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ORIGINAL ARTICLE

Deguelin inhibits epithelial-to-mesenchymal transition and metastasis of human non-small cell lung cancer cells by regulating NIMA-related kinase 2

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Keywords
Deguelin; EMT; metastasis; NEK2.

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Abstract

Background: Non-small cell lung cancer is a lethal malignancy with a high mortality rate. Deguelin displays an anti-tumor effect and inhibits metastasis in various cancers. The aberrant expression of NIMA-related kinase 2 (NEK2) indicates poor prognosis and induces epithelial-to-mesenchymal transition (EMT) and metastasis processes. However, the underlying mechanism between deguelin and NEK2 has remained elusive.

Methods: NSCLC cell lines were treated with deguelin. Wound-healing and invasion assays were applied to study the inhibitory effect of deguelin on NSCLC cells. EMT markers, E-cadherin and Vimentin, were also detected by Western blot. NEK2 protein and messenger RNA expression levels were evaluated when NSCLC cells were treated with different concentrations of deguelin. The effect of NEK2 on NSCLC cell metastasis was evaluated through NEK2 knockdown. To investigate whether deguelin induced EMT by regulating NEK2, we overexpressed NEK2 in both NCI-H520 and SK-MES-1 cell lines, and then used real time-PCR to study the E-cadherin and Vimentin messenger RNA expression in both NSCLC cells.

Results: Deguelin inhibited migration and invasion processes in NSCLC cell lines and decreased NEK2 expression in a concentration-dependent manner. Furthermore, NEK2 knockdown inhibited NSCLC cell migration and invasion. Finally, overexpressing NEK2 in NCI-H520 and SK-MES-1 cells could restore the inhibition of metastasis induced by deguelin.

Conclusions: Deguelin could inhibit EMT and metastasis, while overexpression of NEK2 promotes these processes. Deguelin could decrease NEK2 expression, while NEK2 overexpression could restore deguelin-induced inhibition of metastasis.

Introduction

Lung cancer is one of the most malignant cancers and is responsible for high mortality around the world.[1] Among lung cancer types, non-small cell lung cancer (NSCLC) is the most common, accounting for approximately 85% of lung cancer cases.[2] Recently, the prognosis and survival rate of patients with NSCLC has significantly improved as a result of advanced and effective treatment strategies. However, cancer metastasis still poses a major challenge for NSCLC treatment in clinical settings. Therefore, exploration of a pharmaceutical agent to intervene in cancer progression and determination of the underlying mechanisms for NSCLC development are urgent tasks.

Deguelin, a rotenoid extracted from *Mundulea sericea*, has been documented to possess anti-tumor activities for several cancers, including pancreatic,[3] lung,[4] breast[5] and colon cancers.[6] Accumulating evidences have shown that deguelin is involved in different biological processes, such as apoptosis,[7] proliferation,[8] and migration/invasion.[9,10] A recent study showed that low-dose deguelin inhibited oral

cancer migration and invasion by regulating tumor necrosis factor-α-induced nuclear factor-κB activity and matrix metallpproteinase-2 (MMP-2), and in human osteosarcoma cells by inhibiting MMP-2/MMP-9 expression.[10,11] Deguelin displayed an anti-proliferation effect and suppressed metastasis by regulating the epithelial-to-mesenchymal transition (EMT) process.[3] As EMT and cancer metastasis play an important role in inducing the development of NSCLC,[12] inhibition of the EMT process is a pivotal step for impeding cancer development. However, the effect of deguelin on NSCLC metastasis and its underlying mechanisms require further study.

NIMA-related kinase 2, known as a tumor oncogene, has the potential to be used as a biological marker for NSCLC prognosis.[13–15] Aberrant NEK2 expression has clinical diagnostic significance for the treatment of NSCLC.[16] NEK2 has also shown prognostic significance in hepatocellular carcinoma[17] and liver cancer progression.[5,18] However, the specific involvement of NEK2 in deguelin-induced EMT and metastasis inhibition needs to be determined. Herein, we investigate the underlying mechanism of deguelin-induced inhibition of NSCLC metastasis and the role of NEK2 in deguelin-induced EMT process.

