Original Paper

Leukocyte Cell-Derived Chemotaxin 2 Retards Non-Small Cell Lung Cancer Progression Through Antagonizing MET and EGFR Activities

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Key Words

LECT2 • EGFR • MET • Tumor growth • Tumor invasion • NSCLC

Abstract

Background/Aims: Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI) therapy is a clinical option for non-small cell lung cancer (NSCLC) harboring activating EGFR mutations or for cancer with wild-type (WT) EGFR when chemotherapy has failed. MET receptor activation or *MET* gene amplification was reported to be a major mechanism of acquired resistance to EGFR-TKI therapy in NSCLC cells. Leukocyte cell-derived chemotaxin 2 (LECT2) is a multifunctional cytokine that was shown to suppress metastasis of hepatocellular carcinoma via inhibiting MET activity. Until now, the biological function responsible for LECT2's action in human NSCLC remains unclear. **Methods:** LECT2-knockout (KO) mice and NOD/SCID/IL2r γ null (NSG) mice were respectively used to investigate the effects of LECT2 on the tumorigenicity and metastasis of murine (Lewis lung carcinoma, LLC) and human (HCC827) lung cancer cells. The effect of LECT2 on *in vitro* cell proliferation was evaluated, using MTS and colony formation assays. The effect of LECT2 on cell motility was evaluated using transwell migration and invasion assays. An enzyme-linked immunosorbent assay was performed to detect

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secreted LECT2 in plasma and media. Co-immunoprecipitation and Western blot assays were used to investigate the underlying mechanisms of LECT2 in NSCLC cells. **Results:** Compared to WT mice, mice with LECT2 deletion exhibited enhanced growth and metastasis of LLC cells, and survival times decreased in LLC-implanted mice. Overexpression of LECT2 in orthotopic human HCC827 xenografts in NSG mice resulted in significant inhibition of tumor growth and metastasis. *In vitro*, overexpression of LECT2 or treatment with a recombinant LECT2 protein impaired the colony-forming ability and motility of NSCLC cells (HCC827 and PC9) harboring high levels of activated EGFR and MET. Mechanistic investigations found that LECT2 bound to MET and EGFR to antagonize their activation and further suppress their common downstream pathways: phosphatidylinositol 3-kinase/Akt and extracellular signal-regulated kinase. **Conclusion:** EGFR-MET signaling is critical for aggressive behaviors of NSCLC and is recognized as a therapeutic target for NSCLC especially for patients with acquired resistance to EGFR-TKI therapy. Our findings demonstrate, for the first time, that LECT2 functions as a suppressor of the progression of NSCLC by targeting EGFR-MET signaling.

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Introduction

Non-small cell lung cancer (NSCLC) accounts about 80%~85% of all lung cancers and presents with different histological features, including squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. The 5-year survival rate is as low as 15% in NSCLC patients who present at an advanced stage [1, 2]. Although chemotherapy only showed minor prolongation in survival compared to a sequential approach, it is still considered the standard approach for patients with advanced disease [3]. However, chemotherapy exhibits significant toxicity and can only be applied to a small subset of patients. The advent of novel therapies, such as epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) including gefitinib and erlotinib, has significantly prolonged the survival of NSCLC patients harboring EGFR mutations. It should be noted that even though most patients with EGFR mutations initially respond to TKI therapy, patients uniformly develop disease recurrence [4, 5]. In addition to the use in NSCLC patients with EGFR mutations, several clinical data also showed survival benefits of EGFR-TKI treatment for patients with wild-type (WT) EGFR and previously treated with chemotherapy but still developed advanced disease [6]. According to these observations, several guidelines also suggested that EGFR-TKIs be applied as second-line treatment for NSCLC patients harboring WT EGFR [7, 8].

Several mechanisms of EGFR-TKI resistance in NSCLC harboring activated EGFR mutations were described, such as a secondary EGFR T790M or C797S mutation, MET amplification, and hepatocyte growth factor (HGF) overexpression, and two major causes are the T790M mutation and MET amplification [4, 5, 9]. MET is also a receptor tyrosine kinase (RTK) and showed dysregulated expression in many tumor types including lung, pancreatic, breast, renal, and gastric cancers [10] and shows high-affinity for HGF to mediate cell proliferation, invasion, migration, and survival [11]. Activation of MET is related to advanced cancer stages and worse patient survival [12] and induces acquired resistance to TKI therapy in NSCLC [13]. EGFR is trans-phosphorylated by MET and promotes survival of cells as heterodimerization partners of MET in lung cancer cells with MET amplification [14]. Moreover, HGF was reported to induce gefitinib resistance of lung adenocarcinomas with activated EGFR mutations or WT EGFR through the phosphatidylinositol-3 kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK) pathways [9, 15]. In current clinical trials, the addition of MET-TKIs to EGFR-TKIs can prolong survival or decrease metastasis in NSCLC patients with MET overexpression [16].

