

Napsin A is negatively associated with EMT-mediated EGFR-TKI resistance in lung cancer cells

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Abstract. Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) have been used as a standard therapy for patients with lung cancer with EGFR-activating mutations. Epithelial-mesenchymal transition (EMT) has been reported to be associated with the development of EGFR-TKI resistance, which limits the clinical efficacy of EGFR-TKI. Therefore, investigating the resistance-associated mechanism is required in order to elucidate an effective therapeutic approach to enhance the sensitivity of lung cancer to EGFR-TKI. In the present study, EGFR-TKI erlotinib-sensitive H358, H322 and H441 lung cancer cells, erlotinib-moderately sensitive A549 cells, and erlotinib-insensitive HCC827 cells with EGFR-mutation (exon 19 deletion) were used to detect the mRNA and protein expression of the EMT-associated proteins E-cadherin and vimentin, and napsin A, by reverse transcription-quantitative polymerase chain reaction analysis and western blotting. It was observed that the E-cadherin expression level in erlotinib-sensitive cells was increased compared with the moderately sensitive A549 cells and HCC827 cells; however, vimentin exhibited opposite expression, suggesting a correlation between EMT and erlotinib sensitivity in lung cancer cells. The napsin A expression level was observed to be positively associated with erlotinib sensitivity. In addition, napsin A highly-expressing H322 cells were used and napsin A-silenced cells were constructed using small interfering RNA (siRNA) technology, and were induced by transforming growth factor (TGF)- β 1. It was observed that TGF- β 1 partially induced the alterations in E-cadherin and vimentin expression and the

occurrence of EMT in napsin A highly-expressing cells, while TGF- β 1 significantly induced EMT via downregulation of E-cadherin and upregulation of vimentin in napsin A-silenced cells; cell proliferation and apoptosis assays demonstrated that TGF- β 1 induced marked resistance to erlotinib in napsin A-silenced cells compared with napsin A-expression cells. These data indicated that napsin A expression may inhibit TGF- β 1-induced EMT and was negatively associated with EMT-mediated erlotinib resistance, suggesting that napsin A expression may improve the sensitivity of lung cancer cells to EGFR-TKI through the inhibition of EMT.

Introduction

Lung cancer is one of the most common malignant tumors in the world, exhibiting a high mortality rate due to abnormal cell proliferation and a high metastasis rate (1). Small-molecule epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI), including erlotinib, exert a marked clinical effect in lung cancer with EGFR-activating mutations, and have been used as a standard therapy in the patients with lung cancer (2,3). However, the majority of patients develop drug resistance following a period of treatment with EGFR-TKI, which markedly limits the therapeutic efficacy of EGFR-TKI (4). Therefore, investigating the resistance-associated mechanism is required to explore an effective therapeutic approach to enhance the sensitivity of lung cancer to EGFR-TKI.

Epithelial-mesenchymal transition (EMT) is known to be a molecular mechanism underlying the acquisition of TKI resistance (5). Cells undergo morphological alterations from an epithelial phenotype to a mesenchymal phenotype during EMT, lose epithelial cell-cell adhesion and are able to move through the extracellular matrix, leading to increased proliferation, invasion and metastasis (6). EMT has been frequently reported to be activated during cancer metastasis in multiple types of human cancer (7,8). EMT is characterized by the loss of cell adhesion molecules, including catenin and E-cadherin, and the acquisition of mesenchymal marker proteins including zinc finger protein SNAI1, fibronectin, type I collagen, and vimentin (9). EMT has been demonstrated to be involved in the sensitivity of cancer cells to conventional chemotherapies (10,11). In addition, sensitivity to EGFR-TKI is additionally regulated by EMT in lung cancer cells (12). TGF- β 1 is frequently used to drive the EMT process and induce

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resistance to EGFR-TKI in lung cancer cells (13). Examining novel factors affecting EMT is important to prevent the development of EGFR-TKI resistance.

