]. Cell membrane rupture can be demonstrated within minutes of exposure to NIR-light in targeted cells [12–15].

Among the organs in the body, the lung is able to transmit NIR-light most effectively because it is mostly filled with air. Thus, small lung metastases are potential targets of NIR-PIT. Here, we investigate the efficacy of NIR-PIT in a murine model of lung metastases.

2. Materials and methods

2.1. Reagents

Water soluble, silicon-phthalocyanine derivative, IRDye 700DX NHS ester and IRDye 800CW NHS ester were obtained from LI-COR Bioscience (Lincoln, NE, USA). Trastuzumab, 95% humanized IgG₁ mAb directed against HER2, was purchased from Genentech (South San Francisco, CA, USA).

2.2. Synthesis of IR700-conjugated trastuzumab, and IR800-conjugated trastuzumab

Conjugation of dyes with mAbs was performed according to previous reports [10,12,16]. Details are provided in supplementary materials and methods. We abbreviate IR700 conjugated to trastuzumab as tra-IR700, and IR800 conjugated to trastuzumab as tra-IR800.

2.3. Cell culture

HER2 and luciferase/GFP-expressing Balb/3T3/HER2-luc-GFP cells (3T3/HER2-luc-GFP) were established with a transfection of RediFect Red-FLuc-GFP (PerkinElmer, Waltham, MA, USA). High GFP and luciferase expression was confirmed with 10 passages. Balb/3T3 cells stably expressing RFP were established with transfection by RFP (EF1a)-Puro lentiviral particles (AMSBIO, Cambridge, MA, USA). High RFP expression was confirmed in the absence of a selection agent with 10 passages. 3T3 cells stably expressing RFP (3T3-RFP) were used as negative controls [14,15]. Cell culture conditions were same as previously.¹⁴

2.4. 3D Spheroid culture

Spheroids were generated according to previous reports [12,17].

2.5. Flow Cytometry

Flow cytometry was performed as previously [12]. 3T3/HER2-luc-GFP cells (1×10^5) were incubated with tra-IR700 for 6 hr at 37°C. Specific binding was examined as reported previously [12].

2.6. Fluorescence microscopy

To detect the antigen specific localization of IR700 conjugates, fluorescence microscopy was performed (IX61 or IX81; Olympus America, Melville, NY, USA) as previously [12]. The filter was set to detect IR700 fluorescence using a 590–650 nm excitation filter, and a 665–740 nm band pass emission filter.

3D reconstructions of the spheroids were done as reported previously [14]. Sections of spheroids were obtained and examined as previously described [14].

Analysis of the images was performed with ImageJ software (<http://rsb.info.nih.gov/ij/>).

2.7. *In vitro* NIR-PIT

In vitro NIR-PIT was performed as previously described [14]. Details are provided in supplementary materials and methods.

2.8. Cytotoxicity/Phototoxicity assay

The cytotoxic effects of NIR-PIT with tra-IR700 were determined by the luciferase activity and flow cytometric [14]. Details are provided in supplementary materials and methods.

2.9. Estimation of GFP fluorescence intensity *in vitro*

After *in vitro* PIT, the cells were again incubated for 1 hr and GFP intensity (total pixels) was evaluated using the same threshold and field, as previously reported [15,18].

Fluorescence from treated cells was also measured using a flow cytometer (FACS Calibur) [15,19].

2.10. Animal and tumor models

Details are provided in supplementary materials and methods.

2.11. *In vivo* fluorescence imaging

Details are provided in supplementary materials and methods.

2.12. Fluorescence thoracoscopy

Fluorescence thoracoscopy was performed as previously described [19]. Details are provided in supplementary materials and methods.

2.13. CT Imaging

CT images were obtained with a Nano-SPECT/CT scanner (Bioscan, Inc., Poway, CA, USA). Data reconstruction and analysis were performed with the ordered-subsets expectation maximization algorithm and InVivoScope1.42 software (Bioscan).

