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Research Articles:

Personalized siRNA-nanoparticle Systemic Therapy using Metastatic Lymph Node Specimens Obtained with EBUS-TBNA in Lung Cancer

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Abstract

Inhibiting specific gene expression with short interfering RNA (siRNA) provides a new therapeutic strategy to tackle many diseases at the molecular level. Recent strategies called high-density lipoprotein (HDL)-mimicking peptide-phospholipid nanoscaffold (HPPS) nanoparticles have been used to induce siRNAs-targeted delivery to scavenger receptor class B type I receptor (SCARB1) expressing cancer cells with high efficiency. Here, eight ideal therapeutic target genes were identified for advanced lung cancer throughout the screenings using endobronchial ultrasonography-guided transbronchial needle aspiration (EBUS-TBNA) and the establishment of a personalized siRNA-nanoparticle therapy. The relevance of these genes were evaluated by means of siRNA experiments in cancer cell growth. To establish a therapeutic model, kinesin family member-11 (KIF11) was selected as a target gene. A total of 356 lung cancers were analyzed immunohistochemically for its clinicopathologic significance. The anti-tumor effect of HPPS-conjugated siRNA was evaluated *in vivo* using xenograft tumor models. Inhibition of gene expression for these targets effectively suppressed lung cancer cell growth. SCARB1 was highly expressed in a subset of tumors from the lung large-cell carcinoma (LCC) and small-cell lung cancer (SCLC) patients. High-level KIF11 expression was identified as an independent prognostic factor in LCC and squamous cell carcinoma (SqCC) patients. Finally, a conjugate of siRNA against KIF11 and HPPS nanoparticles induced downregulation of KIF11 expression and mediated dramatic inhibition of tumor growth *in vivo*.

Implications:

This approach showed delivering personalized cancer-specific siRNAs via the

appropriate nanocarrier may be a novel therapeutic option for patients with advanced lung cancer.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (1). In particular, the 5-year survival for patients with regional lymph node (LN) spread shows very poor prognosis (2). The analysis of metastatic LN samples from advanced lung cancer patients can shed some light on the underlying mechanisms of this disease. The use of minimally invasive techniques like endobronchial ultrasound guided transbronchial needle aspiration (EBUS-TBNA) represents an important tool for the collection of metastatic LN samples (3-5). In an effort to identify relevant molecular targets for diagnosis and/or treatment of lung cancer, we have analyzed expression profiles of our previous performed microarray using EBUS-TBNA samples (5) and various types of database (6-9). Throughout these screenings, confirmatory quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was performed against 122 possible candidate genes using samples obtained by EBUS-TBNA.

One of the greatest benefits of nanotechnological applications in medicine is its potential to enhance delivery and activity of bioactive and imaging agents into relevant cell types in vivo in a manner that minimizes toxicity to patients through enhanced target specificity (10). Small interfering RNA (siRNA) is a revolutionary tool for gene therapy and gene function analysis. Despite its promise, a major challenge in siRNA therapy is the transport of siRNAs to the cytoplasm of targeted cells safely and efficiently, since the naked siRNA will be dissolved rapidly post-intravenous injection elimination due to kidney filtration and serum degradation (11). An ideal delivery system should be able to encapsulate and protect the siRNA cargo from serum proteins, exhibit target tissue and cell specificity, penetrate the cell membrane, and

release its cargo in the desired intracellular compartment. siRNA delivery systems via nanoparticles can promote efficient intracellular delivery. However, despite showing promise in many preclinical studies and potential in some clinical trials, siRNA still has poor cytosolic delivery efficiency. Thus novel delivery strategies, from carrier design to formulation, are needed in order to overcome the transport barriers (10). A high-density lipoprotein (HDL)-mimicking peptide–phospholipid nanoscaffold (HPPS) nanoparticle composed of the cholesteryl oleate, phospholipid and an apolipoprotein A-I (ApoA-1) mimetic peptide has recently been developed (11-15). This nanoparticle has a favorable monodisperse size (<30 nm), long circulation half-life (15 hours), excellent biocompatibility as confirmed by its systemic tolerability in mice, and is capable of delivering cholesterol-modified siRNA (chol-siRNA) directly into the cytosol of the target cells in vitro via the scavenger receptor class B type I receptor (SCARB1; alias SR-B1) (11, 13, 14). The SCARB1 targeting of HPPS plays an important role in efficient delivery of siRNA because the direct cytosolic delivery allows siRNAs to reach the action site of the cytosol, thus bypassing endosomal trafficking, which normally induce siRNA degradation in lysosomes (16). We have previously demonstrated that targeting siRNA delivery with the HPPS nanoparticle using a fluorescent dye labelled model siRNA in both cells study (16) and animal model (18). After systemic administration in SCARB1 overexpressed KB tumor-bearing mice, we demonstrated that HPPS prolongs siRNA circulation in bloodstream, improves its biodistribution, and facilitates KB tumor uptake (18). This study is an extension of the application study of using the HPPS-siRNA platform for lung cancer treatment combined with selection of therapeutic genes by analyzing specific gene expression pattern using EBUS-TBNA sample.