Napsin A has been identified to be a novel member of the aspartate protease family (14), and was observed to be correlated with the maturation of the spleen, kidney and lung, in addition to surfactant synthesis in the lung (15-17). Napsin A was demonstrated to be expressed in normal lung tissue and was detected in lung adenocarcinoma (18-20). It was observed that napsin A was negatively associated with the degree of transformation of cancer cells (20-22). Additionally, cells with low napsin A expression or without napsin A expression appear to be susceptible to EMT (23). The present study hypothesized that the expression of napsin A may affect EMT-mediated TKI resistance in lung cancer cells.

The present study employed a number of lung cancer cell lines, differentially-sensitive to the EGFR-TKI erlotinib. The expression of E-cadherin and vimentin, associated with EMT, in addition to napsin A was detected in lung cancer cells prior to and following the induction of TGF- β 1. Lung cancer H322 cells with high napsin A expression were selected for the investigation of the effect of napsin A silencing on the sensitivity of lung cancer cells to erlotinib, through TGF- β 1 induction. It was observed that napsin A-silenced cells exhibited increased resistance to erlotinib under the conditions of TGF- β 1 induction compared with napsin A-expression cells, suggesting that napsin A served an important role in the sensitivity of EMT-mediated resistant lung cancer cells to TKI.

Materials and methods

Cell culture and reagents. The lung cancer cell lines (H358, H322, H441, A549 and HCC827) were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Lonza Group, Ltd., Basel, Switzerland), 5 mmol/l L-glutamine, 5 mmol/l non-essential amino acids and 100 U/ml penicillin and streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), in a humidified 5% CO₂ incubator at 37°C. Erlotinib (Tarceva®) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). A 10-mmol/l erlotinib stock solution was prepared in dimethyl sulfoxide (DMSO).

RNA interference for napsin A. siRNA against napsin A and the negative control were designed and chemically synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The target sequence of siRNA against napsin A was as follows: AATCTTAAACCCACTGAATGG. The small interference RNA of negative control (siCtrl): Sense, 5'-UUCUCCGAACGUGUCACGUTT-3'; Antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. A total of 2x10⁴ H322 cells were seeded into each well of a 12-well plate and were cultured to 80% confluence. Cell transfections were performed using 100 nmol siRNA and 5 μ l Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Cells were further cultured for 48 h following transfection, and cells were subsequently lysed and analyzed for the protein expression of napsin A by western blotting. In addition, cells were treated

with 0, 1, 5, 10 and 15 ng/ml TGF- β 1 for 60 h, and then cell proliferation and the EMT-associated protein levels were detected.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted using an RNA isolation kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's protocol. cDNA was obtained by RT using the RevertAid™ First Strand cDNA synthesis kit (Fermentas, Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) and was amplified using a TaqMan® Gene Expression Assay (Applied biosystems, Thermo Fisher Scientific, Inc.) with fluorogenic Carboxyfluorescein-labeled probes using specific primers for target proteins. The specific primers for PCR were forward, 5'-GGATTGCAAATTCCTGCCATTC-3' and reverse, 5'-AACGTTGTCCCGGGTGTCA-3' for E-cadherin; forward, 5'-GGAAGGCGAGGAGAGCAGGATT-3' and reverse, 5'-TTCAAGGTCATCGTGATGCTGAGAAG-3' for vimentin; forward, 5'-GGAGCCTGAGGAGGCC-3' and reverse, 5'-GGACTTGGGATTAATGCG-3' for napsin A; and forward, 5'-GATCCCTCCAAAATCAAGTG-3' and reverse, 5'-GAGTCCTTCCACGATACCAA-3' for GAPDH. Real-time fluorescence detection was performed with the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Thermo Fisher Scientific, Inc.). PCR involved 40 amplification cycles of 94°C for 10 sec, 53°C for 30 sec and 72°C for 40 sec, followed by final extension at 72°C for 10 min. mRNA expression of target proteins was calculated using the formula 2^{- $\Delta\Delta C_q$} (24) and was normalized to the level of GAPDH. The relative mRNA level was presented as a percentage of the control.