2.14. Characterization of the lung metastasis mouse model

Both the lung metastasis model and the subcutaneous bilateral flank models received 100 μ g of tra-IR700 or tra-IR800 i.v. (tra-IR800 was used to avoid auto-fluorescence). Serial images were obtained as previously described [19].

2.15. *In vivo* NIR-PIT

Details are provided in supplementary materials and methods.

2.16. Histological analysis

Histological analysis was performed as previously described [19].

2.17. Statistical Analysis

Data are expressed as means $\pm$ s.e.m. from a minimum of four experiments, unless otherwise indicated. Statistical analyses were carried out using a statistics program (GraphPad Prism; GraphPad Software, La Jolla, CA, USA). For multiple comparisons, a one-way analysis of variance (ANOVA) with Tukey's test was used. The cumulative probability of survival, determined herein as the tumor diameter failing to reach 2 cm in flank model, natural death in lung metastasis model was estimated in each group with the use of the Kaplan-Meier

survival curve analysis, and the results were compared with the log-rank test and Wilcoxon test. Student's *t* test was also used to compare the two *in vitro* 2D culture studies. $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Evaluation of NIR-PIT effect *in vitro* 2D culture

To monitor the effect of NIR-PIT, Balb/3T3 cell line expressing HER2 (3T3/HER2)[20] was genetically modified to express GFP and luciferase (3T3/HER2-luc-GFP)(Supplementary Fig. S1A). The fluorescence signals obtained with tra-IR700 on 3T3/HER2-luc-GFP cells were evaluated by FACS. After 6 hr incubation with tra-IR700, the cells exhibited detectable fluorescence with tra-IR700, but this could be completely blocked by the addition of excess trastuzumab, suggesting specific binding (Supplementary Fig. S1B). Serial fluorescence microscopy of 3T3/HER2-luc-GFP cells was performed before and after NIR-PIT. After exposure to NIR-light (2 J/cm^2) cellular swelling, bleb formation and rupture of the lysosome were observed (Fig. 1A). Time-lapse image analysis showed acute morphologic changes in the cell membrane and influx of PI indicating cell death (Supplementary Videos S1, S2). Most of these cellular changes were observed within 30 min after light exposure, indicating rapid induction of necrotic cell death after NIR-PIT. No significant changes were observed in HER2-negative 3T3 cells after exposure to the same energy of NIR-light suggesting high selectivity (Supplementary Fig. S2). In order to quantitate the effect of *in vitro* NIR-PIT, we performed a cytotoxicity assay 1 hr after NIR-light irradiation using PI staining and luciferase activity. Based on incorporation of PI, the cell death percentage increased in a light-dose dependent manner. No significant cytotoxicity was observed with NIR-light exposure alone or with APC alone (Fig. 1B). Bioluminescence showed significant decreases of relative light units (RLU) in NIR-PIT treated cells (Fig. 1C, D). BLI also showed a decrease of luciferase activity in a light-dose dependent manner (Fig. 1D). GFP fluorescence intensity was greatly reduced in dead cells (stained positive with PI), while GFP fluorescence was preserved in surviving cells (Fig. 1E). GFP fluorescence was likely reduced after PIT because the GFP was extruded from the cytoplasm after membrane rupture and thus, markedly diluted and/or denatured. The GFP fluorescence ratio (before: 1 hr after PIT) as determined by FACS, decreased in a light dose dependent manner, while no decrease was detected with NIR light exposure alone or APC alone (Fig. 1F). To investigate the change in GFP fluorescence by microscopy, we compared the total GFP pixels ratio (before: 1hr after PIT) in the same field and demonstrated that it decreased in a light-dose dependent manner while no decrease was detected with NIR-light exposure alone or APC alone (Fig. 1G and Supplementary Fig. S3). Taken together, the effects of NIR-PIT on 3T3/HER2-luc-GFP could be monitored with both GFP fluorescence and bioluminescence.

