Enoxaparin Sensitizes Human Non–Small-Cell Lung Carcinomas to Gefitinib by Inhibiting DOCK1 Expression, Vimentin Phosphorylation, and Akt Activation

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ABSTRACT

Gefitinib is widely used for the treatment of lung cancer in patients with sensitizing epidermal growth factor receptor mutations, but patients tend to develop resistance after an average of 10 months. Low molecular weight heparins, such as enoxaparin, potently inhibit experimental metastasis. This study aimed to determine the potential of combined enoxaparin and gefitinib (enoxaparin + gefitinib) treatment to inhibit tumor resistance to gefitinib both in vitro and in vivo. A549 and H1975 cell migration was analyzed in wound closure and Transwell assays. Akt and extracellular signal–related kinase 1/2 signaling pathways were identified, and a proteomics analysis was conducted using SDS-PAGE/liquid chromatography–tandem mass spectrometry analysis. Molecular interaction networks were visualized using the Cytoscape bioinformatics platform. Protein expression of dedicator of cytokinesis 1 (DOCK1) and cytoskeleton intermediate filament vimentin were identified using an enzyme-linked immunosorbent assay, Western blot, and small interfering RNA transfection of A549 cells. In xenograft A549-luc-C8 tumors in nude mice, enoxaparin + gefitinib inhibited tumor growth and reduced lung colony formation compared with gefitinib alone. Furthermore, the combination had stronger inhibitory effects on cell migration than either agent used individually. Additional enoxaparin administration resulted in better effective inhibition of Akt activity compared with gefitinib alone. Proteomics and network analysis implicated DOCK1 as the key node molecule. Western blot verified the effective inhibition of the expression of DOCK1 and vimentin phosphorylation by enoxaparin + gefitinib compared with gefitinib alone. DOCK1 knockdown confirmed its role in cell migration, Akt expression, and vimentin phosphorylation. Our data indicate that enoxaparin sensitizes gefitinib antitumor and antimigration activity in lung cancer by suppressing DOCK1 expression, Akt activity, and vimentin phosphorylation.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, and its incidence in China has emerged in recent years as the first among various types of cancer. According to the 2012 China Cancer Registry Annual Report statistics, the incidence of lung cancer accounted for 22.1 and 14.6% of the national cancer incidence for men and women, respectively, while mortality accounted for 27.2 and 21.9%. Non–small-cell lung carcinoma (NSCLC) represents the majority of lung cancers, and although recently there have been many advances in NSCLC therapies, its median survival rate is still less than 12 months, and <15% of patients with NSCLC survive beyond 5 years from initial diagnosis (Spiro and Porter, 2002; Girnun et al., 2008). The epidermal growth factor receptor (EGFR) is an important signal transduction molecule related to NSCLC (Mukohara et al., 2003). In recent years, small molecule tyrosine kinase inhibitors (EGFR-TKIs) have been used in a subpopulation of NSCLC patients with sensitizing EGFR mutations (Asian, female, nonsmoker, and adenocarcinoma) and have shown beneficial effects (Lynch et al., 2004; Paez et al., 2004). Gefitinib, one of the EGFR-TKIs, is currently used for the treatment of patients with advanced NSCLC. Unfortunately, despite the dramatic response to gefitinib, these tumors invariably develop drug resistance within an average of 9–12 months (Kobayashi et al., 2005; Girnun et al., 2008). Thus, it is necessary to sensitize human lung cancer cells to gefitinib and avoid the development of drug resistance.

The concept of using a combination of agents for cancer therapy has gained much attention. Combinations of low doses...
of anticancer agents that have different mechanisms of action may have significantly improved efficacy in inhibiting tumor formation and tumor growth when compared with individual anticancer substances alone (Chen et al., 2012). Although low molecular weight heparins (LMWHs) (e.g., enoxaparin, dalteparin, reviparin, and tinzaparin) have been widely studied as antitumor drugs and it has been shown that LMWH has an antimitotic effect on multiple neoplasms with improved animal survival rates (Mousa and Petersen, 2009; Pan et al., 2011; Walenga and Lyman, 2013), the anticancer effects of LMWH in combination with gefitinib in NSCLC A549 and H1975 cells as well as xenograft lung tumors in immune-deficient mice are not known. Gefitinib targets and inhibits EGFR–tyrosine kinase, leading to constitutive inhibition of downstream signaling transduction pathways, including RAF–extracellular signal-related kinase 1/2 (Erk1/2) and phosphoinositide 3-kinase (PI3K)–Akt. We therefore investigated the effect of LMWH in combination with gefitinib on lung cancer cells and tumorigenic capacity in vivo. Furthermore, we investigated the effects of this combination on those two EGFR downstream signaling pathways.

