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Research paper

Nanoparticle targeted folate receptor 1-enhanced photodynamic therapy for lung cancer

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ABSTRACT

Objective: Despite modest improvements, the prognosis of lung cancer patients has still remained poor and new treatment are urgently needed. Photodynamic therapy (PDT), the use of light-activated compounds (photosensitizers) is a treatment option but its use has been restricted to central airway lesions. Here, we report the use of novel porphyrin-lipid nanoparticles (porphysomes) targeted to folate receptor 1 (FOLR1) to enhance the efficacy and specificity of PDT that may translate into a minimally-invasive intervention for peripheral lung cancer and metastatic lymph nodes of advanced lung cancer.

Materials and methods: The frequency of FOLR1 expression in primary lung cancer and metastatic lymph nodes was first analyzed by human tissue samples from surgery and endobronchial ultrasonography-guided transbronchial needle aspiration (EBUS-TBNA). Confocal fluorescence microscopy was then used to confirm the cellular uptake and fluorescence activation in lung cancer cells, and the photocytotoxicity was evaluated using a cell viability assay. *In vivo* fluorescence activation and quantification of uptake were investigated in mouse lung orthotopic tumor models, followed by the evaluation of *in vivo* PDT efficacy.

Results: FOLR1 was highly expressed in metastatic lymph node samples from patients with advanced lung cancer and was mainly expressed in lung adenocarcinomas in primary lung cancer. Expression of FOLR1 in lung cancer cell lines corresponded with the intracellular uptake of folate-porphysomes *in vitro*. When irradiated with a 671 nm laser at a dose of 10 J/cm^2, folate-porphysomes showed marked therapeutic efficacy compared with untargeted porphysomes (28% *vs.* 83% and 24% *vs.* 99% cell viability in A549 and SBC5 lung cancer cells, respectively). Systemically-administered folate-porphysomes accumulated in lung tumors with significantly enhanced disease-to-normal tissue contrast. Folate-porphysomes mediated PDT successfully inhibited tumor cell proliferation and activated tumor cell apoptosis.

Conclusion: Folate-porphysome based PDT shows promise in selectively ablating lung cancer based on FOLR1 expression in these preclinical models.

1. Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide [1]. Currently, more lung cancer patients are being diagnosed at an earlier stage due to improved diagnostic imaging [2]. Although surgical resection has always been considered the standard treatment for patients with early-stage NSCLC, some patients are excluded due to significant co-morbidities [3]. Hence non-surgical

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treatment options have evolved significantly over the past decade, including stereotactic body radiotherapy (SBRT) and radiofrequency ablation (RFA). However, SBRT has imperfect local-regional control and cannot be used for local recurrent disease included in the primary radiation field [4]. Percutaneous RFA has low accessibility to many cancers and is inferior in outcome to surgery [5]. Photodynamic therapy, PDT, has been performed as an alternative treatment in lung cancer [6–8]. Successful PDT ablation with minimal invasiveness is highly dependent on precise light delivery and tumor-specific accumulation of photosensitizers. However, access for light delivery is restricted by the location of the tumor, which limits the treatment to carcinoma *in-situ* and palliation of the advanced obstructing lung cancer in the central airway [9,10]. Technical advances in minimally-invasive PDT treatment promise a shift towards using PDT as first treatment choice for early-stage lung cancer.

Radial probe endobronchial ultrasound (EBUS) has been considered a useful technique in addition to conventional bronchoscopy to improve the efficacy of PDT in patients with centrally-located early-stage lung cancer [6,11]. We have previously integrated an ultra-thin optical fiber into TBNA needle and demonstrated transbronchial PDT under navigation bronchoscopy [12,13]. Besides the precise light delivery, tumor targeting of the PDT photosensitizer adds specificity to the treatment. Different strategies have been explored for this purpose to achieve tumor-preferential accumulation of photosensitizer and the tumor-specific activatable photosensitizers. For the latter, after initial accumulation in the tumor the photosensitizers are 'photodynamically inactive' due to photophysical suppression (quenching) of the cytotoxic singlet-oxygen generation. However photoactivity is regained through mechanisms such as enzymatic-, nucleic acid- [14,15] or microenvironmental activation (pH, hydrophobicity, *etc.*) [16,17]. This enables a degree of selectivity to kill only the target cancer cells.

In this study, we introduce an activatable photosensitizer for tumor-specific PDT, named folate receptor-targeted porphysomes. Porphysomes are all-organic bilayer liposome-like nanoparticles that self-assemble from porphyrin-lipid conjugates. They have intrinsic multimodal capabilities for both imaging and therapy [18,19]. Because of the extremely high porphyrin packing density (> 80,000 per particle) in the phospholipid bilayer, both fluorescence and singlet oxygen generation are highly quenched (> 99%) in intact porphyrins [19] but these are restored once the nanoparticles are internalized into the cancer cells. Recently, based on the finding that folate receptor 1 (FOLR1) is overexpressed in various cancers, we have integrated folic acid into the nanostructure to generate 'folate-porphysomes' (FP) to enhance tumor targeting that also re-activates the PDT efficacy [20]. Hence, FP-PDT has emerged as a promising new strategy to treat lung cancers with over-expression of FOLR1. Based on these findings, the aim of the present study is to evaluate FPs in preclinical lung cancer models as a potential minimally-invasive treatment for lung cancer.