Western blot analysis. Cells were cultured to 80% confluence. The cells were washed twice with PBS and proteins were extracted using M-PER Mammalian Protein Extraction Reagent (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following centrifugation at 12,000 x g for 10 min, the supernatant was collected and quantified using a Bicinchoninic acid (BCA) quantification kit (Beyotime Institute of Biotechnology, Haimen, China). The proteins (50 μ g) were separated by SDS-PAGE on a 12% gel (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dried milk in TBS-Tween 20 for 1 h, and incubated with specific primary antibodies overnight at 4°C. Mouse monoclonal anti-E-cadherin (cat. no. sc-21791; 1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse monoclonal anti-vimentin (cat. no. sc-6260; 1:2,000; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-N-cadherin (cat. no. sc-8424; 1:2,000; Santa Cruz Biotechnology, Inc.), anti-GAPDH antibody (cat. no. sc-365062; 1:3,000; Santa Cruz Biotechnology, Inc.) and rabbit monoclonal anti-napsin A antibody (cat. no. ab133249; 1:10,000; Abcam, Cambridge, UK) were used, followed by horseradish peroxidase-conjugated secondary antibodies goat anti-mouse (cat. no. sc-2005; 1:2,000; Santa Cruz Biotechnology, Inc.) and anti-rabbit immunoglobulin G (cat. no. sc-2004; 1:2,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Development was performed using an enhanced chemiluminescence detecting reagent (GE Healthcare Life Sciences, Little

Chalfont, UK). The protein blots were quantified by densitometry using QuantityOne software version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the amounts were expressed relative to the internal reference GAPDH.

Proliferation assay. Cell proliferation was evaluated using MTT (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). A total of 2,000 cells were seeded into each well of a 96-well plate in 100 μ l medium and incubated with or without varying concentrations of erlotinib for different times, at 37°C in a 5% CO₂ incubator. Subsequently, cells were incubated with 20 μ l 5 mg/ml MTT for 4 h, and cells were lysed for 10 min by the addition of 200 μ l DMSO (OriGen Biomedical, Inc., Austin, Texas, USA). Absorbance was measured at 490 nm using a Rainbow microplate reader (Tecan Group, Ltd., Männedorf, Switzerland). Cell proliferation was expressed as a percentage of the untreated control.

Apoptosis assay. Cells were cultured to 80% confluence and treated with 1 μ mol/l erlotinib for 48 h. Apoptosis was analyzed using annexin V-fluorescein isothiocyanate/propidium iodide (PI) assay following the manufacturer's instructions. The amount of phosphatidylserine on the outer surface of the plasma membrane (a biochemical alteration unique to the membranes of apoptotic cells) and the amount of PI, a dye that readily enters dead cells or cells in the late stages of apoptosis and binds DNA, although it does not bind to the plasma membrane of viable cells, were detected. Fluorescence was detected using a FACSCalibur flow cytometer by fluorescence activated cell sorting analysis, and data were analyzed using CellQuest version 3.2 software (BD Biosciences, San Jose, CA, USA). Cells with phosphatidylserine on the surface were considered to be apoptotic.

Statistical analysis. Data were obtained from at least three experiments. Values are expressed as the mean \pm standard error of the mean. Statistical analysis was performed using SPSS version 16.0 for MicroSoft™ Windows. One-way analysis of variance was used to assess differences between groups. Duncan method was employed for pairwise comparison followed by Bonferroni correction. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

EMT and napsin A are associated with the sensitivity of lung cancer cells to the EGFR-TKI erlotinib. Lung cancer cells H322, H358, H441, A549 (wild-type EGFR) cells (25) and HCC827 (EGFR exon 19 deletion) were cultured to 80% confluence and exposed to different concentrations of erlotinib for 48 h. Analysis of erlotinib sensitivity was performed by cell growth inhibition evaluation using an MTT assay and cell growth curves were drawn. The half-maximal inhibitory concentration (IC₅₀) values of these cells were respectively calculated to be 1.0, 1.71, 1.72, 5.65 and 13.6 μ mol/l (Fig. 1A). Sensitivity was defined as $>50\%$ *in vitro* growth inhibition at an erlotinib concentration of <5 μ mol/l; moderate sensitivity was defined as the IC₅₀ at an erlotinib concentration between 5 and 10 μ mol/l; insensitivity was defined as the IC₅₀ at an erlotinib concentration of >10 μ mol/l. The results indicated