Mass spectrometry (MS)–based proteomics technology has been shown as a powerful tool for large-scale protein identification and quantitation (Cox and Mann, 2008; Cox et al., 2011). With reproducible and accurate quantification strategies, differentially expressed proteins in a complex biologic system are investigated. In the present research, we used whole proteomics technology with linear trap quadrupole (LTQ) MS, combined with the bioinformatic tool of the Cytoscape database, an efficient protocol that enables the analysis of proteomes. We identified the key proteins playing an important role in the combination of enoxaparin with gefitinib compared with gefitinib used alone.

Materials and Methods

Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco (Grand Island, NY). The human lung adenocarcinoma cell lines A549 and H1975 were from the American Type Culture Collection (Manassas, VA). The A549-luc-C8 cell line was purchased from Caliper Life Sciences (Hopkinton, MA). Low molecular weight heparin, enoxaparin sodium, and gefitinib were purchased from Dalian Meilun Biotech Co., Ltd. (Dalian, Liaoning, China). The vimentin-specific antibody was from Santa Cruz Biotechnology (Shanghai) Co., Ltd. (Shanghai, China). The antiphosphorylated vimentin antibody (Ser 82) was bought from Medical and Biologic Laboratories Co., Ltd. (Nagoya, Japan). The β-actin and glyceraldehyde-3-phosphate dehydrogenase antibodies were from Sigma-Aldrich (St. Louis, MO). The other antibodies, including pAkt (Ser 473), Akt, phosphorylated extracellular signal-related kinase 1/2 (pErk1/2) (Thr202/Tyr204), and Erk1/2 were purchased from Cell Signaling (Beverly, MA). Dedicator of cytokinesis 1 (DOCK1) antibody was bought from Santa Cruz Biotechnology (Shanghai) Co., Ltd. (Shanghai, China). Human and mouse DOCK1 enzyme-linked immunosorbent assay (ELISA) kits were bought from Cusabio (Wuhan, China).

Cell Culture. A549 cells were cultured in DMEM, and H1975 cells were cultured in RPMI-1640 containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 5% CO₂ in air at 37°C. A549-luc-C8 cells were cultured in an RPMI-1640 medium containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 5% CO₂ in air at 37°C. The cells were treated with different concentrations of gefitinib and enoxaparin for 24 hours and then evaluated as described later.

Wound Closure Assay. Cell migration was assessed by the ability of the cells to migrate into a cell-free area. Briefly, 1 × 10⁶ cells/well in a 2-ml growth medium on six-well plates were grown for 12 or 24 hours to reach confluence. The monolayers were then wounded by scratching with a plastic 200-μl yellow pipette tip. After washing, the cells were incubated for 24 hours in growth medium with or without any treatment and observed under a microscope (Liu et al., 2006). Wound closure was estimated as the inhibitory rate (percentage) compared with that in the control group. Experiments were repeated three times.

Transwell Assay. Cell migration was also assessed using a Transwell apparatus (8.0-μm pore size; Corning, New York, NY) (Rüster et al., 2005). The lower compartment was filled with DMEM containing 10% FBS. The upper compartment was filled with A549 or H1975 cells resuspended in serum-free DMEM. After incubation at 37°C for 24 hours, cells were fixed with 0.5% crystal violet in 20% ethanol (Beyotime, Haimen, China). The migrating cells were photographed in each experiment under a phase contrast microscope using light microscopy. The numbers of the migrating cells were counted by melting crystal violet with 33% glacial acetic acid and detected an optical density value under 570 nm on a microplate reader.

In Vivo Xenograft Experiments. A549-luc-C8 cells that had been engineered to stably express firefly luciferase (Xenogen Corporation, Alameda, CA) were injected into the left flank fold (5 × 10⁶ cells/100 μl per mouse) of 6-week-old BALB/c nude mice weighing 18–20 g, which had been purchased from the Experimental Animal Center of Peking University (Grade II, Certificate No. 11-00-0004). For bioluminescence imaging, mice were anesthetized with 1–3% isoflurane (Abbott Laboratories, Chicago, IL) and given 150 mg/kg of α-luciferin in phosphate buffered saline (PBS) by intraperitoneal injection. A total of 10 minutes after injection, bioluminescence was imaged with a charge-coupled device camera (IVIS, Xenogen). Bioluminescence from relative optical intensity was defined manually, and data were expressed as photon flux normalized to background photon flux, which was defined from relative optical intensity obtained from a control mouse not injected with luciferin. Gefitinib was administered orally via a gastric probe for 26 days, and LMWH was administered subcutaneously three times every week after the inoculation of tumor cells. Mice were randomly divided into four groups (n = 6 per group) and respectively received the vehicle, gefitinib 100 mg/kg per day, enoxaparin 15 mg/kg three times every week, or enoxaparin in combination with gefitinib. On day 26, after anesthesia with ether and scarification, primary tumors and lungs were removed and weighed. The sections of primary tumor tissue used for Western blot were rinsed with cold PBS, frozen in liquid nitrogen, and finally stored at −80°C. Lung colony formation was photographed under microscopy and numbers were calculated.

Animal handling and procedures were approved by the Institutional Animal Care and Use Committee of the Peking University Health Science Center. All animal studies conformed to the principles outlined in the Declaration of Helsinki (Liu et al., 2011).