Methods

Cell lines and reagent

Human NSCLC cell lines NCI-H520 and SK-MES-1 were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 100 U/mL of penicillin/100 μg/mL of streptomycin (Gibco, Carlsbad, CA, USA) in a humidified atmosphere in a 5% CO_2 incubator at 37°C. Deguelin reagent was purchased from Sigma (San Francisco, CA, USA).

RNA isolation and quantitative real time-PCR

Total RNA was extracted from NSCLC cell lines using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription of RNA into complementary DNA was carried out using a PrimeScript first Strand cDNA synthesis Kit (Takara, Dalian, China) and then real-time-PCR was performed using a QuantiFast SYBR Green Kit (Qiagen). The expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers for NEK2 and EMT markers are listed in Table 1. The $2^{-\Delta\Delta Ct}$ method was applied to calculate relative messenger (m)RNA expression. The experiments were repeated in triplicate.

Table 1 Primers for quantitative real time-PCR assay

Genes	Primer sequence
NEK2	Forward: 5′-TGCTTCGTGAACTGAAACATCC -3′
	Reverse: 5′-CCAGAGTCAACTGAGTCATCACT-3′
E-cadherin	Forward: 5′- CGAGAGCTACACGTTCACGG-3′
	Reverse: 5′-GGGTGTCGAGGGAAAAATAGG-3′
Vimentin	Forward: 5′-AGTCCACTGAGTACCGGAGAC-3′
	Reverse: 5′-CATTTCACGCATCTGGCGTTC-3′
GAPDH	Forward: 5′- ACAACTTTGGTATCGTGGAAGG-3′
	Reverse: 5′-GCCATCACGCCACAGTTTC-3′

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NEK2, NIMA-related kinase 2.

Western blot analysis

Cells were washed with cold phosphate buffered saline twice and lysed with radioimmunoprecipitation assay lysis buffer (Thermo Scientific, Waltham, MA, USA) at 4°C for 30 minutes. The cells lysis was centrifuged at 12 000 × *g* for10 minutes at 4°C and then the supernatant was collected and boiled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer for 10 minutes. Proteins with equal amounts were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred into polyvinylidene fluoride membrane (Millipore, Boston, MA, USA). The membranes were blocked with 5% non-fat milk dissolved in phosphate buffered saline supplemented with 0.05% Tween-20 and were then incubated with primary antibodies at 4°C overnight. The primary antibodies against E-cadherin (14472S), Vimentin (5741S) and GAPDH (5174S) were purchased from Cell Signaling Technology (Danvers, MA, USA), and anti-NEK2 antibody (TA349610) was obtained from OriGene (Rockville, MD, USA). After incubation with the appropriate luciferase-conjugated secondary antibody for one hour at room temperature, the membranes were visualized using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, Nebraska, USA). GAPDH protein intensity was used as an internal control.

Cell transfection of NIMA-related kinase 2 (NEK2) plasmid and NEK2 small interfering RNA

Human NEK2 gene cDNA clone expression plasmid (HG10054-ACG) was purchased from Sino Biological Inc. (Beijing, China) and NEK2 small interfering (si)RNA was purchased from RiboBio (Guangzhou, China) to determine NEK2 overexpression or knockdown in NCI-H520 and SK-MES-1 cells, respectively. The cells were plated in 12-well plates and grown into 80% confluence for transfection. The NEK2 plasmids or siRNA and negative control groups were transfected into cells using Lipofectamine 2000

(Invitrogen, Carlsbad, CA, USA). After 48 hours of transfection, the cells were collected for further experimentation.