Here, we report the successful screening therapeutic target genes for the treatment of advanced lung cancer using LN samples from EBUS-TBNA. We examined the effect of targeting on of these genes by means of systemic delivery of siRNA into tumors using HPPS nanoparticles *in vivo*. This treatment approach resulted in a significant targeted siRNA-mediated tumor growth inhibition, therefore demonstrating the utility of EBUS-TBNA sampling as a tool for personalized medicine, and the efficacy of patient-specific siRNA therapeutics via specific a nanocarrier for the treatment of patients with advanced lung cancer.

Materials and methods

Lung cancer and normal tissue samples

EBUS-TBNA samples were obtained via from patients with written informed consent at Toronto General Hospital (Toronto, Canada) (study number: 11-0109-CE). A total of 353 NSCLC samples for immunostaining on tissue microarray (TMA) and additional statistical analysis were obtained from patients who underwent surgery at Hokkaido University and its affiliated hospitals with informed consent (16-18). Histological diagnoses were based on the 4rd Edition of World Health Organization Classification (19). All tumors were staged according to the pathological tumor/node/metastasis (pTNM) classification of the International Union against Cancer (7th Edition) (20). Total RNA of 21 normal human tissues (Human Total RNA Master Panel II) were purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA).

EBUS-TBNA sample preparation

EBUS-TBNA was performed in the usual manner. Briefly, a dedicated 22-gauge

needle was used (NA-201SX-4022; Olympus, Tokyo, Japan). After confirmation of adequate sampling for cytological evaluation, an additional pass was performed for the preservation of RNA. The aspirate was mixed with Allprotect Tissue Reagent® (Qiagen, Valencia, CA, USA) following the manufacturer's instructions and stored at -80°C. The QIAzol Lysis Reagent (Qiagen) and one 5-mm stainless steel Bead (Qiagen) were added before homogenizing with a TissueLyser Adapter Set (Qiagen) for 2 minutes at 20 Hz. Total RNA was then purified using a miRNeasy Mini Kit (Qiagen). The amount and purity were measured using a spectrophotometer (NanoDrop; Thermo Scientific, Wilmington, DE, USA).

Lung cancer cell lines

The human lung cancer cell lines used in this study were as follows: lung ADC DFC1024, DFC1032, NCI-H2228, NCI-H1975, NCI-H3255, NCI-H4006, NCI-H1650, NCI-H1819, NCI-H2009, NCI-H2030, NCI-H2122, NCI-H23, NCI-H2405, NCI-H1437, A549, HCC827, and HCC2935; lung adeno-squamous carcinoma (ASC) NCI-H647; lung SqCC H226, H2170, HCC15, and MGH7; lung large cell carcinoma (LCC) NCI-H460, and NCI-H661; and SCLC H69, H889, SBC-1, H69AR, H1688, SBC3, and SBC-5. NCI-H460SM, that has higher invasive potential activity *in vitro* than parental NCI-H460, was kindly given by Dr. Ming-Sound Tsao. All cancer cells were grown in monolayers in appropriate medium supplemented with 10% FCS and were maintained at 37°C in atmospheres of humidified air with 5% CO₂.

RNAi and cell viability assay

All siRNA oligonucleotide sequences for this study were purchased from Qiagen.

Negative Control siRNA and AllStar Negative Control siRNA (Qiagen) were used as the negative control (NC-siRNAs-#1, -#2). siRNAs with a final concentration of 5-10 nM were incubated with HiPerFect® Transfection Reagent (Qiagen) according to the manufacturer's instructions. The CellTiter96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used for the evaluation of the number of viable cells, and measured using a microplate spectrophotometer (µQuant; Bio-Tek inc., Winooski, VT, USA). Each experiment was performed in triplicates.

The primer sequences and quantitative RT-PCR analysis

The primers were designed as follows: for KIF11, forward primer, 5'-acagcctgagctgttaatgatg-3', and reverse primer, 5'-gatggctcttgacttagagggtc-3'; for KIF23, forward primer, 5'-tggttcctacattcagaaatgaga-3', and reverse primer, 5'-cgttctgatcaggttgaaagagta-3'; for NUF2, forward primer, 5'-gagaaactgaagtcaggagaaat-3', and reverse primer, 5'-ctgatacttcattcgcttcaac-3'; for CDCA5, forward primer, 5'-cgccagagacttggaatgt-3', and reverse primer, 5'-gtttctgtttctcgggtggt-3'; for CASC5, forward primer, 5'-cagcctattatccatctgtacca-3', and reverse primer, 5'-cagtggcactttagatagaatgg-3'; for PLK1, forward primer, 5'-cccctcacagtcctcaataa-3', and reverse primer, 5'-tgtccgaatagtccaccc-3'; for MAGE-A2,A2B, forward primer, 5'-gggacaggctgacaagtagg-3', and reverse primer 5'-ttgcagtgtgactcctctg-3'; for NDC80, forward primer, 5'-actatccaaaagctccatgta-3', and reverse primer 5'-atcaaataaaggtgagctttct-3'; for SCARB1, forward primer, 5'-gcctaaactgacatcatcctatg-3', and reverse primer 5'-attccagtagaaaagggtcacag-3'; for actin, beta (ACTB), forward primer, 5'-gaaatcgctgcgtgacattaa-3', and reverse primer, 5'-aaggaaggctggaagagtg-3'; for glyceraldehyde-3-phosphate dehydrogenase