Whole Proteomics Analysis: One-Dimensional SDS-PAGE. The cells were lysed in a radio-immunoprecipitation assay buffer (Applygen, Beijing, China) and sonicated, and protein concentration was determined by bicinchoninic acid protein assay (Pierce/Thermo Fisher Scientific, Waltham, MA). Protein samples (100 μg) were load on a homemade one-dimensional 10% SDS-PAGE gel (10 × 10 cm, 1.5-mm thick) to fractionate the protein sample. After electrophoresis, the gel was briefly stained with Coomassie Blue. For replicate and comparative analyses, the samples were processed on adjacent migration lanes that were cut simultaneously with a long razor blade. To evaluate gel-to-gel repeatability, different gels were prepared and migrated in parallel, and the same number of homogeneous gel slices was cut and examined. In each experiment under a phase contrast microscope using light microscopy, the proteins were digested overnight with modified trypsin (Promega, Madison, WI) at 37°C. The tryptic peptides were extracted with 5% trifluoroacetic acid/60% acetonitrile. After lyophilization, the
extracted peptides were redissolved in a 0.1% trifluoroacetic acid solution.

Liquid Chromatography–Tandem Mass Spectrometry Analysis. The MS analysis experiments were performed on a nanoflow high-performance liquid chromatography system (Easy-nLC II; Thermo Fisher Scientific) connected to a linear quadrupole ion trap–orbitrap mass analyzer (LTQ-Orbitrap Velos Pro) mass spectrometer (Thermo Fisher Scientific) equipped with a Nanospray Flex Ion Source (Thermo Fisher Scientific). The peptide mixtures were injected (5 μl) at a flow rate of 5 μl/min onto a precolumn (Easy-Column C18-A1, 100-μm i.d. × 20 mm, 5 μm; Thermo Fisher Scientific). The chromatographic separation was performed on a reversed phase C18 column (Easy-Column C18-A2, 75-μm i.d. × 100 mm, 3 μm; Thermo Fisher Scientific) at a flow rate of 300 n/min with a 60-minute gradient of 2–40% acetonitrile in 0.1% formic acid. The electrospray voltage was maintained at 2.2 kV, and the capillary temperature was set at 250°C. The LTQ-Orbitrap was operated in a data-dependent mode to simultaneously measure full-scan MS spectra (m/z 350–2000) in the Orbitrap, with a mass resolution of 60,000 at m/z 400. After the completion of the full-scan survey, the 15 most abundant ions detected in the full MS scan were measured in the LTQ part by collision-induced dissociation. Each sample was analyzed in triplicate (Luber et al., 2010; Wisniewski et al., 2012).

Data Processing and Analysis. The data analysis was performed with MaxQuant software (version 1.4.1.2; Max Planck Institute of Biochemistry, Martinsried, Germany). For protein identification, the MS/MS data were submitted to the Uniprot human protein database using the Andromeda search engine (Cox and Mann, 2008) with the following settings: trypsin cleavage; fixed modification of carboxymethylation of cysteine; variable modifications of oxidation of methionine; and a maximum of two missed cleavages. The false discovery rate was calculated by decoy database searching. Other parameters were set as default. The results were imported into Microsoft Excel (Microsoft, Seattle, WA) for further analysis (Cox and Mann, 2008).

Label-free quantitation was performed using significance analysis of microarrays (Grace and Nacheva, 2012). The minimum ratio count for label-free quantitation was set at 2, and the match-between-runs option was enabled. The delta value was set as 0.06. Other parameters were set to their defaults. The upregulated or downregulated proteins were defined as having a protein ratio with 2-fold change and <5% false discovery rate.

Network Construction. The protein–protein interaction network among those experimentally different proteins obtained from proteomics analysis between the group-administered enoxaparin in combination with gefitinib and the group administered gefitinib alone was initially constructed using the BioGenet plugin of Cytoscape (http://www.cytoscape.org) (Bauer-Mehren, 2013). The direct partners that interacted with the different proteins gained from proteomics analyses were further used as a new query seed to extract another round of partner proteins, and the network was visualized in the Cytoscape environment (http://www.cytoscape.org) (Bauer-Mehren, 2013). Furthermore, duplicated edges and interactions were removed from the network using NetworkAnalyzer plugin of Cytoscape. Finally, the entire network was clustered using the molecular complex detection (MCODE) plugin of Cytoscape based on the MCODE algorithm, as previously reported (Fan et al., 2013).

Western Blot Analysis. Total protein was extracted with a radiolabeled cell lysate buffer and equal amounts of proteins were subjected to SDS-PAGE. The gels were then transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). The membranes were fixed and incubated with primary antibodies overnight at 4°C with gentle agitation, followed by secondary antibody reactions with Dylight 800 conjugated goat anti-mouse or rabbit immunoglobulin G (Cell Signaling).