Wound healing, migration, and invasion assay

Wound healing assay was performed to quantify cell motility. Cells were seeded in six-well plates and grown into a subconfluent monolayer, and wound healing was scratched in the middle of each well using 200 μL pipette tips. The wound healing width was observed in at least three different fields. For migration and invasion, 1×10^5 cells diluted in 200 μL serum-free RPMI 1640 medium were inoculated into the upper chamber of the transwell while the bottom chamber was filled with 500 μL complete RPMI 1640 culture medium. The cells in the upper chamber were wiped out with a cotton swab after culture for 48 hours and those in the bottom chamber were stained with 1% crystal violet. The chamber was pre-coated with Matrigel (BD Bioscience, San Jose, CA, USA) for cell invasion. The cells were counted in at least three random fields.

Statistical analyses

All statistical analysis were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). The results were expressed as mean ± standard deviation. All experiments were performed independently in triplicate, and a P value < 0.05 was considered statistically significant.

Results

Deguelin decreased NEK2 expression

To investigate the regulative effect of deguelin on NEK2 expression, we used different concentrations of deguelin (0, 1, 10, 25, 50, 100 μM) to culture the NSCLC cells for 24 hours. The results showed that deguelin could reduce NEK2 expression in both protein and mRNA levels in NCI-H520 cells (Fig 1a,b) and SK-MES-1 cells (Fig 1c,d).

Deguelin inhibited metastasis and epithelial-to-mesenchymal transition (EMT) in non-small cell lung cancer (NSCLC) cells

In order to investigate the effect of deguelin on NSCLC cells, we applied wound-healing assay to study the migration function of the cells (Fig 2a,c). The administration of deguelin could inhibit cell migration in both NCI-H520 and SK-MES-1 cells. Furthermore, we examined the effect of deguelin (50 μM) on NSCLC cells invasion. After 24 hours of treatment with deguelin, there were less invasive cells in both NCI-H520 and SK-MES-1 cells than in the control groups (Fig 2b,d). We used Western blotting to detect the protein levels of EMT markers, E-cadherin and Vimentin. The group treated with deguelin (50 μM) for 24 hours had increased E-cadherin and decreased Vimentin protein levels in both NCI-H520 and SK-MES-1 cells (Fig 2e,f).