Leukocyte cell-derived chemotaxin 2 (LECT2) is mainly produced in the liver and was originally identified as a chemotactic factor for neutrophils [17]. Increasing evidence has shown that dysregulation of LECT2 is involved in many pathologic conditions, including renal amyloidosis [18], severe liver injury [19], and sepsis [20]. Recently, accumulating evidence indicated that LECT2 is not only a prognostic factor of liver cancer, but can also

suppress the progression of hepatocellular carcinoma (HCC) [21, 22]. Chen et al. dissected the mechanisms involved in LECT2-mediated suppression of HCC and found that LECT2 directly binds to the MET receptor on the extracellular HxGxD motif and recruits protein tyrosine phosphatase 1B (PTP-1B) to inhibit MET activation [22]. Until now, the effects of LECT2 on other tumor types are largely unknown. Most recently, LECT2 was reported to be an important predictor for both breast carcinoma recurrence and mortality among smokers [23]. Herein, we evaluated the effect of secreted LECT2 on modulating the behaviors of NSCLC cells and found that LECT2 could be a potential suppressor of tumor progression of NSCLC cells by targeting both MET and EGFR and their common downstream pathways, Akt and ERK.

Materials and Methods

Chemicals and reagents

Gefitinib (Irrisa) was purchased from Selleckchem (Houston, TX). The recombinant human HGF and murine HGF were purchased from R&D Systems (Minneapolis, MN). Fetal bovine serum (FBS), antibiotics, molecular weight standards, trypsin-EDTA, trypan blue stain, and all medium additives were obtained from Life Technologies (Gaithersburg, MD). Antibodies specific for the unphosphorylated and corresponding phosphorylated (p-) forms of EGFR, MET, Akt, and EKR1/2 were purchased from Cell Signaling Technology (Danvers, MA). Anti-LECT2, anti- β -actin, and anti- α -tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-HA antibody and unless otherwise specified, other chemicals used in this study were purchased from Sigma (St. Louis, MO).

Purification of recombinant LECT2

To express human recombinant (hr)-LECT2 and murine recombinant (mr)-LECT2 proteins, plasmids were transfected into CHO-K1 cells for transient expression using the HyFect DNA transfection reagent (Leadgene Biomedical, Taiwan). In brief, 3×10^6 cells were seeded in T-75-cm² flasks 16 h before transfection. For DNA transfection, each flask used 5 μ g of plasmids for transfection following instructions in the product information. Cells were continuously cultured in CD-CHO medium (Thermo Fisher Scientific) for 96 h. Recombinant proteins in CD-CHO medium were purified using protein A agarose (Thermo Fisher Scientific) and dialysis in phosphate-buffered saline (PBS). Proteins were then filtered with a sterile 0.2- μ m polyethersulfone syringe filter and stored at -20 °C until use.

LECT2-knockout (KO) mice

LECT2-KO mice were produced as described previously [19] and kindly provided by Dr. Yamagoe (National Institute of Infectious Diseases, Tokyo, Japan). All experimental mice were generated from intercrosses between heterozygous mice in the National Laboratory Animal Center (Tainan, Taiwan), and littermates were divided into groups.

Cell lines and cell culture

The A549, H460, H1975, and HCC827 NSCLC cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). PC9 cells were developed by Lee and colleagues at National Cancer Center Hospital (Tokyo, Japan) [24]. All NSCLC cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine. The 293T human embryonic kidney and mouse Lewis lung carcinoma (LLC) cell lines were also purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin-glutamine. All cells were incubated at 37°C in a humidified 5% CO₂ atmosphere.

Preparation of total cell extracts and Western blot analysis

Protein lysates were prepared as described previously [25]. Equal amounts of protein extracts (20–30 μ g) were subjected to 10% or 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene fluoride membranes (Millipore, Belford, MA). After blocking, blots were incubated with primary antibodies in PBS containing Triton X-100 (PBST) for 1h followed by two

washes (for 15 min each) in PBST. Samples were then incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min. After washing, blots were incubated for 1 min with the enhanced chemiluminescent Western blotting reagent (Millipore, Billerica, MA), and chemiluminescence was detected by a chemiluminescence imaging system, MultiGel-21, (TOP BIO, New Taipei City, Taiwan) for 0.5~3 min. Furthermore, the same blots were stripped with stripping buffer (TOOLS, New Taipei City, Taiwan) and reprobed with β -actin or α -tubulin antibodies as the internal control.

Cell viability assay (MTS assay)

Lung cancer cells (5×10^3) were seeded in 96-well plates, treated with 100 ng/mL recombinant LECT2 for different time points, and then subjected to a cell-viability assay (MTS assay; Promega, Madison, WI) according to the manufacturer's instructions.

Transwell migration and invasion assays

Cells (3×10^4) were plated in an uncoated top chamber (24-well insert; pore size, 8 μ m; Corning Costar, Corning, NY) for the migration assay, while the invasion assay was performed in a top chamber coated with Matrigel (BD Biosciences, Bedford, MA). In these assays, LECT2-treated cells or LECT2-overexpressing cells were plated in serum-free medium, supplemented with serum or HGF (40 ng/ml), which was used as a chemoattractant in the lower chamber. After 24 h of incubation, cells on the lower surface of the membrane were fixed with methanol and stained with 0.2% crystal violet. The number of cells migrating through or invading through the membrane was counted under a light microscope ($\times 100$, three random fields per well).