(GAPDH), forward primer, 5'- tgcaccaccaactgcttagc-3', and reverse primer, 5'-ggcatggactgtggcatgag-3'. The thermal cycler conditions were as follows: 5 min at 95.0 °C for denaturation, 45 cycles at 95°C for 10 s, 56°C for 20 s, and 72°C for 10-13 s for PCR amplification, and 1 min at 65°C for melting. The threshold cycle value was defined as the value obtained in the PCR cycle when the fluorescence signal increased above the background threshold. The fold-change of each gene in different cells or tissues were calculated using standard delta-delta-Ct method. PCR reactions were carried out in duplicates.

qRT-PCR analysis and western blotting

The cDNA was synthesized using QuantiTect® Reverse Transcription Kit (Qiagen). qRT-PCR analysis was performed using LightCycler480® SYBR Green I Master and LightCycler480® system (Roche, South San Francisco, CA, USA). For western blot analysis, cell lysates were prepared with RIPA buffer plus complete protease inhibitors (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined by BCA assay (Pierce Biotechnology, Rockford, IL, USA) and immunoblotted using antibodies specific for SCARB1 (Anti-Scavenging Receptor SR-B1 antibody: EP1556Y, 1 : 1000, Abcam Inc. Cambridge, MA, USA) and KIF11 (Eg5 Antibody-10C7/Eg5: sc-53691, 1 : 1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunoreactive proteins were detected using Goat anti-mouse horseradish peroxidase-conjugated secondary antibody (GenScript, Piscataway, NJ, USA) and Clarity Western ECL (Bio-Rad Laboratories Ltd., Ontario, Canada). The membranes were stripped and immunoblotted with a mouse monoclonal antibody against b-actin (Sigma, 1: 5000). Imaging was carried out using a Gel Logic 2200 Imaging System

(Kodak, Rochester, NY, USA).

Tissue microarray construction and immunohistochemistry

Tissue areas for sampling were selected based on visual alignment with the corresponding hematoxylin and eosin (HE)-stained sections on slides. A core (diameter, 2 mm; height, 3-5 mm) taken from each donor-tumor block was placed into a recipient block using a tissue microprocessor (Azumaya Medical Instruments, Tokyo, Japan). To confirm the TMA quality, AE1/AE3 common cytokeratin and rabbit normal IgG were used as a positive and negative control, respectively (Supplementary Fig. S1). KIF11 immunostaining were performed using an automated IHC platform (Autostainer Plus, DAKO Corporation, Carpinteria, CA). Antigen retrieval was performed in pH 9.0 for 20 mins. EnVision™+ Dual Link (K4063, DAKO) was used for detection, with post-primary incubation for 60 mins at room temperature (RT). Anti-KIF11 polyclonal antibody (GTX109054; GeneTex, Inc, Irvine, CA, 1/1,500) was diluted using mixed antibody diluent (DAKO: S2022 Antibody Diluent). A polymer-based detection system (EnVision™+ Dual Link #K4063, DAKO) was used with 3', 3-Diaminobenzidine (DAB) as the chromogen. The positive control included a sample of testis, and normal lung samples were used as negative controls. For cleaved caspase-3 and Ki-67 staining, heat Induced Epitope Retrieval refers to microwaving tissue sections in a medium for antigen retrieval, a 10 mM citrate buffer at pH 6.0. Endogenous peroxidase blocked with 3% hydrogen peroxide. Sections were drained and incubated accordingly at RT with the appropriate primary antibodies using conditions (Cleaved Caspase-3, Cell Signaling CS#9661, 1/600 Overnight, and Ki67, Novus, NB110-90592, 1/700 , 1 h) previously optimized. This was followed with a biotin

labeled anti-mouse secondary (Vector labs) for 30 mins and horseradish peroxidase-conjugated ultrastreptavidin labeling reagent (ID labs.) for 30 mins. After washing well in TBS, color development was done with freshly prepared DAB (Vector Labs Cat# SK4105). Slides were dehydrated and placed on coverslips. For TUNEL staining, paraffin-embedded tumor and normal mice tissue sections were deparaffinized, rehydrated and pretreated for protease with 1% pepsin (Sigma) in 0.01N HCl at pH 2.0. After block endogenous peroxidase using 3% aqueous hydrogen peroxide and endogenous Biotin activity using avidin/biotin blocking kit (Vector labs), slides were treated with Buffer A for 10 mins. After incubating sections with Biotin-nucleotide cocktail in water bath at 37°C for 30 mins to 1 h. Apply Ultra Streptavidin Horseradish Peroxidase Labeling Reagent (ID Labs Inc.) for 30 mins at RT, staining was developed with freshly prepared DAB (Dako).