We firstly examined the expression of FOLR1 on multiple lung cancer cell lines, and evaluated the specificity of FPs in targeting cell lines with overexpressed FOLR1. The therapeutic efficacy of FP-PDT was then evaluated both *in vitro* using lung cancer cell lines and *in vivo* on the mice bearing subcutaneous lung tumors. Finally, the specificity of FP-PDT was evaluated in A549 lung tumors grown orthotopically in mice. The quantitative biodistribution of FPs was also analyzed in the orthotopic model to assess the potential of future translational studies in treating peripheral lung cancer.

2. Materials and methods

2.1. Lung cancer tissue samples and cell lines

Seventeen samples taken from metastatic lymph nodes from lung cancers were obtained via EBUS-TBNA from patients at Toronto General Hospital and a total of 333 NSCLC samples for tissue microarray (TMA) were obtained at Hokkaido University and affiliated hospitals with informed consents [21–23] (Table 1). All specimens were fixed in formalin and embedded in paraffin wax. Representative blocks were selected (based primarily on greatest dimensions of each tumor), and serial 4 μm-thick sections were examined by immunohistochemistry. Histological diagnosis was based on the World Health Organization Classification (4th Ed.) [24]. All tumors were staged according to the pathological tumor/node/metastasis (pTNM) classification of the International Union against Cancer (7th edition) [25].

Table 1
Immunopositivity of folate receptor 1 (FOLR1) protein in lung cancers (n = 333)

Histology	FOLR1-negative	FOLR1-positive	FOLR1 positive rate (%)
Adenocarcinoma (ADC)	125	91	42.1
Squamous Cell Carcinoma (SqCC)	82	5	5.7
Large Cell Carcinoma (LCC)	16	3	15.8
Adeno-Squamous Carcinoma (ASC)	2	1	33.3
Small Cell Lung Cancer (SCLC)	4	0	0.0
Mucoepidermoid Carcinoma	0	2	100.0
Carcinosarcoma	1	0	0.0
Atypical Carcinoid	1	0	0.0
Total	231	102	30.6%

A series of human lung cancer cell lines were used: ADC DFC1024, DFC1032, NCI-H2228, NCI-H1975, NCI-H3255, NCI-H4006, NCI-H1650, NCI-H1819, NCI-H2009, NCI-H2030, NCI-H2122, NCI-H2405, NCI-H1437, A549, HCC827, HCC2279, HCC2935, HCC4011, and HCC4019; lung ASC NCI-H647; lung SqCC MGH7; lung large cell carcinoma (LCC) NCI-H460, H460SM, and NCI-H661. SCLC SBC-5, EBUS-060 and KB cells were kindly provided by Dr. Ming-Sound Tsao (UHN, Toronto). Cells were grown in monolayers in appropriate medium supplemented with 10% FCS and maintained at 37 °C in humidified air with 5% CO_2.

2.2. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

cDNA was synthesized from 2 μg total RNA using a QuantiTect® Reverse Transcription Kit (Qiagen). The primers were designed as follows: for FOLR1, forward primer, 5′-aagtgcgcagtgggagct-3′, and reverse primer 5′-cattgcacagaacagtgggtg-3′; for actin, beta (ACTB), forward primer, 5′-gaaatcgtgcgtgacattaa-3′, and reverse primer, 5′-aaggaaggctggaagagtg-3′. qRT-PCR analysis was performed using LightCycler480® SYBR Green I Master Ready-to-use hot start reaction mix and LightCycler480® system (Roche, South San Francisco, CA, USA). The thermal cycler conditions were 5 min at 95.0 °C for denaturation, 45 cycles at 95 °C for 10 s, 56 °C for 10 s, 72 °C for 10 s for PCR amplification and 1 min at 65 °C for melting. The threshold cycle value was defined as the value when the fluorescence signal increased above the background threshold. PCR reactions were carried out in triplicates.

2.3. FOLR1 immunohistochemistry

FOLR1 immunostaining was performed using an automated IHC platform (Autostainer Plus, DAKO Corp., Carpinteria, CA, USA). Anti-FRA monoclonal antibody (Novocastra™ Liquid Mouse Monoclonal Antibody, Folate Receptor Alpha, Leica, Newcastle, UK) was diluted 30-fold using mixed antibody diluent (DAKO: S2022 Antibody Diluent). After incubation with the primary antibody at 4 °C overnight, a polymer-based detection system (EnVision™ + Dual Link #K4063, DAKO) was used with 3′, 3-Diaminobenzidine (DAB) as the chromogen. Positive controls included a sample of kidney, while normal lung samples were used as negative controls. Slides were dehydrated and