ELISA. ELISA was performed using a human or mouse DOCK1 ELISA kit according to the manufacturer’s instructions (Cusabio). In brief, the cells were seeded in 12-well plates and cultured to 90–100% confluence. Cells were cultured in a fresh culture medium in the presence of a control solvent or different doses of enoxaparin and gefitinib. After incubation for 24 hours, the cells were lysed and collected. Protein concentration was determined by the bicinchoninic acid assay method. DOCK1 in the cell lysis (100 μl) was determined. Then, optical densities were read at 450 nm, and the level of DOCK1 expression was calculated.

Immunofluorescence Staining. Subconfluent cells grown on a glass coverslip were fixed with 3.7% formaldehyde in PBS for 10 minutes at room temperature. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and washed three times with PBS. To visualize vimentin filaments, cells were stained with vimentin or p-vimentin (Ser82) antibodies overnight at 4°C and observed under a confocal laser scanning microscope (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany).

Small Interfering RNA Transfection. DOCK1 small interfering RNA (siRNA) (Santa Cruz Biotechnology, Dallas, TX) and negative control siRNA were transiently transfected into A549 cells using a siRNA transfection medium and siRNA transfection reagent (Santa Cruz, TX) according to the manufacturer’s instructions. In brief, 2 × 10^6 (2 ml) cells were seeded in six-well plates in a non-FBS fresh culture medium and cultured for 24 hours. Then, siRNA was prepared with the transfection medium and siRNA transfection reagent with a non-FBS, nonantibody culture medium. These were added to cells and 6 hours later changed to a fresh culture medium. After incubation for another 66 hours, the cells were lysed and collected for the detection of DOCK1 expression. To assess cell migration, cells were incubated for a further 24 hours after transfection.

Statistical Analysis. Experiments were repeated a minimum of three times. Data are expressed as mean ± S.E.M. Statistical significance of differences between means was determined by one-way analysis of variance followed by Tukey’s test to compare all pairs of groups (GraphPad Prism version 5 software; GraphPad, La Jolla, CA). A value of P < 0.05 was considered to be statistically significant.

Results

Effect of Enoxaparin and Gefitinib on Tumor Growth and Lung Colony Formation of A549-Luc-C8 Xenograft Tumor-Harboring Nude Mice. It was critical to determine whether the combination of enoxaparin and gefitinib could exert more effective antitumor effects in a human lung cancer A549-luc-C8 model in vivo. As shown in Fig. 1, A and B, throughout the animal experiment, the average tumor size in the group administered enoxaparin (15 mg/kg three times per week) + gefitinib (100 mg/kg per day) was significantly smaller than that in the groups administered enoxaparin alone (15 mg/kg three times per week) or gefitinib alone (100 mg/kg per day) or the control group (each P < 0.01). These results suggest that enoxaparin + gefitinib could produce a more effective inhibition of NSCLC A549 tumor growth in vivo. Importantly, there was no significant difference in the body weight of mice between the control group and any of the treatment groups (P > 0.05), suggesting that the doses of enoxaparin and gefitinib in the current animal experiment were within the safe range (data not shown). Moreover, enoxaparin + gefitinib could inhibit lung colony formation more strongly than either drug administered alone. The number of lung colonies formed decreased more in the combination group than in the group administered gefitinib alone (P < 0.05) and the control group (P < 0.01; Fig. 1, C and D).

Effects of Enoxaparin and Gefitinib in Combination on Cell Migration in Human Lung Cancer A549 and H1975 Cells. The migratory ability of A549 and H1975 cells after treatment with enoxaparin, gefitinib, or combination treatment was assessed using wound closure analysis. As shown in Fig. 2A, in A549 cells, the migration inhibitory rate in the group administered enoxaparin (5–500 μg/ml) + gefitinib
(10^{-6} to 10^{-5} \text{ M}) exceeded 50\%, which was higher than that in the enoxaparin alone, gefitinib alone, and control groups (each P < 0.01) by wound closure analysis. This migration inhibition result was also verified by the Transwell migration experiment with 8-\mu m pore Transwell chambers. The percentages of migrating A549 cells in the group administered a combination of enoxaparin (50 \mu g/ml) + gefitinib (10^{-5} \text{ M}) decreased more than in groups administered either gefitinib alone or enoxaparin alone (each P < 0.05; Fig. 2B). Figure 2, C (wound closure analysis) and D (Transwell migration method) shows the inhibitory effects of enoxaparin combined with gefitinib on the migration of H1975 cells. In the group administered enoxaparin (50 \mu g/ml) + gefitinib (10^{-5} \text{ M}), the migration rate of tumor cells was reduced by approximately 40\%, which was higher than in the enoxaparin alone, gefitinib alone, and control groups (P < 0.01 each).