3.2. Characterization of *in vitro* 3D culture and evaluation of NIR-PIT effect on *in vitro* 3D culture

Next, we evaluated the efficacy of NIR-PIT on 3D spheroids (Supplementary Fig. S4A). Fluorescence images of frozen sections revealed that cells were evenly dispersed throughout the spheroid (Supplementary Fig. S4B). Three-dimensional confocal microscopy showed the spheroids grew (Supplementary Fig. S4C) as large as approximately 500 μm in diameter

(Supplementary Fig. S4D). The APC gradually permeated into spheroids from the perimeter as depicted on IR700-fluorescence microscopy; the stained area gradually spread toward the center of the spheroid (Supplementary Fig. S4E, F). NIR-PIT caused necrotic cell death in the APC-bound layer of the spheroid (Fig. 2A). After NIR exposure, the spheroid immediately expanded and stained by PI (Supplementary Videos S3, S4). These changes occurred within only 25 min after light exposure. NIR-PIT killing effects on 3T3/HER2-luc-GFP spheroid were monitored with luciferase activity, which showed a light-dose dependent response (Fig. 2B and Supplementary Fig. S4G). Daily repeated NIR-PIT (Fig. 2C) achieved complete eradication of 3T3/HER2-luc-GFP spheroids, while controls continued to grow (Fig. 2D), as confirmed by BLI and luciferase activity ($**p = 0.0075 < 0.01$, $***p < 0.0001$ vs. APC, Tukey's test with ANOVA)(Fig. 2E, F). These results suggest that repeated NIR-PIT could eradicate target-expressing cells growing in 3T3/HER2-luc-GFP 3D spheroids.

3.3. *In vivo* NIR-PIT reduced tumor volume and luciferase activity in flank xenograft model

To assess the effect of single shot NIR-PIT on 3T3/HER2-luc-GFP tumors *in vivo* fluorescence and BLI imaging were obtained as shown (Supplementary Fig. S5A, B and S7A). While treatment groups showed a decrease in fluorescence and BLI, the RLU of tumor in other groups showed a gradual increase due to tumor growth. In contrast, luciferase activity decreased 1 day after NIR-PIT ($*p = 0.0086 < 0.01$, $**p = 0.0162 < 0.05$ vs. APC, Tukey's test with ANOVA)(Supplementary Fig. S5C). Tumor volume was reduced significantly in the PIT group compared with other control groups ($***p = 0.0141 < 0.05$ vs. APC, Tukey's test with ANOVA)(Supplementary Fig. S5E). No toxicity was observed and body weight (BW) was maintained (Supplementary Fig. S5D). Survival was prolonged significantly in the PIT group ($****p < 0.0001$, Log-rank test and Wilcoxon test) (Supplementary Fig. S5F). Since bioluminescence is more sensitive for cell killing, it was the earliest hallmark of tumor response whereas the tumor volume took the longest to show an effect. Collectively, NIR-PIT caused significant tumor reduction and prolonged survival in an *in vivo* flank tumor model.

3.4. Characterization of lung metastasis mouse model with fluorescence, BLI, fluorescence thoracoscopy, and CT

To determine the natural history of the lung metastasis mouse model, serial fluorescence imaging, BLI, fluorescence thoracoscopy and CT were performed without treatment. The implanted tumors demonstrated high activity with fluorescence imaging based on IR700, IR800 and GFP, but also high activity on bioluminescence, all of which co-localized with each other (Fig. 3A)(flank tumor model: see Supplementary Fig. S6). Fluorescence thoracoscopy indicated that multiple lung metastases were scattered in the lung and there were visible differences in IR700 fluorescence between tumors and intrathoracic organs (Fig. 3A, left and Supplementary Videos S5, S6). CT confirmed the presence of pulmonary nodules (Fig. 3B). These data suggest that this lung metastasis mouse model, which uses 3T3/HER2-luc-GFP cells, was successfully established and that intravenously injected APCs could reach the metastases, providing sufficient uptake to differentiate normal from abnormal regions during thoracoscopy.