Evaluation of immunohistochemical staining

Digital images of IHC-stained slides were obtained using a whole slide scanner (ScanScope CS, Leica Microsystems Inc., ON, Canada). Aperio's annotation software were used to analyze and quantify the expression of KIF11, Ki-67, cleaved caspase-3, and TUNEL staining. For Ki-67 and TUNEL, the 'Percent Positive Nuclei' was calculated by Nuclear v9 with default setting. KIF11 expression was quantified by immunohistochemical scoring, which summated the percentage of area stained at each intensity level multiplied by the weighted intensity (0, 1, 2, or 3) reported in other studies (21). Initially, the weighted intensity of staining was graded as follows; grade 0 (negative), 1+ (weak positive: Intensity Threshold WEAK (upper Limit)=240, (lower Limit)=220), 2+ (moderate positive: MEDIUM (upper)=220, (lower)=180)), and 3+

(strong positive: STRONG (upper)=180, (lower)=0) according to the Positive Pixel Count v9. KIF11 expression was then finally divided into two groups (the threshold leading to the lowest *P* value in log-rank test): low-level KIF11 expression (KIF11-L, with an IHC score <0.25) and high-level KIF11 expression (KIF11-H, IHC score $\geq$ 0.25). KIF11 immunoreactivity was assessed for association with clinicopathologic variables using the χ^2 test for variables.

HPPS nanoparticle preparation and characterization

The HPPS was prepared as previously described (12). Briefly, a mixture of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (3 mmol), cholesterol oleate (0.1 mmol) in chloroform was dried under nitrogen and placed under vacuum for 1 h. PBS buffer (0.1 M, 2 mL, 0.1 M NaCl, pH 7.5) was then added to the dried residue and the mixture was vortexed for 5 min. The turbid emulsion was subsequently sonicated for 60 min at 48°C under nitrogen and AP (0.87 mmol) suspended in PBS buffer (2 mL) was added to the mixture. The turbid emulsion immediately became transparent upon the addition of a short apoA-1 mimetic peptide. The resulting heterogeneous complex peptide-associated lipid nanoparticle solution was stored at 4°C overnight. This complex was then isolated by filtration (0.2 mm) and purified by gel filtration chromatography using the Akta FPLC system (Amersham Biosciences, USA) equipped with a HiLoad 16/60 Superdex 200 pg column. The resulting nanoparticles were eluted with Tris-buffered saline (10 mM Tris-HCl, containing 0.15 M NaCl, 1 mM EDTA, pH 7.5) at a flow rate of 1 mL min⁻¹. The size of the eluted particles was negatively correlated with their respective retention time. FNC particles eluted at a retention time of approximately 60 min and were collected and concentrated to 1 μ M by

using a centrifugal filter device (10,000 MW, Amicon, Millipore). Chol-si-KIF11 (50 μ M) was prepared in RNase-free water. The chol-si-KIF11 and HPPS were mixed at a ratio of 1 to 3 and incubated for 30 min at RT.

***In vivo* RNAi study using xenograft models**

All animal studies were conducted in the Animal Resource Center of the University Health Network in accordance with protocols approved by the Animal Care Committee (AUP 4154). Nude mice (male, 5–7 weeks old) were inoculated with 2×10^6 H460SM cells (in 50 μ l Matrigel®, Corning, Wilmington, NC, USA) subcutaneously in the right flanks. Mice were subjected to start treatment when the tumor volume reached 40–60 mm³ on day 5 after the inoculation. As our previous study demonstrated that neither HPPS nanoparticle alone nor siRNA alone has any therapeutic or side effect *in vivo* (15), we investigated this *in vivo* RNAi study by three treatment groups. Mice were administered intravenously with saline control (group 1; n = 10), HPPS-chol-siRNA-scramble (group 2; n = 6), and HPPS-chol-siRNA-KIF11 (group 3; n = 6), respectively. Each treatment group received tail vein injections of the following dose every other day for a total three doses: saline (200 μ l), HPPS-chol-siRNA-scramble (containing 10 mg/kg of siRNA and 41.12 nmol/ml of HPPS in 200 μ l saline), HPPS-chol-siRNA-KIF11 (containing 10 mg/kg of siRNA and 41.12 nmol/ml of HPPS in 200 μ l saline). All siRNAs were synthesized by Genepharma Co. (Shanghai, China). Cholesterol-conjugated siRNA-KIF11 (chol-siRNA-KIF11) consisted of the sense strand 5'-chol-fCfUfCGGGAAGfCfUGGAAAfUAfUAA-dTsdTs-3' and antisense strand 5'-fUfUAfUAfUfUfUfCfCAGfCfUfUfCfCfCGAG-dTsdT-3'. Cholesterol-conjugated