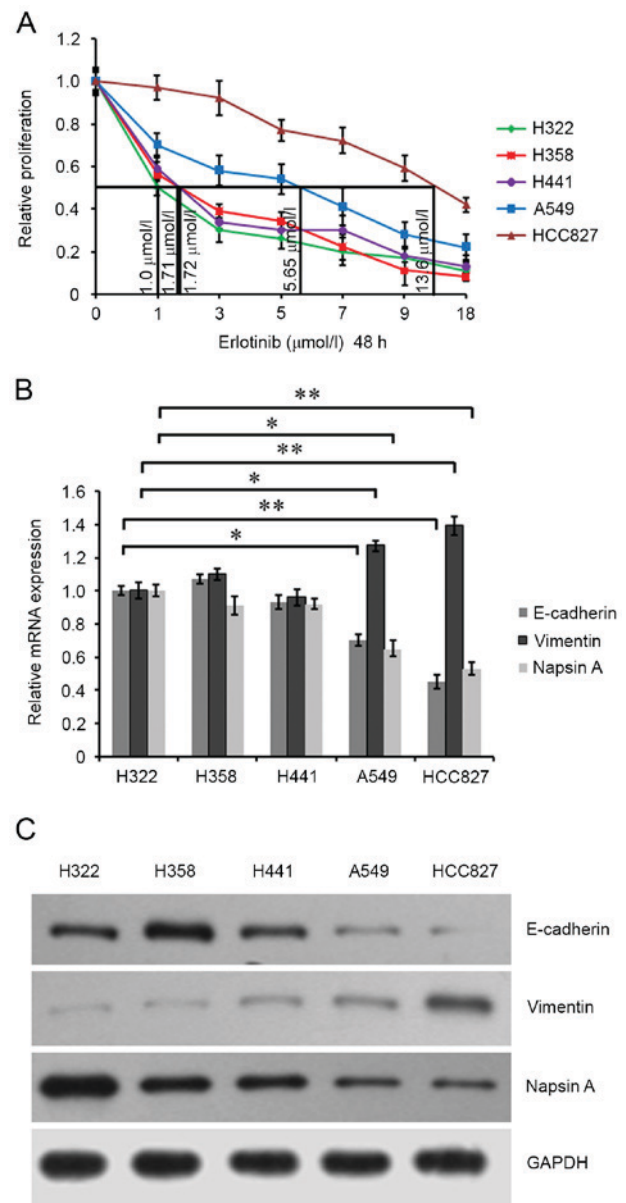


Figure 1. Characterization of lung cancer H322, H358, H441, A549 and HCC827 cells. (A) Cells were maintained in 100 μ l medium in 96-well plates for 24 h and exposed to 0, 1, 3, 5, 7, 9 and 18 μ mol/l erlotinib for 48 h. Cell proliferation was evaluated by MTT assay. Growth curves of the cells were drawn. Half-maximal inhibitory concentration values of the cells were calculated. (B) The mRNA expression of E-cadherin, vimentin and napsin A in these lung cancer cells were assessed by reverse transcription-quantitative polymerase chain reaction analysis using the corresponding primers. GAPDH was detected as an internal standard. (C) The protein expression of E-cadherin, vimentin and napsin A in cells was assessed by western blot analysis using anti-E-cadherin, anti-vimentin and anti-napsin A antibodies. GAPDH was detected as an internal standard. * $P < 0.05$; ** $P < 0.01$.

that lung cancer cells H322, H358 and H441 (wild-type EGFR) were sensitive to erlotinib, A549 (wild-type EGFR) cells were moderately sensitive, and HCC827 (EGFR exon19 deletion) cells exhibited lower sensitivity to erlotinib. Additionally, cells were treated with 1 μ mol/l erlotinib for 48 h, and the expression of EMT-associated proteins, including E-cadherin and vimentin, was detected. RT-qPCR analysis and western blotting demonstrated that erlotinib-sensitive H322, H358 and H441 cells exhibited increased E-cadherin mRNA and protein

expression levels compared with erlotinib-moderately sensitive A549 cells and erlotinib-insensitive HCC827 cells. However, vimentin exhibited opposite expression (Fig. 1B and C). These data suggested that EMT may be associated with the sensitivity of lung cancer cells to erlotinib. Additionally, napsin A mRNA and protein expression in erlotinib-sensitive H322, H358 and H441 cells was demonstrated to be increased compared with erlotinib-moderately sensitive A549 cells, and erlotinib-insensitive HCC827 cells exhibited the lowest napsin A level, suggesting that napsin A maybe positively associated with the sensitivity of lung cancer cells to erlotinib.