LECT2 measured by an enzyme-linked immunosorbent assay (ELISA)

Cultured cancer cells (6×10^6 cells/10-cm plate) were rinsed twice with PBS and cultured in 10 ml of serum-free medium for 24 or 48 h. Cancer cell conditioned medium (CM) was collected and clarified by centrifugation (5000 rpm at 4 °C for 5 min) to remove cell debris. Moreover, serum from LECT2-KO and WT mice were also collected. LECT2 levels in CM and serum were respectively determined with a human LECT2 ELISA kit (Cusabio Biotech, Wuhan, China) and a mouse LECT2 ELISA kit (MBL, Nagoya, Japan), according to the manufacturers' instructions.

Lentiviral production and infection

The lentiviral vector and its packaging vectors were transfected into 293T packaging cells by calcium phosphate transfection. Briefly, 10^6 293T cells were subcultured on 10-cm culture dishes overnight before transfection. Then, cells were transfected with 10 μ g pWPI-LECT2 or pWPI-control together with 10 μ g of pCMV Δ R8.91 (the packaging vector) and 1 μ g of pMD.G (the envelope vector). After 5 h of incubation, the transfection medium was replaced with fresh culture medium. After 48 h, lentivirus-containing medium was collected from the transfection and spun down at 1500 rpm for 5 min to pelletize the cell debris. The supernatant was then filtered through a 0.45- μ m filter, and target cells were infected with fresh lentivirus-containing medium (supplemented with 8 μ g/ml polybrene) for 24 h and subjected to different functional assays.

Plate colony-formation assay

WT or LECT-overexpressing NSCLC cells (10^3) were seeded in six-well plates and incubated for 24 h. Thereafter, the medium was changed every 2 days; 10 days later, cells were stained with crystal violet, and colonies with more than 50 cells were counted.

DNA transfection

All of the constructs were generated by standard protocols. The genes and/or their fragments, including LECT2, MET, and EGFR, were amplified by a PCR and cloned into the pCDNA3.1 vector (Invitrogen). Expression vectors were transiently transfected into test cells with the Lipofectamine 2000 transfection reagent.

Immunoprecipitation (IP)

293T cells were transfected with indicated plasmids and lysed on ice with IP lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, and 1% NP-40). Equal amounts of cell lysates were immunoprecipitated with an anti-hemoagglutinin (HA) antibody combined with protein A agarose beads. The collected protein complexes were washed four times with IP buffer and eluted by boiling with protein sample buffer under reducing conditions. Then, proteins were resolved by SDS-PAGE and analyzed by Western blotting with antibodies for MET, EGFR, HA, and LECT2.

In vivo antitumor activity of LECT2

Male NSG (NOD-scid IL2r^{null}) and LECT-KO mice were used for the orthotopic metastasis assays. Totals of 10⁶ and 5 × 10⁵ luciferase-expressing human NSCLC cells (HCC827 and A549) and murine LLC cells were resuspended in a 1:1 mixture of PBS and Growth Factor-Reduced (GFR)-Matrigel. This mixture was then injected into the left lateral thorax of each NSG or LECT2-KO mouse. The tumor size and location were monitored and quantified with a noninvasive bioluminescence system (Xenogen IVIS-200 system, PerkinElmer, Waltham, MA). After 21 and 17 days, the HCC827-injected and LLC-injected mice were respectively sacrificed, and luciferase activities in the excised lungs were further determined using the IVIS-200 system. Mouse lungs were also fixed, sectioned, and stained with hematoxylin and eosin (H&E).

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using the Statistical Package for Social Science software, vers. 16 (SPSS, Chicago, IL). A non-parametric test was used for comparisons between two groups due to small sample size, i.e., The Median test was applied to examine the differences between group medians. Differences were considered significant at $p < 0.05$. Kaplan-Meier nonparametric model plots were conducted and the log rank test was used to test the differences in the survival between wild-type and LECT2-KO mice injected with luciferase-expressing murine LLC cells. $p < 0.05$ was considered to represent a statistically significant difference.

Results

Genetic depletion of LECT2 promotes tumor progression and decreases the survival rate in an LLC orthotopic graft model

To evaluate the role of LECT2 in the progression of lung cancer in mice, systemic KO mice of LECT2 (LECT2 ^{-/-} mice) were used. No serum LECT2 was detected in LECT2 ^{-/-} mice and around 54 ng/ml of LECT2 was detected in WT mice using an ELISA (Fig. 1A). Seventeen days after inoculation with luciferase-tagged LLC cells into the left lateral thorax, the tumorigenic ability had increased in LECT2 ^{-/-} mice compared to WT mice by photon emission detection *in vivo* (Fig. 1B). After the mice were sacrificed, ex vivo imaging of their lungs exhibited a higher photon intensity in LECT2 ^{-/-} mice compared to WT mice, in both orthotopic tumors (left lung) and metastatic tumors (right lung) (Fig. 1C). A parallel study in a different animal cohort was also performed and showed that LECT2 ^{-/-} mice with LLC tumors had significantly shorter survival times compared to WT mice with tumors (Fig. 1D). These results revealed that deletion of LECT2 enhances progression of murine lung tumors in mice and suggested that LECT2 functions as a circulating protein.