3.5. *In vivo* NIR-PIT effect on lung metastasis mouse model

To monitor targeted tumor killing induced by single-shot NIR-PIT, bioluminescence was used (Fig. 4A). The NIR-PIT treatment resulted in decreases in bioluminescence and fluorescence (Fig. 4B and Supplementary Fig. S7B). RLU of tumor in other groups showed a gradual increase due to rapid tumor growth. In contrast, luciferase activity decreased 1 day after NIR-PIT (* $p = 0.0117 < 0.05$ vs. APC, Tukey's test with ANOVA)(Fig. 4C). Despite the effectiveness of NIR-PIT measured by bioluminescence, the survival wasn't significantly prolonged in the single shot NIR-PIT group (** $P = 0.2674$, ns, Log-rank test and Wilcoxon test)(Fig. 4D). These data indicated that single-shot NIR-PIT, while demonstrating a significant effect, did not prolong survival since 3T3/HER2-luc-GFP tumors in the lung grew very rapidly after the therapy. The BW ratio showed the feasibility of NIR-PIT, and no remarkable acute toxicity was observed (Fig. 4E). Taken together, single-shot NIR-PIT caused significant tumor reduction in an *in vivo* lung metastasis model without affecting survival.

3.6. *In vivo* multiple NIR-PIT effect on lung metastasis mouse model

To investigate whether multiple NIR-PIT exposures could prolong survival, multiple NIR-PIT was performed after intravenous injection of the APC (Fig. 5A). The NIR-PIT treatment effect was confirmed with bioluminescence and fluorescence both of which decreased (Fig. 5B and Supplementary Fig. S7C). Multiple NIR-PIT induced lower RLU than other groups (* $p = 0.0491 < 0.05$, ** $p = 0.0376 < 0.05$ vs. APC, Tukey's test with ANOVA)(Fig. 5C). Survival was prolonged significantly in the multiple PIT group (Fig. 5D)(*** $p < 0.0001$, Log-rank test and Wilcoxon test). During multiple PIT, no significant loss of weight was observed, yet the BW was maintained compared to other groups (**** $p = 0.0146 < 0.05$ vs. APC, Tukey's test with ANOVA)(Fig. 5E). This maintenance of BW was observed even while mice in the non-PIT-treated group lost weight (Supplementary Fig. S8). Collectively, multiple NIR-PIT caused significant anti-tumor effects and prolonged the survival.

3.7. *In vivo* NIR-PIT effect assessed with GFP fluorescence imaging

Finally, to assess the effect of NIR-PIT on 3T3/HER2-luc-GFP tumors *in vivo*, GFP fluorescence imaging was performed for the flank model and lung metastasis model (Fig. 6A). With the flank model, the decrease of both GFP/IR700 fluorescence and bioluminescence was detected 1 day after NIR-PIT, which was confirmed by *ex vivo* tumor analysis (Supplementary Fig. S9). In the lung metastasis model, decrease of both the bioluminescence and IR700 fluorescence was observed in the flank model (Fig. 6B left). *Ex vivo* BLI confirmed the decrease of luciferase activity. *Ex vivo* GFP fluorescence imaging confirmed the result of BLI (Fig. 6B right). Moreover, no apparent damage to the normal lung by NIR-PIT was observed. The changes seen with single shot NIR-PIT were confirmed by histological analysis (Fig. 6C), which revealed that well-packed metastatic tumor nodules containing scattered clusters of necrotic cells were surrounded by layers of necrotic tissue after NIR-PIT with 100 J/cm^2 , while the alveolar structures were intact, suggesting that normal lung was not affected. (Fig. 4).