siRNA bearing a scrambled sequence (chol-siRNA-scramble), consisted of the sense strand 5'-chol-GAfcGfUAAfCGGfCfCAfUAGfUfCfU-dTsdTs-3' and the antisense strand 5'-AGAfCfUafUGGfCfCGfUfUafCGfUfCdTsdT-3' as a control (abbreviations as follows: chol, cholesterol; fC and fU, 2'-deoxy-2'-fluoro cytidine and uridine, respectively; 's', phosphorothioate linkage). Tumor dimensions were measured with Vernier calipers and volumes were calculated as follows: tumor volume (mm³) = width² (mm²) × length (mm)/2 on the first day of treatment (day 0), and day 2, 4, 7, 9, 11, and 15 after treatment. For the confirmation of KIF11 mRNA and protein knockdown, the mice were sacrificed on day 5 after the start of injection, and quickly frozen in liquid nitrogen until used.

Adverse effects of HPPS-chol-si-KIF11

10 mg/kg HPPS-chol-si-KIF11, HPPS-chol-si-Scramble, and saline were administered intravenously in healthy mice (male, 6–8 weeks old) with every other days. After injection, mice behaviors were monitored and the body weight was measured every 2 days. At 6 days post first injection, the mice were sacrificed and their vital organs (the lungs, the heart, the liver, and the kidneys), the adrenal gland, and the testis were excised and stained for histological analysis.

Statistical analysis

The Kaplan-Meier method was used to generate survival curves, and survival differences were analyzed with the log-rank test, based on the status of KIF11 expression. Uni- and multivariate analyses were performed using Cox's proportional hazard regression model. Values of $P < 0.05$ were considered statistically significant. All

analyses were performed using StatView version 5.0 software (SAS Institute, Cary, NC, USA). *In vivo* experiments, tumors treated with KIF11 vs. saline and HPPS-chol-siRNA-scramble were analyzed by paired t-test and repeated measure one-way ANOVA.

Results

Expression of therapeutic candidate genes

To identify the molecular targeted genes for advanced lung cancer, we examined 122 genes by qRT-PCR. These genes are i) overexpressed in the majority of EBUS-TBNA samples, ii) overexpressed at least in one lung cancer cell line for siRNA screening, and iii) expressed only in the testis and less expressed in other human vital organs, which provides further evidence supporting these genes as promising molecular targets (Fig. 1). The expression of candidate genes was significantly higher in samples from patients with advanced lung cancer with higher frequency compared with the expression of normal lung and no malignant (negative) LN tissues (Fig. 2A). qRT-PCR analysis using cDNA panel containing normal human tissues also identified these genes as being expressed only in the testis and thymus, with almost no expression in the other vital organs (Supplementary Fig. S2). We also confirmed high expressions of candidate genes using 21 lung cancer cell lines. This step also allowed identification of relevant cell lines for RNAi experiments (data not shown).

Growth inhibition by specific siRNA

To assess whether candidate genes are essential for growth or survival of lung-cancer cells, we transfected at least 2-4 different types of target-specific siRNAs against 67

genes as well as two different negative control siRNAs into appropriate lung cancer cell lines (Fig. 1). qRT-PCR showed that the mRNA levels transfected with independent siRNAs was significantly decreased (Fig. 2B_upper). The proliferation was evaluated, resulting in the identification of 8 potential therapeutic candidate genes (Supplementary Table S1). Gene knockdown in lung cancer cell lines identified growth inhibition following knockdown of each candidate genes (Fig. 2B_lower), suggesting up-regulation of these genes can be associated with growth or survival of lung cancer cells.

Expression of SCARB1 in lung tumors

To investigate possible nanocarrier HPPS for the delivery of siRNAs, we examined the expression of SCARB1 (natural receptor gene for HDL cholesterol and which allows for targeted delivery by means of HPPS). SCARB1 is highly expressed mainly in lung large cell carcinoma (LCC) or small cell lung cancer (SCLC) (Fig. 3A). We found that SCARB1 is the highest expressed in the H460SM lung LCC cell line (Fig. 3B). SCARB1 is mainly expressed in the adrenal gland, the liver and the other steroidogenic tissues, such as the placenta and testis, as reported previously (Fig. 3C) (22, 23). We also found that *KIF11*, one of therapeutic genes, is highly-expressed in H460SM (Fig. 3D). Therefore, we decided to pursue *KIF11* as a potential therapeutic gene for the treatment of H460SM xenograft model.