mr-LECT2 suppresses migration and invasion of LLC cells by downregulating MET activation

Previous studies indicated that MET is an important target of LECT2 to suppress HCC progression, and MET activation also plays a critical role in modulating NSCLC progression [22, 26]. To determine whether the LECT2 protein interferes with MET activation and cell behaviors such as migration, invasion, and proliferation in LLC cells, we purified the mr-LECT2 protein and performed Western blot, migration/invasion, and MTS assays. The addition of murine HGF (40 ng/mL) to starvation medium significantly induced phosphorylation of MET and its downstream signals, Akt and ERK, and activation of the HGF/

Fig. 1. Leukocyte cell-derived chemotaxin 2 (LECT2) deletion enhances tumor progression and decreases survival rates in mice with Lewis lung carcinoma (LLC) orthotopic grafts. (A) Serum levels of LECT2 in LECT-deficient and wild-type (WT) mice with implanted LLC tumors after 1 week. (B) Left panel, Xenogen IVIS spectrum bioluminescence images are shown with the orthotopic LLC tumor location and growth. A quantitative analysis of the Xenogen imaging signal intensity from tumor tissues on days 0, 7, 14, and 17 after tumor implantation is shown in the right panel. * $p < 0.05$, ** $p < 0.01$ compared to the WT groups. (C) Lungs were isolated and examined at the end of this spontaneous metastasis assay. Left panel: Cancer metastasis from the left lung to the right lung was imaged with bioluminescence at the end of the study. Right panel: Signal intensities from primary tumors (left lung) and metastatic tumors (right lung) were bioluminescently captured at the end of the study, with the mean signal for each group indicated. (D) An overall survival curve was produced for WT and LECT2-depleted mice ($n = 30$) implanted with LLC tumors using the Kaplan-Meier method. p values were determined using a log-rank test.

Fig. 2. Effect of murine recombinant (mr)-LECT2 on hepatocyte growth factor (HGF)-induced activation of MET, Akt, and extracellular signal-regulated kinase 1/2 (ERK1/2) signals and cell motility in Lewis lung carcinoma (LLC) cells. (A) Serum-starved LLC cells were treated with HGF or cotreated with HGF and indicated concentrations of mr-LECT2 for 30 min. Lysates were collected from cells and subjected to a Western blot analysis to detect phosphorylation levels of MET, Akt, and ERK1/2. (B-C) LLC cells were treated with 100 ng/mL mr-LECT2 with or without HGF for the transwell migration and Matrigel invasion assays (B). Results are expressed as multiples of the control. Data are expressed as the mean $\pm$ SD of three independent experiments. * $p < 0.05$ compared to control group. # $p < 0.05$ compared to the HGF treatment-only group (C). (D) Proliferation rate of LLC cells seeded in a 96-well plate and treated with HGF (40 ng/mL) alone or combined with 100 ng/mL mr-LECT2 protein for 24 h. Cell proliferation was measured with an MTS assay.

Fig. 3. Human recombinant (hr)-LECT2 inhibits the MET activation-mediated cell motility increase in human non-small cell lung cancer (NSCLC) cells harboring the wild-type (WT) and mutant epithelial growth factor receptor (EGFR). (A) Detection of protein levels of MET and p-MET by Western blotting in different NSCLC cell lines harboring the WT (A549 and H460) and mutant EGFR (HCC827, PC9, and H1975). (B, D) Serum-starved A549 cells were treated with various concentrations of hr-LECT2 with or without cotreatment with hepatocyte growth factor (HGF) (B), or HCC827 cells were only treated with various concentrations of hr-LECT2 (D) for 30 min, and a Western blot analysis was performed to detect phosphorylation of MET, Akt, and extracellular signal-regulated kinase 1/2 (ERK1/2) in both cells. (C, E) Effects of hr-LECT2 on HGF-induced motility in A549 cells (C) and the endogenous migratory and invasive activities in HCC827 cells (E) were measured by transwell migration and Matrigel invasion assays. Results are expressed as multiples of the control. Data are expressed as the mean $\pm$ SD of three independent experiments. * $p < 0.05$ compared to the control group. # $p < 0.05$ compared to the HGF treatment-only group.

MET axis was significantly suppressed by co-treatment with different concentrations of mr-LECT2 (50~200 ng/mL) (Fig. 2A). Moreover, the HGF-induced increases in the migratory and invasive abilities of LLC cells were also significantly inhibited by treatment with the mr-LECT2 protein (Fig. 2B-C). Although previous studies indicated that HGF/MET signaling

Fig. 4. Neither treatment with HGF or hr-LECT2 only, nor cotreatment with HGF and hr-LECT2 alters the proliferation rate of non-small cell lung cancer (NSCLC) cells. Proliferation rates of HCC827 and A549 cells seeded in a 96-well plate and treated with HGF, HGF + hr-LECT2, or hr-LECT2 as indicated for 24, 48, and 72 h. Cell proliferation was measured with the MTS assay.

