Overexpression of Napsin A resensitizes drug-resistant lung cancer A549 cells to gefitinib by inhibiting EMT

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Abstract. Lung cancer is one of the most common malignant tumors and also the leading cause of cancer-related deaths in the world. Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI), such as gefitinib, have been used in the therapy of lung cancer. However, the acquisition of drug resistance is a major limitation in the clinical efficiency of EGFR-TKIs. Epithelial-mesenchymal transition (EMT) has been demonstrated to be an underlying mechanism of acquired resistance. A previous study has reported that Napsin A expression can inhibit EMT in lung cancer cells. The present study therefore investigated the effect of Napsin A on the sensitivity of EGFR-TKI-resistant lung cancer cells. First, a drug-resistant lung cancer cell line was generated using the EGFR-TKI gefitinib on A549 cells (termed here A549-GFT). EMT was demonstrated to be induced in the drug resistant A549-GFT cells, evidenced by reduced E-cadherin expression and increased Vimentin expression compared with control A549 cells. Next, Napsin A was overexpressed in the cells by transfection of the Napsin A-expression vector, PLJM1-Napsin A. Western blot analysis confirmed that the protein expression levels of Napsin A were significantly elevated in the Napsin A-overexpressing cells. Cell proliferation and apoptosis assays were performed to evaluate the effect of Napsin A overexpression on resistant A549 cells. The results of MTT assay demonstrated that Napsin A overexpression inhibited the proliferation of A549 and drug-resistant A549-GFT cells and that the proliferation of Napsin A-overexpressing A549-GFT cells was significantly inhibited by gefitinib treatment compared with control A549-GFT cells. The results from the Annexin V/propidium iodide double staining apoptosis assay indicated that Napsin A overexpression enhanced gefitinib-induced apoptosis in A549-GFT cells. Additionally, EMT was reversed following Napsin A expression in A549-GFT cells, as evidenced by the restoration of E-cadherin and downregulation of Vimentin expression. Further investigation demonstrated that Napsin A overexpression resulted in inhibition of focal adhesion kinase, a critical factor in integrin signaling, in the resistant A549-GFT cells. These data suggested that Napsin A resensitized the drug-resistant A549-GFT cells to gefitinib, possibly by reversing EMT via integrin signaling inhibition. Therefore, Napsin A combined with a TKI may be a more effective treatment strategy for lung cancer.

Introduction

Lung cancer is one of the most common malignant tumors, exhibiting the highest mortality worldwide due to the uncontrolled cell growth in tissues of the lung and high metastatic ability (1). Non-small cell lung cancer (NSCLC) accounts for 80-85% of lung cancer (2). EGFR receptor tyrosine kinase inhibitors (EGFR-TKI), such as gefitinib, have demonstrated notable therapeutic efficacy in non-small cell lung cancer patients (3,4). However, nearly all patients develop drug resistance after a period of EGFR-TKI treatment, which eventually limits the application of EGFR-TKI (5). Therefore, developing strategies to overcome drug resistance is significant for improving prognosis and survival in the future.

A variety of molecular mechanisms are involved in the acquisition of TKI resistance, including epithelial-mesenchymal transition (EMT) (6). EMT is a cellular process characterized by morphologic changes from an epithelial phenotype to a mesenchymal phenotype, by which cells lose epithelial cell-cell adhesions and gain the ability to move through the extracellular matrix. EMT is often associated with enhanced proliferation, invasion and metastasis in cancer cells (7). During EMT, cells exhibit the acquisition of mesenchymal markers, including Vimentin, type I collagen, fibronectin and Snail, and the inhibition of cell adhesion molecules, such as E-cadherin and catenin (8). Additionally, EMT is correlated with chemotherapeutic sensitivity in several types of cancer and sensitivity to EGFR-TKI in lung cancer cells, in particular (9-11).
Materials and methods

Cell culture. The human NSCLC cell line A549 was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences Institute (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Lonza Group, Ltd., Basel, Switzerland), 5 mM L-glutamine, 5 mM non-essential amino-acids, 100 U/ml penicillin and streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), in a humidified 5% CO₂ incubator at 37˚C. In preliminary experiments, A549 cells were detected to be sensitive to gefitinib and exhibited E-cadherin and vimentin expression, which facilitates the analysis of the EMT phenotype in response to Napsin A overexpression; whereas vimentin expression in H322, H358 and H441 lung cancer cells was negative or in trace amounts (data not shown). In addition, Napsin A expression levels in parental A549 cells are lower compared with other lung cancer cell lines (data not shown). Therefore, the A549 cell line was selected for the present study, with the hypothesis that exogenous overexpression of Napsin A in A549 cells might result in more significant change in biological function.