Prognostic significance of KIF11 expression as a therapeutic target gene

To determine the clinical relevance of *KIF11* genes, we assessed KIF11 expression using TMA analysis. We categorized KIF11 expression according to the

immunohistochemical (IHC) score described previously (24, 25). The representative staining and its IHC score are shown in Fig. 4A. Positive staining of tumor cells generally showed a cytoplasmic pattern. High-level KIF11 expression (KIF11-H) was observed in 68.0% (Data in detail are shown in Supplementary Table S2) and no significant association between KIF11-H in lung cancers with all histology or adenocarcinoma (ADC) patients and overall 5-year survival ($P=0.7693$ and $P=0.1104$, respectively, Supplementary Fig. S3). However, interestingly, SqCC and LCC patients with KIF11-H revealed significantly shorter overall survival than those with low-level KIF11 expression (KIF11-L) ($P=0.0143$, Fig. 4B). Although there were no significant correlations between KIF11 expression and any other clinicopathological variables (Table 1A), advanced pT-, pN-, pleural invasion status, and KIF11 status were significantly associated with poor prognosis in univariate analysis (Table 1B). KIF11 expression was also identified as an independent prognostic factor of lung SqCC and LCC ($P=0.0185$) by multivariate analysis, as was pN status ($P=0.0173$).

Therapeutic efficacy of HPPS-cho-si-KIF11

We confirmed that *in vitro* delivery of KIF11 targeting chol-siRNA by means of HPPS (HPPS-chol-siRNA-KIF11) enhanced KIF11 knockdown in H460SM cells when compared with HPPS-chol-siRNA-scramble or control groups (Supplementary Fig. S4). *In vivo*, H460SM tumor-bearing mice were treated with HPPS-chol-siRNA-KIF11 ($n = 6$) once every 2 days for 3 times by intravenous injection. The representative cases were shown in Fig. 5A. The actual tumor volume of the HPPS-chol-siRNA-KIF11 treatment group was significantly lower than control groups (KIF11 vs. saline and HPPS-chol-siRNA-scramble, $P=0.008$ and $P=0.012$, respectively by paired t-test, Fig.

5B). The relative changes in tumor volume after the last injection was also significantly reduced in HPPS-chol-siRNA-KIF11 treatment group (Supplementary Fig. S5, $P<0.0001$). After the three-dose regime, the tumors were excised to determine the siRNA knockdown effects. Importantly, upon HPPS-chol-siRNA-KIF11 treatment, both KIF11 mRNA and protein expression were significantly decreased, whereas no significant decrease was observed for the control groups (Figs. 5C, D). The quantified area for positive Ki-67 cells showed a dramatic decrease, and increasing apoptosis, which was confirmed by cleaved caspase-3 and TUNEL positivity in the HPPS-chol-siRNA-KIF11 group compared to control groups (Figs. 5E, F).

Adverse effects of HPPS-chol-si-KIF11

There was no significant pathologic abnormality in histology of vital organs between HPPS-chol-siKIF11 and control groups (Fig. 6A). The HPPS-chol-siRNA-KIF11 treated tumor showed no difference in Ki-67, cleaved caspase-3, and TUNEL positivity compared with control tumor in the adrenal gland as a steroidogenic organ (SCAR1 is highly expressed) and the testis (KIF11 is highly expressed) (Fig. 6B). There was no significant adverse effect of HPPS-chol-si-KIF11 during treatment. Collectively, our studies provide convincing evidence that HPPS is not only able to efficiently deliver siRNA to target tumor *in vivo*, but is also capable of facilitating less side-effect and tailor-made therapy on advanced lung cancer by conjugating patients-specific siRNAs based on the result of analyzing EBUS-TBNA samples.

Discussion

Despite a modest improvement in survival observed after the introduction of

cisplatin-based systemic treatment, the prognosis of advanced lung cancer has remained poor (26). EBUS-TBNA is a minimally invasive procedure with a high yield for LN staging in patients with NSCLC (27). EBUS-TBNA enables molecular analysis of biopsy samples, which is clinically significant as it increases molecular targeted strategies (28, 29). It is possible that the expression of these genes in metastatic LNs may be different from primary tumors due to the tumor heterogeneity and its metastatic potential because of growth factors or other molecules that are differentially expressed (30, 31). Mutation status of metastatic lymph node rather than the one from the primary tumor is a predictive marker of the response to epidermal growth factor receptor (EGFR)- tyrosine kinase inhibitor (TKI) therapy in patients with recurrent NSCLC after surgical resection (32). The differences between molecular features of the primary lesion and its metastases may be responsible for failure of systemic therapy in patients with discordant phenotype between primary and metastatic disease. Therefore, we believe that biopsying specimen from the metastatic site is more essential not only for the diagnosis but also for further investigation into potential genes involved in advanced tumorigenesis. We have demonstrated here that 8 therapeutic genes led to growth inhibition in lung cell lines, in line with the well-characterized roles of these genes in cancer biology. An involvement in lung cancer cell survival has previously been demonstrated for several genes (Supplementary Table S1). Taken together, these observations support our qRT-PCR and RNAi-based screens to identify molecular targets in advanced lung cancer.