Napsin A silencing enhances TGF- β 1-induced EMT. To investigate the correlation between napsin A expression and EMT in lung cancer cells, the napsin A-expressing H322 cells were used and napsin A was knocked down using siRNA technology. The mRNA and protein expression of napsin A was evaluated by RT-qPCR analysis and western blotting. The results demonstrated that napsin A expression in napsin A-silenced cells was significantly decreased compared with non-silenced control cells (Fig. 2A and B). Subsequently, cells were treated with 0, 1, 5, 10 and 15 ng/ml TGF- β 1 for 60 h, and a concentration response curve for TGF- β 1 was performed. It was observed that cellular morphology began to alter when the TGF- β 1 concentration reached 5 ng/ml. In addition, of the five concentrations, 5 ng/ml TGF- β 1 stimulated cell proliferation most rapidly and the EMT-associated protein levels were altered (data not shown). Therefore, 5 ng/ml TGF- β 1 was selected to induce EMT. It was demonstrated that napsin A silencing significantly enhanced the TGF- β 1-induced EMT phenotype characterized by decreased E-cadherin and increased vimentin expression in napsin A-silenced H322 cells compared with highly napsin A-expressing control H322 cells (Fig. 2C). N-cadherin expression in lung cancer cells was additionally detected to be positively associated with vimentin expression in this study (data not shown).

Napsin A silencing promotes EMT-mediated erlotinib resistance. Napsin A-silenced H322 cells and control cells were respectively induced with or without 5 ng/ml TGF- β 1 for 60 h, and subsequently treated with 1 μ mol/l erlotinib for 48 h. The cell proliferation assay demonstrated that TGF- β 1-induced EMT mediated the increased cell growth rate and resistance to erlotinib in H322 cells (Fig. 3A). However, napsin A silencing enhanced the EMT-mediated erlotinib resistance of H322 cells (Fig. 3A). In addition, the cellular apoptosis assay demonstrated that napsin A silencing attenuated the inducing effect of erlotinib on apoptosis in TGF- β 1-treated cells compared with napsin A-expressing control cells (Fig. 3B), suggesting that the expression of napsin A may inhibit EMT-mediated erlotinib resistance. These data indicated that napsin A was a potential target for improving the sensitivity of EMT-induced resistant lung cancer cells to the EGFR-TKI erlotinib.

Discussion

Lung cancer is a common malignancy with a high mortality rate, which is a severe threat to human health (1). EGFR-TKIs, including Gefitinib and erlotinib, have been used as the standard therapy in lung cancer with EGFR-activating mutations (2,3). However, the majority of patients eventually