**Effects of Enoxaparin and Gefitinib in Combination or Alone on Akt and Erk1/2 Protein Expression in Human Lung Cancer A549 and H1975 cells.** Gefitinib is known to be an EGFR–tyrosine kinase inhibitor, and therefore we detected two EGFR-related signal pathways, PI3K/Akt and Raf/Erk1/2, which control tumor cell growth and migration (Ono et al., 2004). Western blot analysis (Fig. 3) showed that gefitinib alone significantly inhibited the pErk1/2 protein
expression of A549 cells ($P < 0.01$) and H1975 cells ($P < 0.01$). Enoxaparin alone had an obvious inhibitory effect on the pErk1/2 protein expression of H1975 cells ($P < 0.01$). However, combined enoxaparin and gefitinib had a greater inhibitory effect on pAkt protein expression than gefitinib alone in both A549 and H1975 cells ($P < 0.05$), while the protein expression of pErk1/2 was not inhibited to a greater extent by the enoxaparin + gefitinib combination than by gefitinib alone ($P > 0.05$) (Fig. 3).

**Quantitative Proteomic Comparison between the Group Administered Enoxaparin in Combination with Gefitinib and the Group Administered Gefitinib Alone.**

A total lysate of A549 cells with drug treatments was used for all experiments and processed in all cases through one-dimensional SDS-PAGE, as shown in Fig. 4A. After separation with SDS-PAGE, gel bands of every sample were cut into 16 bands along the migration lanes, perpendicular to the migration direction. The resulting tryptic digests were analyzed by nanoliquid chromatography–tandem mass spectrometry on an Orbitrap Velos instrument with high sequencing speed. Database searches using MS/MS sequencing data were performed through Mascot (Matrix Science Inc, Boston, MA). Altogether, we identified almost 6000 peptides or proteins in SDS-PAGE gels of samples administered gefitinib alone or enoxaparin in combination with gefitinib. By significance analysis of microarray (Fig. 4B), we identified 16 significantly changed proteins (twofold upregulation or downregulation)
between those two groups: five downregulated proteins and 11 upregulated proteins (Table 1).

**DOCK1 Is a Regulator of the Positive Phenotype of Enoxaparin.** Using Cytoscape software (version 2.8.3), we processed the network construction. The results of the expanded network related to the 16 different proteins that were identified during the proteomics experiment and set as the seed proteins in this network (Fig. 4C). The entire network obtained from the proteomics study was further clustered using the MCODE algorithm. As shown in Fig. 4, D and E, we constructed the subnetwork of clusters in which the cluster rank was counted accounting to the score (cluster 1 scored 3.667, and cluster 2 scored 2.685). In our present study, cluster 1 was identified as a negative subnetwork because of its poor connections with our proteomic seed or node. In comparison with cluster 1, there seemed to be more associations with the positive phenotype of enoxaparin in A549 cells, including DOCK1, GEMIN4, and MRPL15. Among them, DOCK1 was the only downregulated protein influenced by the combination of enoxaparin and gefitinib. This protein has previously been reported to play an important role in tumor cell growth, migration, and metastasis (Jarzynka et al., 2007; Laurin et al., 2013).

**Effects of Enoxaparin and Gefitinib in Combination or Alone on DOCK1 Expression in Tumor Tissues of Nude Mice with A549-Luc-C8 Cells and A549 Cells.** Proteomics and bioinformational analysis showed that DOCK1 may be the key node protein specially regulated by enoxaparin in combination with gefitinib. Then, using ELISA, we verified its downregulation in the group administered enoxaparin + gefitinib compared with the group administered gefitinib alone, both in vivo and in vitro (Fig. 5). In tumor tissue of mice xenografted with A549-luc-C8 cells, the group administered enoxaparin + gefitinib had more effective inhibition of DOCK1 expression than the control group and the gefitinib alone group. In A549 cells, it was also found that the enoxaparin + gefitinib group had more effective inhibition of DOCK1 expression than the control group and the gefitinib alone group.
group (each $P < 0.05$). In A549 cells, gefitinib alone had no inhibitory effect on DOCK1 expression ($P > 0.05$).

**Effects of Enoxaparin and Gefitinib in Combination or Alone on Vimentin Protein Expression and Distribution in A549 Cells.** DOCK proteins regulate the cytoskeleton, cell migration, and epithelial-to-mesenchymal transition (EMT). DOCK1 has been found to be important in tumor cell morphology, invasion, and migration (Cote and Vuori, 2007; Yan et al., 2013). Vimentin, a major constituent of the cytoskeleton intermediate filament (IF) family of proteins, plays an integral role in the progression of lung cancers, including cell morphology, invasion, migration, and EMT. In our previous studies, we...
found that vimentin could be regulated by LMWH and played a critical role in the antiangiogenesis and antimetastasis effects of LMWH (Pan et al., 2011). In the present study, we found that vimentin activity was influenced in the group administered enoxaparin with gefitinib in A549 cells (Fig. 6). That is, p-vimentin (Ser82) expression was decreased significantly in enoxaparin + gefitinib compared with either the group administered gefitinib alone or the control group (P < 0.05), although there was no more significant decrease in vimentin expression between the enoxaparin + gefitinib combination and gefitinib administered alone (P > 0.05). According to immunofluorescence studies, in control A549 lung cells, vimentin formed an extended network from the perinuclear region to the cell periphery. After treatment with gefitinib, a marked change in the organization of the vimentin network was observed (Fig. 6B). Vimentin filaments were aggregated into more dense structures in cells rather than being spread throughout the untreated control cells. Immunofluorescence studies showed that the dispersed p-vimentin filaments present in untreated cells aggregated into dense structures in the perinuclear area after enoxaparin + gefitinib treatment (Fig. 6B).