Fig. 5. Overexpression of LECT2 in human non-small cell lung cancer (NSCLC) cells inhibits MET activation. (A-B) LECT2 protein levels in A549 and HCC827 cells infected with a lentivirus carrying the control (A549/vector and HCC827/vector) or hemoagglutinin (HA)-tagged LECT2 expression vector (A549/LECT2 and HCC827/LECT2). After 24 or 48 h of infection, cell lysates and conditioned medium were collected to measure LECT2 protein levels by Western blotting (A) and an ELISA (B), respectively. (C) Cell lysates from serum-starved A549/LECT2 and A549/vector cells which were treated with vehicle or hepatocyte growth factor (HGF) as indicated for 30 min or cell lysates from HCC827/LECT2 and HCC827/vector cells were all subjected to a Western blot analysis to detect MET activation.

Fig. 6. Overexpression of LECT2 in human non-small cell lung cancer (NSCLC) cells suppresses their ability to migrate and invade in vitro. (A-B) A549/LECT2 and A549/vector cells with vehicle or HGF treatment (A) or HCC827/LECT2 and HCC827/vector cells with no treatment (B) were all subjected to transwell migration and Matrigel invasion assays. Results are expressed as multiples of the control. Data are expressed as the mean $\pm$ SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to the control group. ## $p < 0.01$ compared to the HGF treatment-only group.

can also promote the proliferation of lung cancer cells [26], our MTS results showed that treatment with HGF or HGF combined with mr-LECT2 for 24 h did not significantly alter the proliferation rate of LLC cells (Fig. 2D). These data suggested that the mr-LECT2 protein inhibits the motility of LLC possibly through suppressing the HGF/MET axis.

hr-LECT2 inhibits MET activation-induced cell motility of human NSCLC cells harboring the WT and activating mutant EGFR

To further confirm the inhibitory effect of LECT2 on MET activation-induced progression of human NSCLC cells, we first evaluated the endogenous protein expression and activation levels of MET in several human NSCLC cell lines harboring the WT (H460 and A549) or activating mutant EGFR (PC9, HCC827, and H1975). We found that A549, H1975, PC9, and HCC827 cells expressed higher levels of the MET protein compared to H460 cells. HCC827 and PC9 cells respectively showed high and moderate endogenous activation of MET (Fig. 3A). Therefore, the HCC827 and A549 cell lines were selected for subsequent *in vitro* and *in vivo* studies. Treatment of A549 with different concentrations of hr-LECT2 effectively suppressed the HGF-induced activation of MET, Akt, and ERK signaling (Fig. 3B) and increases in the migratory and invasive abilities (Fig. 3C). In the absence of HGF, we found that hr-LECT2 (100~200 ng/ml) still suppressed the endogenous activity of MET and the migratory and invasive abilities of HCC827 cells (Fig. 3D-E), suggesting that LECT2 inhibits MET-mediated cell motility possibly not through competing with HGF binding. Similar to LLC cells, Neither HGF or hr-LECT2 treatment only, nor cotreatment with HGF and hr-LECT2 altered the proliferation rate of A549 or HCC827 cells (Fig. 4).

Overexpression of LECT2 in human A549 and HCC827 cells inhibited MET activation and their ability to migrate and invade in vitro

Due to the half-life of LECT2 protein (16 kDa) is short, we further overexpressed LECT2 in A549 and HCC827 cells to examine the chronic action of LECT2, such as its anticancer effects in an *in vivo* model. Expression of endogenous LECT2 was negligible in A549 and HCC827 cells infected with the control vector (Fig. 5A-B). After infection with the LECT2 expression vector (pWPI-LECT2), overexpressed protein in cells and secreted LECT2 protein in the culture medium were confirmed (Fig. 5A-B). Our results showed that overexpression

Fig. 7. Ectopic LECT2 expression suppresses growth and metastasis of a HCC827 orthotopic graft model by suppressing MET activation. Luciferase-tagged HCC827/LECT2 and HCC827/vector cells were orthotopically injected into NSG mice (n = 6). The tumor size was monitored by bioluminescence imaging. (A) Upper panel: Xenogen IVIS spectrum bioluminescence images of orthotopic lung tumor growth. A quantitative analysis of imaging signal intensity from tumor tissues on days 0, 4, 8, 15, and 21 after tumor implantation is shown in the lower panel. * p<0.05, ** p<0.01 compared to the control groups. (B-D) Lungs were isolated and examined at the end of this spontaneous metastasis assay. (B) Upper panel: Cancer metastasis from the left lung to the right lung was imaged with bioluminescence. Lower panel: Signal intensities from primary tumors (left lung) and metastatic tumors (right lung) were bioluminescently captured at the end of the study, with the mean signal for each group indicated. *** p<0.001 compared to the control group. (C) Representative pictures of lung tissues isolated from each group of mice, and metastatic colonies are indicated by an arrow in the right lungs. (D) H&E staining of lungs and the metastatic colonies are indicated by an arrow in the right lungs. (E) Colony-forming abilities of HCC827 cells overexpressing LECT2 or control vector. Data are expressed as the mean ± SD of three independent experiments. * p<0.05 compared to the control group. (F) HCC827/vector and HCC827/LECT2 orthotopic tumors were isolated for protein extraction at the end of the experiment, and a Western blot analysis was performed. Quantitative p-MET levels were respectively adjusted to MET and β-actin levels. Values are presented as the mean ± SD. *** p<0.001 compared to the control group.