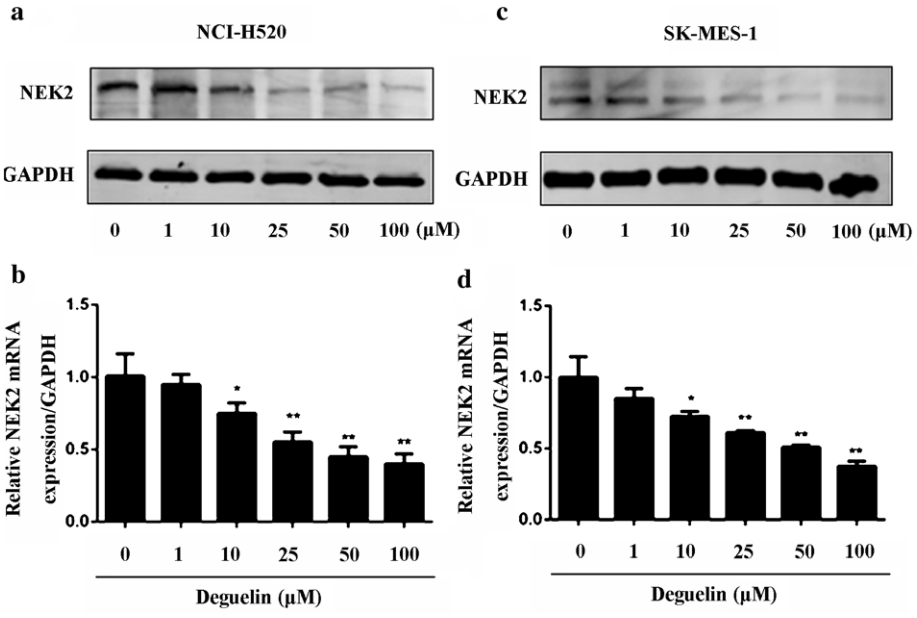

Figure 1 Deguelin decreased NIMA-related kinase 2 (NEK2) expression. Western blot analysis of NEK2 expression treated with deguelin at different concentrations (0, 1, 10, 25, 50, 100 μM) in (**a**) NCI-H520 and (**c**) SK-MES-1 cells. Real time-PCR analysis of messenger (m)RNA expression of NEK2 in (**b**) NCI-H520 and (**d**) SK-MES-1 cells treated with different concentrations (0, 1, 10, 25, 50, 100 μM) of deguelin. *P < 0.05, **P < 0.01. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. All experiments were repeated in triplicate.