Effects of DOCK1 siRNA on A549 Cell Migration, AKT, and Vimentin Expression. To confirm a causal link between DOCK1 expression downregulation mediated by the combination of enoxaparin with gefitinib and decreased migration, A549 cells were transfected with DOCK1 siRNA. Figure 7A shows that 10 μM DOCK1 siRNA decreased DOCK1 expression by 60% compared with the controls (P < 0.01). The nonsilencing siRNA had no effect on DOCK1 expression. Both DOCK1 siRNA and the nonsilencing siRNA exerted no cytotoxic effect on A549 cells (data not shown). Notably, knockdown of DOCK1 expression led to a significant decrease of migration in A549 cells (P < 0.05), suggesting that enoxaparin + gefitinib prevents A549 cell migration through downregulation of DOCK1 (Fig. 7B).

We had already shown the downregulation of p-AKT and p-vimentin expression in A549 cells treated with enoxaparin + gefitinib. Therefore, we investigated whether this down-regulation was linked with DOCK1 expression. Knockdown of DOCK1 expression led to the significant decrease of p-AKT (P < 0.01) and p-vimentin (P < 0.05) expression in A549 cells (Fig. 7C).
In this study, we demonstrated that the combined administration of enoxaparin and gefitinib inhibited cell migration of A549 and H1975 cells in vitro and tumor growth of A549-luc-C8 xenograft tumors in immunodeficient mice in vivo.

Drug resistance remains a major obstacle to successful NSCLC treatment with EGFR-TKI-based therapies, such as gefitinib, including de novo resistance and acquired resistance (Girnun et al., 2008; Park et al., 2010). LMWHs, including enoxaparin, are anticoagulants. Preliminary clinical studies and meta-analyses suggest that beyond preventing venous thromboembolism accompanied with cancer, the use of LMWH confers a potential survival benefit in cancer populations. Preclinical data support the effects of LMWH on processes involved in tumor growth, angiogenesis, and metastasis (Walenga and Lyman, 2013). In the present study, we investigated whether enoxaparin enhances the antitumoral effect of gefitinib in human NSCLC A549 and H1975 cells. The combined treatment decreased the migration of A549 and H1975 cells in vitro. A combination of 50 μg/ml enoxaparin and 10^{-5} M gefitinib caused 60% inhibition of A549 cell migration, which is higher than the 17% for 50 μg/ml enoxaparin alone or the 11% for 10^{-5} M gefitinib alone. No changes in A549 viability or shape were observed at this dose, indicating that this dose is not cytotoxic (data not shown). The same inhibitory effects occurred with H1975 cell migration. Strikingly, in vivo, enoxaparin + gefitinib had a stronger inhibitory effect on the growth of A549-luc-C8 xenograft tumors in immunodeficient mice than did the individual agent alone, suggesting that enoxaparin + gefitinib exerts more effective antitumor activity than monotherapy. This combination may be an effective strategy for the sensitization of gefitinib treatment and the prevention of NSCLCs in vivo.

In this study, enoxaparin + gefitinib did not cause systemic toxicity since both the body weights and overall appearance of animals being administered the combination of drugs were similar to the mice receiving gefitinib alone.

**Fig. 6.** Effects of enoxaparin (L), gefitinib (G), and their combination (L+G) on the expression and distribution of vimentin and p-vimentin (Ser82). (A) A549 cells were treated for 24 hours with 50 μg/ml enoxaparin, 1 × 10^{-5} M gefitinib, or their combination. The changes in vimentin and p-vimentin were analyzed by Western blot. Mean ± S.E.M. *P < 0.05 compared with the control group; **P < 0.05 compared with the gefitinib group. The experiment was performed three times. (B) Immunofluorescence analysis of vimentin (red fluorescence) and p-vimentin (green fluorescence) in A549 cells after treatment of 24 hours with 50 μg/ml enoxaparin, 1 × 10^{-5} M gefitinib, or their combination. C, control.
Previous findings indicated that unfractionated heparin and LMWH has a potential role in reducing multidrug resistance in cancer patients by modulation of drug transport (Chen et al., 2014). LMWH combined with cisplatin overcame cisplatin resistance both in vitro and in vivo (Niu et al., 2012). However, our data demonstrate that enoxaparin substantially enhances the efficacy of gefitinib, potentially causing tumor inhibition, and may demonstrate a significant advance in EGFR-TKI treatment of NSCLCs.