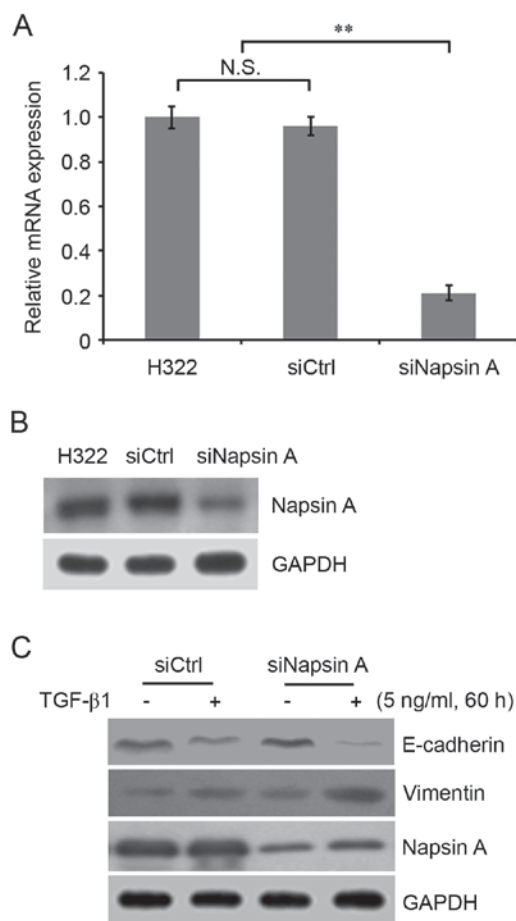


Figure 2. Effect of napsin A silencing on epithelial-mesenchymal transition-associated proteins. Cells were transfected with siCtrl or sinapsin A for 48 h and the (A) mRNA and (B) protein expression of napsin A cells were analyzed respectively by reverse transcription-quantitative polymerase chain reaction and western blot analyses. GAPDH was detected as an internal standard. ** $P < 0.01$. (C) napsin A-silenced and non-silenced H322 cells were treated with or without 5 ng/ml TGF- β 1 for 60 h. The protein expression of E-cadherin, vimentin and napsin A in cells was assessed western blot analysis using anti-E-cadherin, anti-vimentin and anti-napsin A antibodies. GAPDH was detected as an internal standard. siCtrl, control small interfering RNA; sinapsin A, small interfering RNA for napsin A; N.S., not significant; TGF- β 1, transforming growth factor- β 1.

succumb to recurrence due to drug resistance; thus, therapeutic efficacy is markedly limited. Therefore, elucidating effective therapeutic strategies is required to overcome the acquisition of EGFR-TKI resistance. The present study demonstrated that the expression of napsin A was able to increase the sensitivity of EMT-mediated resistant lung cancer cells to erlotinib compared with napsin A-silenced cells.

EMT, defined by the combined loss of E-cadherin and the gain of mesenchymal lineage marker expression, negatively affected cellular responses to EGFR inhibitors (26). In the present study, three EGFR-TKI erlotinib-sensitive lung cancer cell lines, H358, H322 and H441, erlotinib-moderately sensitive A549 cells, and erlotinib-insensitive HCC827 cells were used and the expression of the EMT-associated proteins E-cadherin and vimentin, and the expression of napsin A, which was reported to inhibit EMT in lung cancer A549 cells (27), was detected. It was observed that E-cadherin mRNA and protein expression levels were positively associated with the sensitivity of lung cancer cells to erlotinib, while vimentin exhibited a negative

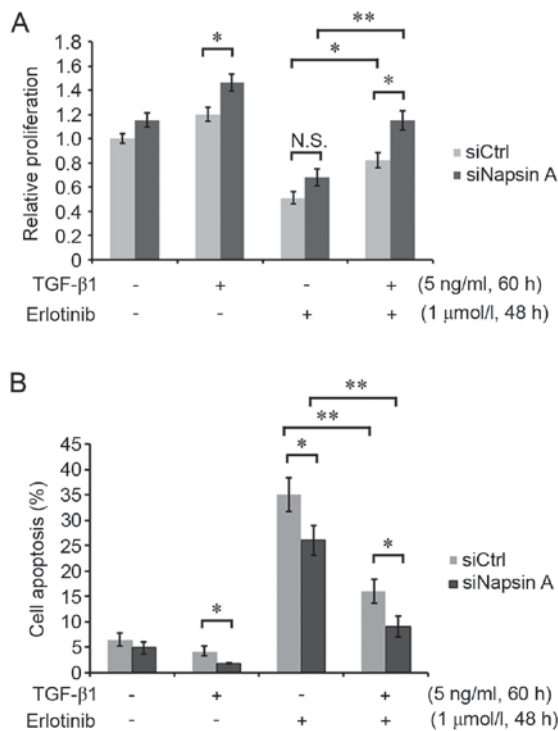


Figure 3. Effect of napsin A silencing on the sensitivity of H322 cells to erlotinib. (A) napsin A-silenced and non-silenced H322 cells were incubated with fresh medium with or without 5 ng/ml TGF-β1 for 60 h, and were subsequently exposed to 1 μmol/l erlotinib for 48 h. Cell proliferation was detected by an MTT assay. (B) Cellular apoptosis was evaluated by an annexin V-fluorescein isothiocyanate/propidium iodide assay. *P<0.05; **P<0.01. TGF-β1, transforming growth factor-β1; N.S., not significant; siCtrl, control small interfering RNA; siNapsin A, small interfering RNA for napsin A.