4. DISCUSSION

In this study, we demonstrate that NIR-PIT can successfully treat lung metastases in a mouse model with acceptable morbidity [21]. In order to non-invasively evaluate the therapeutic effects of NIR-PIT *in vivo*, we used fluorescence and bioluminescence reporters [22,23]. GFP fluorescence imaging enabled depiction of the time course of response to NIR-PIT [15]. The high level of GFP tumor fluorescence enabled imaging and quantification of tumor growth and dissemination without using additional injections of contrast agents or substrates [24]. By employing GFP expressing cells, antitumor effects induced by NIR-PIT could be clearly monitored [15]. BLI was also used to monitor NIR-PIT [12,14,25]. BLI requires both oxygen and ATP to actively transport the substrate luciferin, and subsequently catalyze the photochemical reaction [26,27]. Since NIR-PIT induces necrotic cell death which leads to the release of ATP, BLI is also a suitable early biomarker for NIR-PIT effects. We also used fluorescence thoracoscopy to confirm the model [28]. The development of a mini-endoscope mimicking thoracoscopy permits fluorescence imaging for detecting disseminated tumors using a highly sensitive EM-CCD camera. This is the likely method by which NIR-PIT could be made practical in humans. Both real-time multiple color capability and direct access to the disseminated tumors, resulted in higher resolution imaging than could be achieved transcutaneously. From the photophysical point of view, the endoscope can minimize light scattering and absorbance caused by overlapping tissue, resulting in more accurate light-targeting of the lesion [28]. We envision that for humans, NIR-light could be administered via thoracoscopy directed at lesions as guided by pre-operative computed tomography. On all techniques, NIR PIT demonstrated successful treatment of the pulmonary metastases with minimal side effects.

We used a lung metastasis model that utilized tail vein cell injection [20]. Surgical orthotopic injection (SOI) is another way of creating this model [29]. Various advantages and disadvantages exist among these models. SOI requires highly trained surgical skills and invasive methods and results in a high perioperative mortality rate, however it is thought to be a more physiological model. The cell-injection model requires less advanced skills, and is less invasive, and shows a very low mortality rate. Due to these considerations and the recent demands of animal wellness, we chose a cell-injection model. Moreover, in this study, we monitored live cell-injection lung metastasis by non-invasive BLI, to quantify the efficacy. It is difficult to evaluate with fluorescence live imaging because mice die due to pneumothorax. To prevent this pneumothorax, a ventilation system would have been necessary, which again requires advanced skills, invasive procedures, and expensive devices [30]. Recognizing that all animal models have flaws, we chose the least invasive methods. Thus, we accessed live cell-injection lung metastasis models by non-invasive BLI and then confirmed our findings with *ex vivo* fluorescence imaging. This data simply provides “proof of concept” that will require *in vivo* testing in humans for validation.

Unlike other therapies used to treat pulmonary metastases, NIR-PIT resulted in no damage to the normal lung, based on both *ex vivo* and histological analysis. This is of potential importance to patients with pulmonary metastases as the normal pulmonary reserve may already be compromised by the metastases and post obstructive collapse. Although not

extensively studied here, treated mice maintained their body weight and did not exhibit the cachexia seen in control animals.

Pulmonary metastases generally signal systemic disease and therefore, local therapies of lung metastases should not be construed as curative [7]. However, because pulmonary metastases can result in debilitating symptoms and lead to death, the ability to locally control them with minimally invasive procedures is appealing and may lead to longer survival with higher quality of life. Therefore, NIR-PIT is not proposed as curative but rather as palliative for pulmonary metastases.

It was possible to treat mice transcutaneously with light. This is clearly not feasible in humans. However, thoracoscopically delivered light could enable rapid, non-surgical treatment of pulmonary metastases. Post mortem thoracoscopy in the mouse model revealed excellent visualization of the lesions. Were the thoracoscope to be equipped with a NIR laser, it could be used as a method of delivering NIR-PIT. Such a procedure could be repeated should the metastases recur or new ones appear. This would be a viable alternative to repeated surgery, chemotherapy and focal radiation.