Fig. 8. LECT2 overexpression in A549 cells does not affect the *in vitro* colony-forming abilities and *in vivo* tumor progression. (A) Colony-forming abilities of A549 cells overexpressing LECT2 or control vector. Upper panel: Representative photomicrographs. Lower panel: Data are expressed as the mean $\pm$ SD of three independent experiments. (B) Luciferase-tagged A549/LECT2 and A549/vector cells were injected into the left lateral thorax of NSG mice ($n = 5$). Upper panel: Primary tumor growth in the left lung and tumor metastasis in the right lung were imaged with bioluminescence at the end of the study. Lower panel: Signal intensities from primary tumors (left lung) and metastatic tumors (right lung) were bioluminescently captured, with the mean signal for each group indicated. NS, not significant.

of LECT2 significantly reversed HGF-induced MET activation in A549 cells and suppressed endogenous MET activity in HCC827 cells (Fig. 5C). Moreover, cells infected with pWPI-LECT2 showed inhibition of HGF-induced cell migration and invasion in A549 cell (Fig. 6A) and decreased basal migratory and invasive abilities in HCC827 cells (Fig. 6B). Taken together, our results suggest that LECT2 derived from the pWPI-LECT2 exhibited an autocrine or paracrine function.

Overexpression of LECT2 in orthotopic HCC827 xenografts in NOD-scid IL2 γ ^{null} (NSG) mice resulted in inhibition of tumor growth and metastasis by suppressing MET activation

To further elucidate the *in vivo* effects of LECT2 expression on human lung tumor progression, an orthotopic lung tumor-bearing model was established by transplanting luciferase-tagged cells, HCC827-luc or HCC827-luc/LECT2, into NSG mice. Three weeks after inoculation with cancer cells, the tumorigenic ability had decreased in LECT2-overexpressing HCC827 cells compared to control HCC827 cells (Fig. 7A). Moreover, after the mice were sacrificed, *ex vivo* imaging of their lungs showed a lower photon intensity in HCC827/LECT2-injected mice compared to control mice, in both orthotopic tumors (left lung) and metastatic tumors (right lung) (Fig. 7B). Furthermore, mice bearing HCC827/LECT2 tumors also developed fewer metastatic nodules (as indicated by a black arrow) in the right lung compared to control mice (Fig. 7C). The histological data also confirmed the appearance of metastatic tumors in lung sections (as indicated by a black arrow) (Fig. 7D). Although our results showed that LECT2 only partially affected the proliferation rate of NSCLC cells in a short period of time (24~72 h) (Fig. 4), comparing the long-term culture (10 days) of

Fig. 9. LECT2 interacts with epidermal growth factor receptor (EGFR) to suppress its activation. (A) 293T cells were transfected with a hemoagglutinin (HA)-LECT2-expressing vector with the control vector, an EGFR-expressing vector, a MET-expressing vector, or an AXL-expressing vector as indicated. Cell lysates were immunoprecipitated with an HA antibody and then subjected to immunoblotting with the indicated antibodies. (B) Treatment of HCC827 cells with the vehicle, gefitinib (0.1 and 1 μ M), or human recombinant (hr)-LECT2 (100 ng/mL) for 30 min, and a Western blot analysis was performed to respectively detect phosphorylation levels of MET and EGFR. (C) HCC827/vector and HCC827/LECT2 orthotopic tumors were isolated for protein extraction at the end of the experiment and subjected to a Western blot analysis. Quantitative p-EGFR levels were respectively adjusted to EGFR and β -actin levels. Values are presented as the mean $\pm$ SD.

HCC827/LECT2 to HCC827/vector cells, we found that overexpression of LECT2 significantly inhibited the colony-forming abilities (Fig. 7E). Next, we further detected LECT2 and p-MET protein levels of HCC827 xenografts harvested from LECT2-overexpressing or control mice and found that tumors from HCC827/LECT2-injected mice still retained high levels of LECT2, while levels of p-MET had decreased in tumors (Fig. 7F). In contrast to HCC827 cells, we found that LECT2 overexpression in A549 cells did not affect the *in vitro* colony-forming abilities or *in vivo* tumor progression (Fig. 8A and B). Taken together, these data indicate that LECT2 overexpression suppressed the growth and metastatic abilities by inhibiting MET activation in an NSCLC xenograft model.