association with erlotinib sensitivity. Like vimentin, N-cadherin is an important marker of EMT, commonly expressed in mesenchymal cells (28). The downregulation of E-cadherin and the upregulation of vimentin and N-cadherin are the characteristics of EMT (9) and EMT is the underlying mechanism of acquisition of TKI resistance (5). Therefore, it was hypothesized that EMT may be involved in the development of TKI resistance in lung cancer cells. In addition, the expression level of napsin A was demonstrated to be positively associated with the sensitivity of lung cancer cells to erlotinib, suggesting that napsin A may serve an adverse role in EMT-mediated drug resistance. Therefore, highly napsin A-expressing H322 cells were used to construct a napsin A-silenced cell line using siRNA technology. Subsequently, TGF-β1 was used to induce cellular EMT, and it was observed that TGF-β1-treated H322 cells were more resistant to erlotinib compared with TGF-β1-untreated cells. In addition, it was observed that napsin A silencing enhanced TGF-β1-induced cellular resistance to erlotinib via proliferation and apoptosis assays, verifying the aforementioned hypothesis.

Napsin A has been reported to contain an Arg-Gly-Asp (RGD) sequence at the carboxyl terminal. The sequence is able to recognize and bind integrins on the cell surface (27). Integrins are able to mediate cell adhesion and signal transduction between cells and the ECM (29), and is an important regulator of cell proliferation, apoptosis, migration and metastasis (30,31). Napsin A may suppress the interaction between Integrins and the ECM by binding Integrins, and thus inhibit the integrin-mediated signaling pathway. We have found that focal

adhesion kinase 1 (FAK) was inhibited by napsin A expression in Gefitinib-resistant A549 cells (Zhou *et al*, unpublished data). FAK serves an important role in integrin signaling (27,32-36), and may be activated by integrin signaling and modulate a number of signaling pathways, including phosphatidylinositol 3-kinase/RAC-α serine/threonine-protein kinase, signal transducer and activator of transcription 1, and Ras-mitogen-activated protein kinase signaling (32,37,38), and thus triggers cell growth and transformation. It was hypothesized that napsin A may repress the interaction between Integrins and ECM through RGD sequence-mediated interaction with integrin, and further inhibit the integrin signaling pathway, and cell proliferation and transformation, by downregulating FAK expression in lung cancer cells. Whether the mechanism is implicated in other lung cancer cell lines requires further investigation. Additionally, napsin A has been demonstrated to be able to suppress cell growth in 293T cells (23), and napsin A expression in systemic anaplastic large cell lymphoma (ALCL) was associated with an increased international prognostic index in malignant lymphoma; napsin A expression predicted a poor prognosis in patients with ALCL and diffuse large B-cell lymphoma (39). Therefore, these data, combined with the present finding that the expression of napsin A augmented the effect of erlotinib on TGF-β1-induced TKI-resistant lung cancer cells, suggested that napsin A may be a promising target for improving the sensitivity of drug resistant cells, and may exhibit clinical potential.

In conclusion, the results of the present study demonstrated that napsin A served an important role in the development of EMT-mediated resistance in lung cancer cells to EGFR-TKI, and napsin A combined with EGFR-TKI may be a more effective way of improving the sensitivity of lung cancer cells to the TKI erlotinib. In order to verify the underlying resistance-associated mechanism to EGFR-TKI *in vivo* in human lung cancer tissues, *in vivo* xenograft models may be constructed by injecting different lung cancer cell lines with or without napsin A silencing into mice, and a napsin A-targeted gene treatment maybe employed in order to further assess the clinical importance and significance of the present study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LZ, XL and ZW were major contributors in the conception and design of the research and revision of the manuscript for important intellectual content. Acquisition of data was performed by JY and YZ. TX was the major contributor in the analysis and interpretation of data and statistical analysis. Drafting of the manuscript was performed by ZW.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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