There are several limitations to this study. First, not all pulmonary metastases overexpress HER2. However, NIR-PIT has proven effective with multiple antibodies including anti-EGFR, anti-PSMA, anti-Mesothelin, anti-CEA, among others [12,14,15,31]. Therefore, we are optimistic that NIR-PIT could be used with a wide range of cancers. The possibility of mixtures of APCs is also feasible in cases where there are mixed populations of cells [32]. Another limitation is that long-term side effects of NIR-PIT were not determined. Short-term studies of the mice demonstrated no observable toxicity after repeated NIR-PIT. The major reason multiple NIR-PIT was needed in this animal model was that the tumor growth was so rapid. Fortunately, tumor growth in humans is considerably slower [21] and this may allow for fewer doses of APC and NIR-light therapy to be given. One potential side effect of NIR-PIT could be toxicity related to the systemic release of necrotic cell components leading to a “post infarction” syndrome. For this reason, it may not be possible to treat high volume metastases at one setting, but rather this may require several sessions. NIR-PIT alone will not be curative as disease is assumed to be systemic with multiple pulmonary metastases. However, local control of pulmonary disease could be palliative and prolong survival. Some types of cancer, such as sarcomas, preferentially metastasize to the lungs and thus, local control of pulmonary metastases could translate into improved outcomes [33]. Interestingly, NIR-PIT may be successfully combined with chemotherapy as it induces marked increases in vascular permeability enabling the accumulation of nano-sized drugs within the tumor beds of treated cancers. Therefore, a combination therapy with NIR-PIT and adjuvant nano-drug chemotherapy could be a promising treatment for patients with lung metastasis [16]. Finally, there may be limits to the size of individual lesions that can be treated with NIR-PIT as even NIR-light cannot penetrate large lesions >3cm in diameter.

In conclusion, this study demonstrates that NIR-PIT may be an alternative method for local treatment of pulmonary metastases. Naturally, translation to humans will require direct thoracoscopy to apply light, but this promises to be a new method of treating pulmonary lesions while preserving normal pulmonary reserve.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PIT	photoimmunotherapy
EGFR	epidermal growth factor receptor
tra	trastuzumab
IR700	IRDye700DX
NIR	near-infrared

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Highlights

- Lung metastases are a leading cause of cancer related deaths.
- The lung is the ideal internal organ for light therapy.
- Small diffuse lung metastases are potential targets of NIR-PIT.
- NIR-PIT is a potential new therapy for the local control of lung metastases.

Fig. 1. Evaluation of NIR-PIT effect on *in vitro* 2D culture

(A) 3T3/HER2-luc-GFP cells were incubated with tra-IR700 for 6 hr, and observed with a microscope before and after irradiation of NIR-light (2 J/cm²). Necrotic cell death was observed after exposure to NIR-light (1 hr after PIT). Bar = 50 μm. (B) Membrane damage and necrosis induced by NIR-PIT was measured by dead cell count using PI staining. Cell killing increased in a NIR-light dose-dependent manner. (n = 4, *p < 0.0001, vs. untreated control, Student's t test) (C) Luciferase activity in 3T3/HER2-luc-GFP cells was measured as relative light unit (RLU), which also decreased in a NIR-light dose-dependent manner. (n = 4, **p < 0.0001, vs. untreated control, Student's t test) (D) Bioluminescence imaging (BLI) of a 10 cm dish demonstrated that luciferase activity in 3T3/HER2-luc-GFP cells decreased in a NIR-light dose-dependent manner. (E) 3T3/HER2-luc-GFP cells were incubated with tra-IR700 for 6 hr and irradiated with NIR-light (0.5 J/cm²). GFP-fluorescence intensity decreased in dead cells (*) stained by PI but was unchanged in living cells at 1 hr after NIR-PIT. Bar = 200 μm. (F) GFP fluorescence intensity decreased after NIR-PIT in a NIR-light dose-dependent manner as measured by FACS. (n = 4, ***p < 0.0001, vs. untreated control, Student's t test) (G) Diminishing GFP-fluorescence intensity at 1 hr after PIT occurred in a manner dependent on the light dose (total pixel of GFP fluorescence in the same)(n = 12 fields)(*p < 0.0001, vs. untreated control, Student's t test).