EGFR, another important RTK involved in NSCLC progression, can be targeted and inhibited by LECT2

Activating mutations in EGFR are commonly found in NSCLC and are important for the progression of NSCLC. According to previous studies, data from liquid chromatography/tandem mass spectrometry (LC-MS/MS) showed that EGFR was also assumed to be bound by LECT2 [22]. We next tested whether LECT2 also inhibits EGFR phosphorylation through binding to EGFR. Co-IP experiments of 293T cells co-transfected with LECT2 and EGFR or MET revealed that LECT2 not only interacted with MET but also was associated with EGFR (Fig. 9A). Moreover, EGFR phosphorylation was markedly inhibited after hr-LECT2

Fig. 10. LECT2 efficiently suppresses EGFR activation and motility in PC9 cells (A) Detection of protein levels of EGFR and p-EGFR by Western blotting in different non-small cell lung cancer (NSCLC) cell lines harboring WT (A549) and mutant EGFR (HCC827, PC9, and H1975). (B) Western blot analysis was used to detect MET and EGFR activation in PC9/vector and PC9/LECT2 cells. (C-D) PC9/LECT2 and PC9/vector cells at different seeding densities (3×10^4 /well and 5×10^4 /well) (C) or PC9 cells were treated with different concentrations of hr-LECT2 (50~200 ng/mL) for 24 h (D) were all subjected to a transwell Matrigel invasion assay. Results are expressed as multiples of the control. Data are expressed as the mean $\pm$ SD of three independent experiments. * $p < 0.05$ compared to the control group. (E) Schematic diagram depicting the antitumor effects of LECT2 on NSCLC through binding with MET and EGFR to antagonize their activation and further suppress their common downstream pathways, PI3K/Akt and Raf-1/ERK1/2.

treatment in HCC827 cells (Fig. 9B). We further detected p-EGFR protein levels of HCC827 xenografts harvested from LECT2-overexpressing or control mice and found that LECT2 overexpression showed a decreasing trend of EGFR phosphorylation at orthotopic tumors sites (Fig. 9C). We further determined endogenous protein expression and activation levels of EGFR in other NSCLC cells and found that another EGFR-activating mutation cell, PC9, also harbored higher endogenous EGFR and MET activities compared to A549 cells (Fig. 3A, 10A). Similar to HCC827 cells, overexpressing LECT2 in PC9 cells significantly reduced EGFR and MET activation and suppressed the invasive ability of PC9 cells (Fig. 10B-C). Moreover, treatment of PC9 cells with different concentrations of hr-LECT2 also attenuated the invasive ability of cells (Fig. 10D). These data suggest that EGFR might be another target of LECT2 in suppressing progression of NSCLC cells.

Discussion

MET amplification is considered a mechanism of acquired resistance to EGFR-TKIs in NSCLC; therefore, combinations of EGFR and MET kinase inhibitors may lead to better therapeutic outcomes [27]. After treatment with EGFR-TKIs, up to 20% of tumor samples presented MET protein overexpression or *MET* gene amplification. The *MET* gene mutation and the corresponding amplification of MET kinase occurred independently of the T790M status [28]. MET amplification was shown to restore the PI3K/Akt pathway mediated by ErbB3 transactivation and thereby induced acquired resistance to gefitinib [13]. In addition to MET amplification, Yano et al. determined that HGF-induced MET activation without a gene mutation or amplification could mediate activation of survival signaling (PI3K/Akt and ERK) independently of ErbB3 against gefitinib [9]. Moreover, Breindel et al. indicated that using gefitinib and U0126 respectively inhibited EGFR and ERK, which reduced MET activation in NSCLC cells harboring either the WT or mutant EGFR. Furthermore, MET signaling promoted EGFR-induced migration and invasion, and EGFR-MET signaling was enhanced in the highly metastatic PC9-BrM3 EGFR-mutant cell line, compared to the parental PC 9 line [29]. Overall, the results mentioned above all indicate that EGFR-MET signaling, including PI3K/Akt and ERK, is critical for the aggressive behavior of NSCLC. LECT2 was demonstrated to be a tumor suppressor in HCC [22], and serum LECT2 could be a potential biomarker in HCC patients [30]. However, it is not yet clear whether LECT2 also regulates the behaviors of NSCLC. In this study, we found that genetic depletion of LECT2 in mice and overexpression of LECT2 in NSCLC cells harboring mutant EGFR respectively promoted and suppressed progression (growth and metastasis) of cancer cells in xenograft mice models and *in vitro* cell models (migration, invasion, and colony formation). We subsequently generated recombinant murine and human LECT2 to investigate whether treatment with LECT2 would affect the behaviors of NSCLC cells and found that treatment of murine and human lung cancer cells with LECT2 suppressed cell motility and the colony-forming ability. These results imply that LECT2 functions as a circulating protein which affects NSCLC behaviors through paracrine effects. In addition to cancer, the circulating level of LECT2 was reported to be correlated with several pathologic conditions. For example, circulating LECT2 was positively correlated with the severity of both obesity and insulin resistance (IR) and was negatively correlated with the severity of sepsis in humans [20, 31]. However, the correlation between circulating LECT2 and NSCLC prognoses should be further investigated in future work.