EGFR-TKIs are thought to function through selective binding to the tyrosine kinase domain on EGFR and suppressing its two major downstream signaling pathways, prosurvival and anti-apoptosis, including Akt and Erk1/2 (Ono et al., 2004). Aberrant signaling through the PI3K/Akt pathway is thought to contribute to gefitinib resistance in NSCLC treatment (Jeannot et al., 2014). Akt activation impairs the efficacy of gefitinib (Wu et al., 2013). Inhibition of Akt signaling by the PI3K inhibitor LY294002 and wortmannin can eliminate the resistance of other NSCLC cells to gefitinib, producing a significant induction of apoptosis and inhibition of cell proliferation (Maseki et al., 2012; Wu et al., 2013). Nevertheless, mitogen-activated protein kinase/Erk1/2 pathway inhibition, using the specific inhibitors PD98059 [2-(2-amino-3-methoxyphenyl)-4H-chromen-4-one] or U0126 [(2Z,3Z)-2,3-bis(amino(2-aminophenylthio)methylene) succinonitrile], did not significantly enhance levels of gefitinib-induced apoptosis (Jeannot et al., 2014), although prolonged exposure to Erk1/2 inhibitors may heighten therapeutic responses to EGFR inhibitors (Buonato and Lazzara, 2014).

Here, enoxaparin + gefitinib inhibit Akt activation in A549 and H1975 cells more effectively than gefitinib alone, while there was no difference in Erk1/2 activation between the enoxaparin + gefitinib combination and gefitinib alone, which also suggested that the decrease in Akt activation is related closely to enoxaparin-sensitized tumor inhibition in A549 and H1975 cells. Akt is a potential intracellular target for the effects of LMWHs and EGFR-TKI on NSCLC tumor treatment.

Fig. 7. SiRNA interferes with DOCK1 expression in A549 cells. Nonsilencing siRNA (si-Control) was used as a negative control. DOCK1-targeted siRNA (si-DOCK1) was transfected into A549 cells. The total protein was extracted with a protein lysis buffer, and protein concentrations were measured using the bicinchoninic acid assay. (A) After transfection for 66 hours, cell were lysed before Western blot analyses or fixed for immunofluorescence analysis to detect the efficiency of the DOCK1 knockdown. (B) The effect of this DOCK1 knockdown on A549 cell migration was detected by Transwell analysis 24 hours after transfection. (C) The influence of this DOCK1 knockdown on A549 cell AKT and vimentin expression was detected by Western blot using the AKT and vimentin antibody after transfection for 24 hours; n = 3; mean ± S.E.M. *P < 0.05 and **P < 0.01 compared with the control; #P < 0.05 and ##P < 0.01 compared with the gefitinib group. Each independent experiment was performed three times. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
In this study, we also used a global and powerful whole proteomics analysis with LC-MS techniques to elucidate the molecular mechanisms by which enoxaparin + gefitinib functions to inhibit cancer cell migration. We identified 16 differentially expressed candidate proteins from the whole proteomic data to elucidate the molecular mechanisms underlying the antimigration activity of enoxaparin in combination with gefitinib. Network analysis with the Cytoscape database identified the guanine nucleotide exchange factor family member DOCK1 (Dock180) protein, which is strongly related to cell invasion and migration (Jarzynka et al., 2007), cell growth, and Akt activation (Dun et al., 2013). DOCK1 is essential in multiple biologic processes involving cell migration and cell survival (Raftopoulou and Hall, 2004; Jarzynka et al., 2007; Li et al., 2013). Furthermore, this effect of DOCK1 in promoting cell migration was confirmed by the oncogenic receptor tyrosine kinase, including EGFR (Laurin et al., 2013). In this study, we reconfirmed the inhibitory effect of enoxaparin + gefitinib on DOCK1 expression in vitro and in vivo and on the basis of proteomic and in silico studies. In this current study, the reduced protein expression of DOCK1 in tumor tissues of mice with A549-luc-C8 cells was significant (Fig. 5A); however, the decrease was only approximately 10–15%. DOCK1 is an important effector protein for many membrane signaling molecules. DOCK1 is activated by and interacts with some receptors and tyrosine kinases in membranes to promote various cellular processes, which suggests that it is a potential treatment target against cancer metastasis. After its binding to ligands, surface receptors recruit the DOCK1 protein through adaptor molecules. When DOCK1 is recruited to the plasma membrane from a cytoplasmic reservoir, changes in cellular morphology occur. Besides this distribution of traffic to the membrane by DOCK1, the phosphorylation of DOCK1 is important to activate related downstream pathways (Laurin et al., 2013; Feng et al., 2014; Gadea and Blangy, 2014). The distribution of DOCK1 and phosphorylated DOCK1 in the membrane and cytoplasm of cancer cells will be clarified in our future work.