Development of the gefitinib-resistant A549 cell line. Gefitinib-resistant A549 cells (A549-GFT) were developed by exposing A549 cells to increasing concentration of gefitinib ([also known as Iressa and ZD1839; Bioruler, Beijing Bioway Biotechnology Co. Ltd., Beijing, China (http://a0002945.casmart.com.cn/)] ranging from 0.2 to 2 µmol/l in complete medium (20)). Following exposure to increasing concentration of gefitinib for at least 3 months, live cells that developed resistance to 5 µmol/l gefitinib were collected, termed A549-GFT and used in subsequent experiments.

Morphological observation of gefitinib-resistant cells. Cells were cultured in 60 mm culture dishes for 24 h, and then treated with or without 2 µmol/l gefitinib for 48 h. The medium was then removed and cells were washed once with RPMI-1640 medium. Cell morphology was observed and photographed using a vertical microscope (Olympus Corporation, Tokyo, Japan).

Western blot analysis. Cells were treated with different doses of gefitinib as indicated. The cells were then washed twice with PBS and total protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's manual. The proteins (50 µg) were separated by 12% SDS-PAGE (Beijing Solarbio Science & Technology Co., Ltd.) and transferred to polyvinylidene fluoride membranes (Millipore; Merck KGaA, Darmstadt, Germany). The membranes were blocked with 5% non-fatty milk in TBST for 1 h, and incubated with the following specific primary antibodies overnight at 4˚C: Mouse monoclonal anti-E-cadherin (cat. no. sc-21791; 1:2,000), mouse monoclonal anti-Vimentin (cat. no. sc-6260; 1:2,000), anti-GAPDH (cat. no. sc-365062; 1:3,000; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit polyclonal anti-FAK (cat. no. 3285; 1:1,000), rabbit monoclonal anti-phospho-FAK (Tyr397; cat. no. 8556; 1:1,000; both Cell Signaling Technology, Inc., Danvers, MA, USA) and rabbit monoclonal anti-Napsin A (cat. no. ab133249; 1:10,000; Abcam, Cambridge, UK). Membrane were then incubated with horseradish peroxidase-conjugated secondary antibodies goat anti-mouse (cat. no. sc-2005; 1:2,000) and goat anti-rabbit IgG (cat. no. sc-2004; 1:2,000; both Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Development was performed using enhanced chemiluminescence detecting reagent (Amersham; GE Healthcare, Chicago, IL, USA). The protein blots were quantified by densitometry using QuantityOne software (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the amounts were expressed relative to the internal reference GAPDH.

Overexpression of Napsin A. The Napsin A-overexpressing plasmid, PLJM1-Napsin A, was constructed by cloning the full-length human Napsin A cDNA into the PLJM1 lentivirus vector (Addgene, Cambridge, CA, USA). cDNA was amplified using reverse transcription-polymerase chain reaction (RT-PCR). The PLJM1-Napsin A or the empty vehicle plasmid was cotransfected with pVSVG and pGag-pol plasmids (Clontech Laboratories, Inc., Mountainview, CA, USA) into HEK293T cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) to produce virus, according to the manufacturer's manual. The lentivirus supernatant was harvested 48 h post-transfection and used to infect A549 cells in order to obtain Napsin A stable overexpressing cells. Puromycin (Sigma-Aldrich; Merck KGaA) was used to screen positive clones.

Proliferation assay. Cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Aldrich; Merck KGaA) assay. A total of 2,000 cells were seeded into each well of a 96-well plate in 100 µl of medium and incubated with or without various concentrations of gefitinib for different time periods at 37˚C in a 5% CO₂ incubator. Then cells were incubated with 20 µl of 5 mg/ml MTT for 4 h, and then cells were lysed for 10 min by addition of 200 µl dimethyl sulfoxide. Absorbance was measured at
490 nm using a Rainbow microplate reader (Tecan Group Ltd., Mannedorf, Switzerland). Cell proliferation was expressed as a % of the untreated control.