Mechanistic investigations showed that LECT2 treatment could enhance phagocytosis of macrophage via the CD209a receptor [32]. LECT2 can lead to IR in skeletal muscle cells by activating c-Jun N-terminal kinase (JNK) [31]. More recently, Hwang et al. showed that LECT2 induced an atherosclerotic inflammatory reaction via CD209-mediated JNK phosphorylation in human endothelial cells [33]. Our results showed that LECT2 can inhibit activation of both MET and EGFR and their common downstream pathways, Akt and ERK, to suppress tumorigenicity and metastasis of NSCLC. Moreover, co-IP data demonstrated that LECT2 directly interacts with MET and EGFR. Previous studies indicated that LECT2 bound to the extracellular domain of MET and did not compete with HGF for the MET receptor. LECT2 was reported to induce an association between PTP-1B and MET to suppress MET activation [22]. PTP-1B as well as other PTPs, such as receptor type of protein tyrosine phosphatase sigma (RPTP σ), LAR, Src homology region 2 domain-containing phosphatase (SHP)-1, and T-cell protein tyrosine phosphatase (TCPTP), were implicated in the dephosphorylation of the EGFR and consequent suppression of EGFR signaling [34]. At present, our results only showed that LECT2 can bind to EGFR and further inhibit EGFR activation. However, the binding site of LECT2 on EGFR and the effect of LECT2 on PTPs should be further investigated.

Interestingly, from the results of the *in vitro* colony-forming ability and *in vivo* tumor xenograft assay, we found that the malignant phenotype of cells harboring activated MET and EGFR (HCC827 and PC9 cells) could be suppressed by LECT2, but cells lacking constitutive activation of MET and EGFR (A549 cells) were not affected by LECT2. These results suggest that dual inhibition of EGFR/MET pathways might be critical for the ability of LECT2 to inhibit

NSCLC progression. Moreover, our study results showed that the growth inhibitory effect of LECT2 against HCC827 xenografts was more dominant than its effect against the colony-forming ability of HCC827 cells, suggesting that LECT2 might target tumor cells and also affect the lung tumor microenvironment. Actually, previous studies indicated that LECT2 can target vascular endothelial growth factor receptor 2 (VEGFR2) of human endothelial cells to show an antiangiogenic effect [35]. Angiogenesis is essential for the processes of primary tumor growth and metastasis of NSCLC [36]. In addition to endothelial cells, LECT2 was also reported to activate macrophages, resulting in increased phagocytic activity and cytokine expressions such as interferon (IFN)- γ and the chemokine INF- γ -inducible protein 10 kDa (CXCL-10) [32]. In a xenograft model of lung adenocarcinoma, the production of CXCL10 was inversely correlated with tumor growth, resulting in a marked reduction in tumor-associated angiogenesis [37, 38]. IFN- γ was shown to be involved in the antiproliferative, antiangiogenic, and proapoptotic effects against cancer cells [39]. Taken together, the angiostatic effect of LECT2 may be partly attributable to its capacity to suppress lung tumor progression *in vivo*. Alveolar macrophages (AMs) are likely to be of central importance in the lung cancer immune response. A dual role of macrophages in lung cancer was suggested with the idea that AMs may both inhibit and/or promote tumor progression [40]. There are two different types of macrophages, including classical M1 and alternative M2 macrophages, which respectively exhibit antitumor and protumor effects. M1 macrophages are activated by IFN- γ treatment, while the M1 macrophage subset was associated more within tumor islets and with extended survival times in patients with NSCLC [41]. The effects of LECT2 on the polarization of AMs, cytokine secretion from AMs, and phagocytic activity of AMs are worthy of investigation in the future.

Conclusion

The present data first demonstrated that LECT2 is a potential therapeutic agent for NSCLC because it inhibits activation of both EGFR and MET and their common downstream signaling pathways, PI3K/Akt and Raf-1/ERK1/2, which are important in promoting NSCLC progression (Fig. 10E). Resistance of NSCLC cells to gefitinib treatment was mediated by MET activation [13]. In addition to MET, AXL, another RTK, was recently reported to contribute to the survival of EGFR-mutant lung cancer cells in a manner similar to that observed for MET [42]. High activation of AXL was observed in NSCLC cells with acquired resistance to erlotinib treatment, and increased AXL expression was found in approximately 20% of patients with acquired resistance to EGFR-TKIs [43]. Interestingly, the LC-MS/MS data from a previous study [22] and the Co-IP data from the present study showed that LECT2 also interacted with AXL (Fig. 9A). Although the effect of LECT2 on AXL activation in NSCLC cells remains unknown, our present findings still support the development of clinical trials to determine whether LECT2 or LECT2 combined with other TKI regimens would be useful in managing human NSCLC, especially NSCLC cells that have acquired resistance to EGFR-TKIs harboring the MET amplification.

Acknowledgements

We would like to thank Dr. Shuang-En Chuang (National Health Research Institutes, Taiwan) for the gift of the AXL-overexpressing plasmid. We also thank Dr. Yen-Kuang Lin (Biostatistics Center, College of Management, Taipei Medical University, Taipei, Taiwan) for the assistance in statistical analysis. This study was supported by grant 107TMU-WFH-13 from Wan Fang Hospital, Taipei Medical University. This study was also supported by the "TMU Research Center of Cancer Translational Medicine" from The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan.

Disclosure Statement

All authors declare that they have no conflicts of interest.

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