KIF11 is a member of the family of kinesin related proteins (33). KIF11 has been implicated in centrosome separation and in the organization of *in vitro* mitotic asters (33). Since a phenotype-based screens identified monastrol as a small

molecule which targets KIF11 and leads to mitotic arrest, many small molecules targeting KIF11 have been developed (34-37). Activation of the spindle checkpoint followed by mitotic slippage initiates apoptosis by activating Bax and caspase-3 in response to KIF11 inhibition (38). In this study, we could confirm that there was significantly increase of cleaved caspase-3 and TUNEL positivity in HPPS-chol-si-KIF11 treated tumor which indicated that it induced apoptosis *in vivo*. Although it has been reported that KIF11 expression might predict a response to antimitotic agents combined with platinum chemotherapy among patients with advanced NSCLC (39), there have been no reports addressing the functional role of KIF11 in lung-cancer prognosis with regards to patients with resectable lung cancer. We demonstrated that KIF11 overexpression is associated with the prognosis in patients with lung SqCC and LCC, suggesting the relevance of KIF11 to malignant potential. Additionally, no- or extremely low-expression were found among normal human tissues including normal lung or vital organs except testis and thymus (Supplementary Fig. S2). Based on these results, specific inhibition of KIF11 may be a promising therapeutic agents for patients with NSCLC, especially in lung SqCC and LCC.

Numerous promising nanoparticles, including liposomes and stable nucleic acid lipid particles (SNALP), have been developed for the delivery of siRNAs to tumors in human (40-42). The clinical trial of siRNA therapy targeting KIF11 and VEGF have proven antitumor activity, including complete regression of liver metastases (41). These data provide proof-of-concept for RNAi therapeutics and form the basis for further development for the novel therapeutics. Efficient delivery of siRNA to tumors *in vivo* not only requires good tumor accumulation, but also requires its efficient

transportation into the cytoplasm of targeted cells. In addition, it is critical to develop efficient as well as safe, biocompatible, and biodegradable delivery systems for the clinical application of siRNA-based cancer therapeutics (10, 43). The SCARB1 targeting of HPPS played an important role in efficient delivery of siRNA. It has been demonstrated that HPPS is a safe nanocarrier evidenced by the absence of adverse effects when 2000 mg/kg of HPPS was administered intravenously (13). Our study further proved that HPPS is an efficient and safe delivery vehicle since no adverse effect was detected during treatment. Although one limitation of our experiment was that the delivery of siRNA via HPPS depended on the expression of SCARB1 of the tumors, our results show that SCARB1 were highly expressed in metastatic LNs from LCC and SCLC tumors. In addition, considering the potential of cancer-specific siRNAs from our expression profiles, we will be able to perform cancer-specific treatment. Our results also indicate that SCARB1 is mainly expressed only in the adrenal gland as well as some steroidal production tissues. However, our screened therapeutic genes have originally almost no expression in these organs, which means that there is almost no knockdown effect against targeted genes by siRNAs even if HPPS-siRNAs are delivered to these organs and the proposed therapy may induce less side-effects as opposed to small molecule inhibitors which will be delivered to any organs via blood stream, that may cause severe side-effect. We could confirm there was no significant morphological change, Ki67 positivity, the cleaved caspase-3, and TUNEL apoptotic index, in the adrenal gland as well as in the testis in which KIF11 is highly expressed. However, from a clinical perspective, the function of hormones such as cortisol, ACTH, and testosterone might be more sensitive to assess adverse effects on these organs. In addition, it is possible that adrenal or gonadal insufficiency would

take longer to develop, therefore, a more comprehensive study evaluating the hormonal safety of the HPPS-KIF11 siRNA should be performed in future studies. We also have additional data demonstrating that two different siRNAs may act synergistically (Supplementary Fig. S6), which means that we will be able to conjugate multiple siRNAs against target genes from our screened gene lists. Finally, this novel therapy will allow the use of multiple HPPS-siRNAs by analyzing customized patient's specific gene expression pattern (Supplementary Fig. S7).

In conclusion, this study revealed that the high level expression of eight candidate genes were observed in majority of metastatic LN tissues from advanced lung cancer using EBUS-TBNA, which are crucial for growth and survival of lung cancer cells by RNAi screening. In particular, a high level of the KIF11 in lung SqCC and LCC is strongly associated with poor survival, suggesting that KIF11 can be a promising molecular target. A conjugate of HPPS nanoparticles and siRNA against KIF11 enhanced inhibition of tumor growth *in vivo*. There was no significant adverse-effect throughout the studies. These results show that delivering siRNA against potential therapeutic target genes via its specific delivery nanoparticle could be the possibility in developing novel strategy for the treatment of advanced lung cancers.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

Author Contributions

Study design: TK, DL HH, WC, JC, GZ, and KY

Sample collection: TK, TN, and MK

Data collection: TK, HU, HW, PP, HPH, KH, TN, and MS

Performed the experiments: TK, DL, HH, and WC

Analyzed the data: TK, DL, HU, and MS

Wrote the paper: TK, DL, WC, and KY

Supervision: KK, YM, GZ, and KY.

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Figure Legends

Figure 1.

Screening of therapeutic candidate genes for lung cancer.

Figure 2.