**Apoptosis assay.** Cells were grown to 80% confluence and treated with 5 µmol/l gefitinib for 48 h. Apoptosis was analyzed using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's instructions. The amount of phosphatidylserine on the outer surface of the plasma membrane (a biochemical alteration unique to membranes of apoptotic cells) and the amount of PI (a dye that easily enters dead cells or cells in the late stages of apoptosis and binds DNA, but does not bind the plasma membrane of viable cells) were detected by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA, USA). Data were analyzed using CellQuest software (BD Biosciences). Cells with phosphatidylserine on their surface were considered to be apoptotic.

**Statistical analysis.** All experiments were repeated at least in triplicate. Statistical analysis was performed with SPSS 11.0 (SPSS, Inc., Chicago, IL). Values are presented as the mean ± standard error of the mean. One-way analysis of variance was used to assess differences between groups. The Duncan's new multiple range test was subsequently employed for pairwise comparisons followed by the Bonferroni correction. *P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Development of gefitinib-resistant A549 lung cancer cell line.** To investigate the mechanism underlying gefitinib resistance in lung cancer, a gefitinib-resistant lung cancer cell model (A549-GFT) was first constructed by exposing the A549 lung cancer cell line to increasing concentrations of gefitinib. Cell growth was assessed using MTT assay and growth curves of A549-GFT and A549 cells were calculated. The data demonstrated that A549-GFT cells grew faster compared with A549 cells at the same time interval (Fig. 1A). Additionally, the IC_{50} values of the two cells were respectively calculated to be 11.5 and 5.1 µmol/l (Fig. 1B). Further analysis of the gefitinib-resistant cells revealed that A549-GFT cells exhibited an EMT phenotype, characterized by spindle-like morphology, decreased E-cadherin expression and increased Vimentin expression (Fig. 1C and D). Finally, the two cell lines were treated with 2 µmol/l gefitinib for 48 h and their morphology was inspected by microscopy. As expected, A549 cells appeared round and with obvious blebbing, which are features of apoptosis (Fig. 1C). However, no significant changes in A549-GFT morphology were observed (Fig. 1C).
following gefitinib treatment, confirming that the cells were gefitinib-resistant.

*Napsin A overexpression resensitizes resistant A549 cells to gefitinib*. To examine the effect of Napsin A on gefitinib resistance in lung cancer cells, the PLJM1-Napsin A expression vector was constructed and used to increase the expression of Napsin A. Western blot analysis confirmed that the Napsin A expression levels in the PLJM1-Napsin A-transduced A549 and A549-GFT cells were markedly elevated compared with the control cells (Fig. 2A). Next, A549 and A549-GFT cells were transiently transduced with empty control vector or PLJM1-Napsin A expression vector, and cultured in the presence or absence of 2 µmol/l gefitinib for 48 h. Cell proliferation was then analyzed by MTT assay. The results demonstrated that cell proliferation of Napsin A-overexpressing A549-GFT cells was significantly suppressed by gefitinib treatment compared with gefitinib-treated control A549-GFT cells (P=0.006; Fig. 2B). The growth of control A549-GFT cells was not nearly affected by gefitinib treatment and exhibited significantly increased survival compared with the parental A549 cells (P=0.019; Fig. 2B). However, under identical experimental conditions, there was no significant difference in the proliferation between Napsin A-overexpressing and control A549 cells (Fig. 2B).

*Napsin A overexpression promotes gefitinib-induced apoptosis in A549-GFT cells*. To clarify the mechanism by which Napsin A overexpression resensitized the resistant A549-GFT cells to gefitinib, apoptosis was evaluated by the Annexin V-FITC/PI assay. A549-GFT and A549 cells were transduced with empty control and Napsin A-expressing vector, and exposed to 2 µmol/l gefitinib for 48 h. The data indicated that gefitinib treatment induced the apoptosis of both Napsin A-overexpressing and control A549 cells compared to the corresponding untreated control cells (P=0.006 and P=0.009, respectively; Fig. 3A). Notably, Napsin A overexpression significantly stimulated the gefitinib-induced apoptosis in A549-GFT cells compared with the A549-GFT cells transduced with the empty vector (P=0.005; Fig. 3A). However, gefitinib treatment did not significantly induce apoptosis in empty vector-transduced A549-GFT cells compared with empty vector-transduced parental A549 cells (Fig. 3A). These results suggest that Napsin A may be correlated with the resistance of lung cancer A549 cells to gefitinib and Napsin A expression may increase the sensitivity of resistant cells to gefitinib by enhancing gefitinib-induced apoptosis.