In siRNA studies of A549 cells, with DOCK1, expression levels were reduced, while cell migration and Akt activation were abrogated. Although some studies have found that DOCK1 is important in tumor cell function, including cell migration and invasion (Wang et al., 2010), this is the first demonstration of the relationship of DOCK1 with cell migration and gefitinib treatment function in NSCLC A549 cells and establishes DOCK1 as a key target for the effects of enoxaparin and gefitinib in the treatment of NSCLC A549. Interestingly, this downregulation of DOCK1 expression by enoxaparin + gefitinib is consistent with the downregulation of pAkt expression. In H9C2 cardiomyocytes, DOCK1 exerts its function via the activation of its downstream signaling molecule Akt (Dun et al., 2013; Yan et al., 2013). Therefore, we proved that enoxaparin substantially enhances the efficacy of gefitinib by inhibiting Akt activation via the suppression of DOCK1 expression.

DOCK1 also regulates cytoskeleton polymerization (Cote and Vuori, 2007; Yan et al., 2013). Vimentin, a major constituent of the cytoskeleton IF family of proteins, plays an integral role in the progression of lung cancers, including cell migration and invasion, EMT, regulation of major signal transduction pathways, the positioning and anchorage of organelles, such as mitochondria, and cell-cell and cell-substrate adhesion (Tzivion et al. 2000; Mendez et al., 2010; Kidd et al., 2014). Our previous studies (Pan et al., 2011) have shown that vimentin intermediate filaments are an important factor in the antiadhesion and migration effects of LMWH in tumor cells that travel to the endothelium. In the present study, enoxaparin + gefitinib produce a better effective inhibitory effect on p-vimentin expression, while the expression of the vimentin protein showed no significant decrease after enoxaparin + gefitinib treatment. Intermediate filaments are highly dynamic structures in living cells. The protein subunits are continually undergoing a reversible cycling of disassembly and reassembly in response to a variety of stimuli. There is accumulating evidence that the organization of intermediate filaments is regulated by phosphorylation. Changes in the phosphorylation of the intermediate filaments have been suggested as a potential mechanism to modulate their assembly and distribution (Steinert et al., 1993; Sin et al., 1998; Valgeirsdottir et al., 1998; Satelli and Li, 2011; Cogli et al., 2013). Using immunofluorescence analyses, we found that enoxaparin + gefitinib treatment redistributed p-vimentin to the perinuclear area. Furthermore, p-vimentin expression levels decreased after enoxaparin + gefitinib treatment to some extent as

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**Fig. 8.** Schematic model depicting the proposed mechanism of enoxaparin + gefitinib–mediated regulation of A549 cell migration. From the present studies, enoxaparin sensitizes the antitumor function of gefitinib by inhibiting DOCK1 expression via the suppression of Akt activation and the vimentin phosphorylation signaling pathway in NSCLC A549 cells.
a result of the inhibition on tumor cell migration by DOCK1. Furthermore, in our DOCK1 siRNA studies, p-vimentin expression, which has been shown to enhance tumor and metastasis growth (Zhu et al., 2011), was reduced in accordance with the siRNA-mediated inhibition of DOCK1 expression.

As other studies have proved, Akt activation induces other tumor cell motility and invasiveness partially via interaction with vimentin. The binding of Akt to vimentin results in vimentin phosphorylation, enhancing the ability of vimentin to induce motility and invasion while protecting vimentin from caspase-induced proteolysis (Zhu et al., 2011). Thus, it can be speculated that the more effective inhibitory effect on p-vimentin produced by enoxaparin + gefitinib involves control of the Akt pathway in NSCLC A549 cells. The IP proteins have recently been recognized as regulators of signal transduction events. Activation of an Akt–14-3-3vimentin phosphorelay pathway mediates multidrug-resistant invasive behavior in other cells (Maxwell et al., 2011). From the results of our study, it can be deduced that enoxaparin sensitizes the antitumor function of gefitinib by inhibiting DOCK1 via suppression of Akt activation and the vimentin phosphorylation signaling pathway in NSCLC A549 cells (Fig. 8).

Our recent study showed that unfractionated heparin does not easily cross the cell membrane (Pan et al., 2011). Therefore, it is possible for enoxaparin to exert its function through proteins present on the cell surface, including DOCK1, which could be translocated across the plasma membrane (Kobayashi et al., 2001). It has been reported that the DOCK1 inhibitor blocked Rac activation and migration, and is therefore implicated as a next-generation drug for clinical use in combination with EGFR-TKI regimens to limit metastasis (Laurin et al., 2013). Enoxaparin regulation of DOCK1, identified by proteomics and network analysis in the present study, is implicated as the key node-targeted protein in the gefitinib-sensitized effect.

In conclusion, our results suggest that the combination of enoxaparin and gefitinib is a promising and useful therapeutic strategy to sensitized the effect of gefitinib on NSCLC A549 and H1975 cells by inhibiting DOCK1 via suppressing Akt tumor cell activation and vimentin phosphorylation. Enoxaparin targets Akt cell growth and migration, thus potentially antagonizing gefitinib resistance and reducing side effects.

Authorship Contributions

Participated in research design: X. J. Li, Pan.
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References


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