Fig. 2. Characterization and evaluation of NIR-PIT *in vitro* 3D culture

(A) 3D spheroid at day 7 after 6hr incubation with tra-IR700, before and after irradiation of NIR-light (0.5 J/cm^2). Necrotic cell death was observed in the layer of IR700 1hr after NIR-light. Bar = 200 μm . (B) Bioluminescence in 3T3/HER2-luc-GFP 3D spheroids was measured as RLU, and decreased in a NIR-light dose-dependent manner. ($n = 5$, $*p < 0.0001$, vs. untreated control, Student's t test) (C) The PIT regimen incorporating repeated NIR-light exposures is shown. (D) Day 7 3T3/HER2-luc-GFP 3D spheroids were divided into 4 groups as shown. Bar = 200 μm . (E) BLI of each group demonstrated that luciferase activity decreased after repeated PIT. Bar = 5 mm. (F) Luciferase activity in 3D spheroids gradually decreased after repeated NIR-PIT leading to complete killing of cells in the spheroid ($n = 8$). ($**p = 0.0075 < 0.01$, $***p < 0.0001$ vs APC, Tukey's test with ANOVA).

Fig. 3. Characterization the lung metastasis tumor model

(A) *In vivo* BLI and fluorescence (GFP/IR700/IR800) imaging of 3T3/HER2-luc-GFP lung metastasis model are shown and indicated colocalization of signal. To avoid auto-fluorescence, tra-IR800 was used as well as tra-IR700 for imaging. Fluorescence thoracoscopy was indicated lung metastasis tumors had both IR700 and GFP fluorescence signals. APCs were intravenously injected 1 day before the imaging. (B) CT image confirmed the multiple metastasis inside lung.

Fig. 4. Evaluation of NIR-PIT effects on lung metastasis model

(A) The regimen of NIR-PIT is shown. Images were obtained at each time point as indicated. (B) *In vivo* BLI and IR700 fluorescence imaging of lung metastasis model. Prior to treatment mice exhibiting approximately the same luciferase activity in the chest were selected. (C) Quantitative RLU in the lung metastasis model showed a significant decrease in the PIT ($n = 8$ mice in each group) ($*p = 0.0117 < 0.05$, vs APC, Tukey's test with ANOVA). (D) NIR-PIT led no significant prolonged survival in 3T3/HER2-luc-GFP lung metastasis model. ($n = 8$ mice in each group) ($**p = 0.2674$, ns, Long-rank test and Wilcoxon test). (E) BW ratio of tumor bearing mice in response to NIR-PIT. No significant difference was detected among the groups ($n = 8$).

Fig. 5. Evaluation of multiple NIR-PIT effects on lung metastasis model

(A) The regimen of multiple NIR-PIT is indicated. Images were obtained at each time point as indicated. (B) *In vivo* BLI and fluorescence imaging of lung metastasis model in response to multiple NIR-PIT. (C) Quantitative RLU in the lung metastasis model showed a significant decrease in the multiple PIT ($n = 8$ mice in each group) ($*p = 0.0491 < 0.05$, $**p = 0.0376 < 0.05$ vs. APC, Tukey's test with ANOVA). (D) Multiple NIR-PIT significantly prolonged survival in lung metastasis model. ($n = 9$ mice in each group) ($***p < 0.0001$, Long-rank test and Wilcoxon test). (E) BW ratio of tumor bearing mice in response to multiple NIR-PIT. No significant difference was detected among the groups ($n = 8$ mice in each group) ($****p = 0.0146 < 0.05$ vs. APC, Tukey's test with ANOVA).

Fig. 6. NIR-PIT effects on flank or lung metastasis model by GFP fluorescence imaging
 (A) The regimen of NIR-PIT is shown. Images were obtained at each time point as indicated. (B) *In vivo* BLI and *ex vivo* fluorescence imaging of lung metastasis model in response to NIR-PIT. Both bioluminescence and IR700 fluorescence intensity was decreased by NIR-PIT. GFP fluorescence of *ex vivo* indicated the decrease with NIR-PIT compared to APC mouse. (C) Histological specimens of lung with metastatic tumors, which were treated with PIT at 0, 100 J/cm^2 , are shown. All specimens are stained with Hematoxylin and Eosin. Well-packed metastatic tumor nodules containing scattered clusters of necrotic cells surrounded by layers of necrotic tissue were shown after PIT with 100 J/cm^2 , while the intact structure of alveolar was sustained. Necrotic tissues especially located around tumor nodules were not found without PIT. Bar indicates 50 μm .