Additionally, the protein expression levels of E-cadherin, Vimentin and FAK were detected, because they serve a critical role in integrin-mediated signaling pathway and are correlated with cell transformation (21-23). Autophosphorylation of FAK at residue Tyr397 results in the activation of the integrin signaling pathway. The results demonstrated that Napsin A overexpression rescued the expression of E-cadherin and blocked the upregulation of Vimentin in A549-GFT cells (Fig. 3B). Furthermore, Napsin A overexpression resulted in an inhibition of FAK expression, as well as its phosphorylation at Tyr397 in resistant A549-GFT cells (Fig. 3B). These findings suggested that Napsin A reversed the EMT and enhanced the sensitivity of A549-GFT cells to gefitinib, potentially through the inhibition of FAK-mediated integrin signaling.

**Discussion**

Lung cancer, a common malignancy with very high mortality, seriously threatens human health (1). The majority of lung cancers are diagnosed as NSCLC (2). EGFR-TKIs, such as gefitinib, have been used as the standard therapy in NSCLCs with EGFR-activating mutations (3,4). However, nearly all patients eventually succumb to recurrence due to developing drug resistance; therefore the therapeutic efficacy of EGFR-TKIs is significantly limited. Therefore, exploring novel therapeutic strategies to overcome the acquisition of EGFR-TKI resistance is necessary. The present study demonstrated that Napsin A was able to attenuate the drug resistance of lung cancer A549 cells to gefitinib and Napsin A in combination with gefitinib significantly inhibited cancer cell growth in vitro.

In the present study, gefitinib-resistant lung cancer A549-GFT cells were firstly generated by simulating the condition of drug resistance development in vivo, using an increasing concentration of gefitinib over a long period of time. Results from a cell proliferation assay revealed that the IC_{50} of gefitinib in the drug-resistant A549-GFT cells at 48 h was markedly increased compared with the parental A549 cells. In addition, gefitinib treatment at 2 µmol/l concentration did not significantly affect the proliferation of A549-GFT cells, while under identical conditions, the proliferation of the parental A549 cells was reduced by ~80%. These findings confirmed that A549-GFT cells were resistant to gefitinib and
A overexpression significantly enhanced the sensitivity to gefitinib in A549-GFT cells. Further investigation needs to be performed in order to test this hypothesis.

In conclusion, the present data demonstrated that Napsin A overexpression in resistant A549-GFT cells. FAK has an important role in integrin signaling (21,25-27). FAK gets activated by integrin and subsequently regulates several signaling pathways, including signal transducer and activator of transcription 1 (STAT1), mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling (21-23), resulting in the initiation of cell proliferation and transformation. Integrin aggregation by ligand binding results in the oligomerization of FAK, which is mediated by talin. Autophosphorylation of FAK at residue Tyr397 results in the binding of the SH2 domain of Src and Fyn, which then phosphorylate a number of FAK-associated proteins, including paxilin, tensin and the docking protein p130CAS (Crk-Associated Substrate). In addition, phosphorylation of Tyr397 leads to the recruitment of other SH2-containing proteins, including PI3K, phospholipase-Cγ and the adapter protein growth factor receptor-bound protein 7 (GRB7) (5), resulting in the activation of the integrin signaling pathway (28). The carboxyl terminal of the Napsin A contains an Arg-Gly-Asp (RGD) sequence, which recognizes and binds integrins on the cell surface (29). Integrins mediate cell adhesion and signal transduction between cells and the extracellular matrix (ECM) (30), and they are an important regulator of cell proliferation, apoptosis, migration, and metastasis (31,32). In the present study, it may be possible that Napsin A repressed the interaction between integrins and ECM through RGD sequence-mediated interaction with integrin, thus hindering integrin signaling pathway and inhibiting cell proliferation and transformation by downstreaming FAK expression and phosphorylation in gefitinib-resistant A549-GFT cells. Further investigation needs to be performed in order to test this hypothesis.

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Consent for publication

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