Expression of the eight therapeutic target genes in lung cancers and effects of short interfering RNAs (siRNAs) against therapeutic target genes on lung cancer cell proliferation *in vitro*. A, Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis in metastatic lymph node samples from advanced lung cancer. The relative expression levels were normalized to the ACTB level in each sample and calculated as the threshold cycle (CT) value in each sample divided by the average CT values in normal lung. Error bar represents the standard error of the mean (SEM) of duplicate. ADC, adenocarcinoma; SqCC, squamous cell carcinoma; LCNEC, large-cell neuroendocrine carcinoma; Small, small-cell lung cancer; Negative, no malignancy lymph node samples. B, (upper) Effects of siRNA on mRNA expressions. qRT-PCR analysis of gene expression in each lung cancer cells treated with negative control siRNAs (negative control-siRNA-1, 2) and different gene-specific siRNAs. Error bar represents the standard error of the mean (SEM) of duplicate. (lower) Effect of each siRNA on lung cancer cell proliferation *in vitro*: Cells were treated with siRNAs for 96 h, and cell viability was determined using a CellTiter96® AQueous One Solution Cell Proliferation Assay. Results shown are mean $\pm$ SD (bars) of three experiments (n=3) (* P <0.05, Student's t-test).

Figure 3.

Expression of SCARB1 genes in lung cancers and normal organs and selection of KIF11 as a therapeutic gene for *in vivo* study. A, qRT-PCR analysis of SCARB1 genes in EBUS-TBNA samples from advanced lung cancer. ADC, adenocarcinoma; SqCC, squamous cell carcinoma; LCC, large cell carcinoma; Small, small-cell lung cancer; LCNEC, large-cell neuroendocrine carcinoma. B, Western blot analysis of SCARB1 expression in lung cancer cell lines. C, qRT-PCR analysis of SCARB1 genes in normal human tissues. D, KIF11 expression in lung cancer cell lines. ADC, adenocarcinoma; ADS, adenosquamous carcinoma; SqCC, squamous cell carcinoma; LCC, large cell carcinoma; Small, small-cell lung cancer; Error bar represents SEM.

Figure 4.

A, Representative examples of KIF11 protein expression in lung squamous cell carcinoma (SqCC) and large cell carcinoma (LCC). Intensity and proportion scores were multiplied together to obtain the immunohistochemical (IHC) score. KIF11 protein was detected by immunohistochemistry using rabbit polyclonal anti-KIF11 antibody, with hematoxylin counterstaining. The IHC core by Imaging Software of each case was described at the bottom of the figure. No staining was observed in normal lung tissue. B, Kaplan-Meier analysis of overall survival in lung SqCC and LCC patients according to KIF11 expression level. The 5-year survival rate was 74.7% for patients with low-level KIF11 expression (KIF11-L) (n=32), whereas 49.3% for patients with high-level KIF11 expression (KIF11-H) (n=78).

Figure 5.

In vivo the knockdown and therapeutic effects of systemic administration of HPPS-chol-si-KIF11 in H460SM xenograft tumor model. A, The representative time-course pictures of each treatment groups. B, H460SM xenograft tumor-bearing mice were systemically administered with saline (n=10), HPPS-chol-siRNA-scramble (n=6), and HPPS-chol-siRNA-KIF11 (n=6), respectively, every other day for a total three doses. Tumor volume was measured from the day of initial treatment (day 0) to at day 15 after treatment, respectively, by blind method. Error bars represent the standard error of the mean (SEM). The student's t-test (two tailed) was used to determine. Significance and p-values less than 0.05 were considered significant ($P<0.05$). C, qRT-PCR in each tumor treated with saline control and HPPS-chol-siRNA-scramble, and HPPS-chol-siRNA-KIF11. D, Western blot analysis. E, Ki-67, cleaved caspase-3, and TUNEL staining of the tumors for different groups. F, H460SM tumor sections treated with KIF11 siRNA have reduced proliferation and increased apoptosis as measured by Immunostaining of Ki-67, cleaved caspase-3, and TUNEL staining, respectively. Cont, saline control; SCR, HPPS-chol-siRNA-scramble; KIF11, HPPS-chol-siRNA-KIF11.

Figure 6. The evaluation of the adverse effect of HPPS-chol-si-KIF11. The healthy nude mice (male, 6–8 weeks old) were intravenously administered with 10 mg/kg HPPS-chol-si-KIF11 (KIF11), HPPS-chol-si-scramble (SCR), and saline (Cont) every other day for a total three dose. All the organs were excised at 7 days after first injection. A, H&E staining of organs (the liver, kidney, heart, lung, testis, and adrenal gland tissue slices for different groups. B, Ki-67, cleaved caspase-3, and TUNEL staining of the testis and the adrenal gland tissues.

Figure 1

Figure 2A

Figure 2B

Figure 3A

Figure 3B

Figure 3C

Figure 3D

Figure 4A

Figure 4B

KIF11 Overall survival for lung SqCC + LCC

Figure 5A

Figure 5B

Figure 5C

Figure 5D

Figure 5E

Figure 5F

* $P < 0.05$ ** $P < 0.001$

Figure 6A

Figure 6B