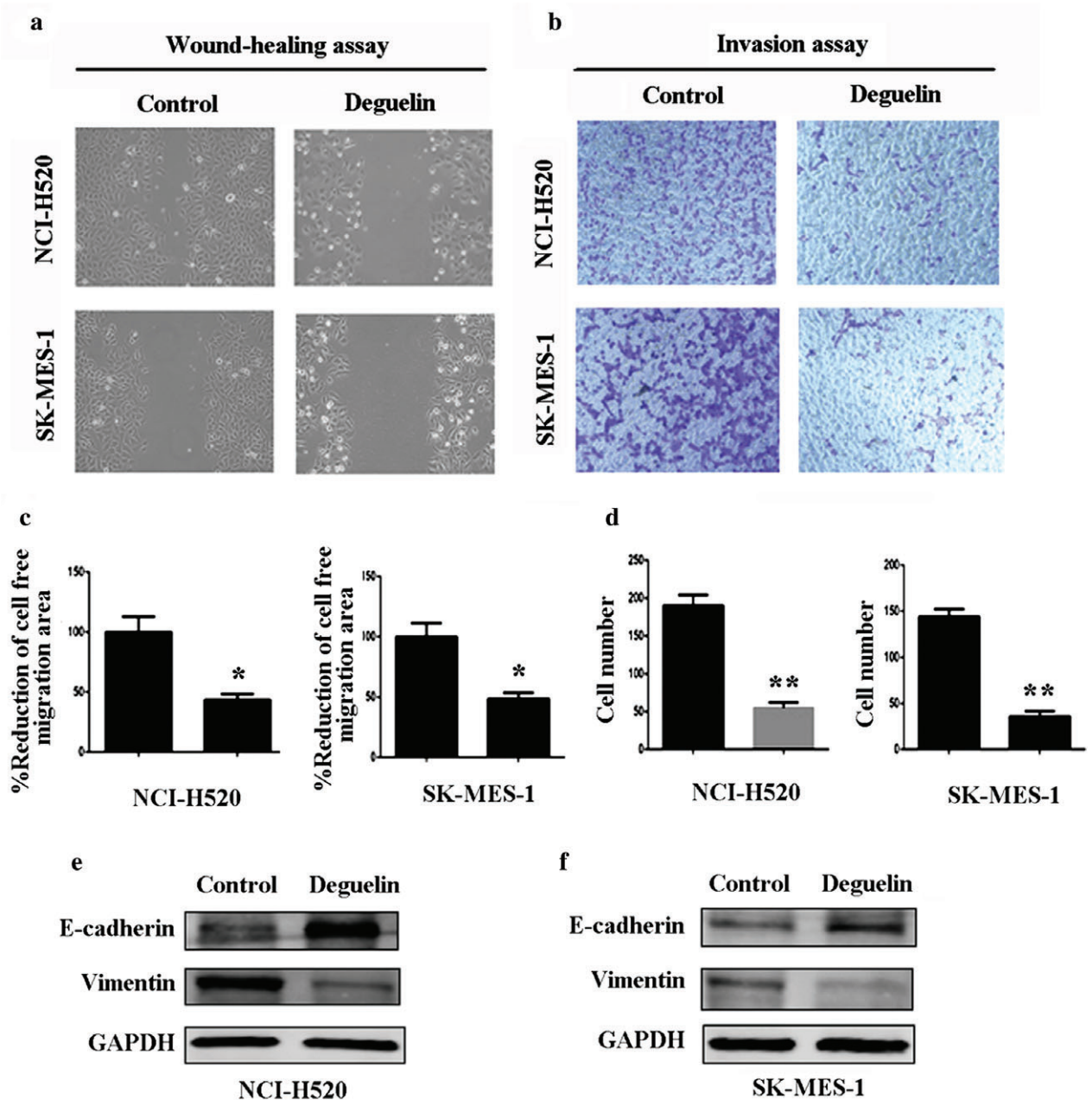

Figure 2 Deguelin inhibited metastasis and epithelial-to-mesenchymal transition (EMT) in NSCLC cells. Wound-healing assay showed that deguelin (50 μM) could significantly inhibit cell migration compared to the control group in both NCI-H520 and SK-MES-1 cells (**a,c**). Invasion assay showed that deguelin (50 μM) could inhibit cell invasion compared to the control group in both NCI-H520 and SK-MES-1 cells (**b,d**). Western blotting analysis showed that deguelin (50 μM) upregulated epithelial marker E-cadherin and downregulated mesenchymal marker Vimentin in NCI-H520 cells (**e**) and increased epithelial marker E-cadherin protein expression and decreased mesenchymal marker Vimentin expression in SK-MES-1 cells (**f**). $*P < 0.05$; $**P < 0.01$. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used an internal control. All experiments were repeated in triplicate.

NEK2 knockdown inhibited NSCLC cell metastasis

In order to understand the role of NEK2 in NSCLC cell metastasis, we first knocked down NEK2 expression through transfection of NEK2 siRNA (Fig 3a). Western blotting and RT-PCR showed successful NEK2 protein knockdown and mRNA levels in both NCI-H520 and SK-MES-1 cells. We then applied invasion assays to investigate

the role of NEK2 in tumor cell invasion, and the results indicated that NEK2 silencing could inhibit the cell invasion process (Fig 3b). Moreover, we used wound-healing assay to study the role of NEK2 in the migration process and we observed that NEK2 knockdown could inhibit the migration of both NSCLC cells compared to the control groups (Fig 3c).

NEK2 overexpression restored the deguelin-induced EMT process

To further study the role of NEK2 in the deguelin-induced EMT process, we firstly overexpressed the NEK2 expression by transfecting NEK2 plasmid in both NCI-H520 and SK-MES-1 cells. Western blotting results showed that NEK2 plasmids were overexpressed (Fig 4a). Furthermore, we applied real-time PCR method to study the E-cadherin and Vimentin mRNA expression levels. In NCI-H520 cells, NEK2 overexpression could restore the deguelin-induced upregulation of E-cadherin and downregulation of Vimentin (Fig 4b, left). We observed similar results in SK-MES-1 cells after real-time PCR analysis (Fig 4b, right).

Discussion

Non-small cell lung cancer accounts for the major percentage of lung cancer cases and has a poor prognosis. In recent years, drug resistance in tumor treatment has been a concern for patients;[19] however, more in-depth studies on anti-tumor mechanisms and components, such as deguelin, are being studied in different cancers.[5,8,20] In this study, we demonstrated that deguelin inhibited NSCLC cell metastasis and EMT by downregulating NEK2 expression.

Deguelin has been reported to have an anti-tumor effect on different tumor types, such as human pancreatic,[8] breast,[5] oral,[21] head and neck squamous cell,[22,23] and non-small cell lung cancers.[24] Deguelin has an effective therapeutic effect on cancer by targeting different oncogenes[4] and tumor signaling pathways.[5] In treatment for NSCLC, deguelin displayed a significant inhibition effect by downregulating galectin-1 expression.[4] Recently, Lee *et al.* reported that a novel deguelin derivative, L80, had inhibited lung cancer tumorigenesis by disrupting the binding between adenosine 5'-triphosphate and heat shock protein 90,[25] while another deguelin analogue, SH-1242, had an

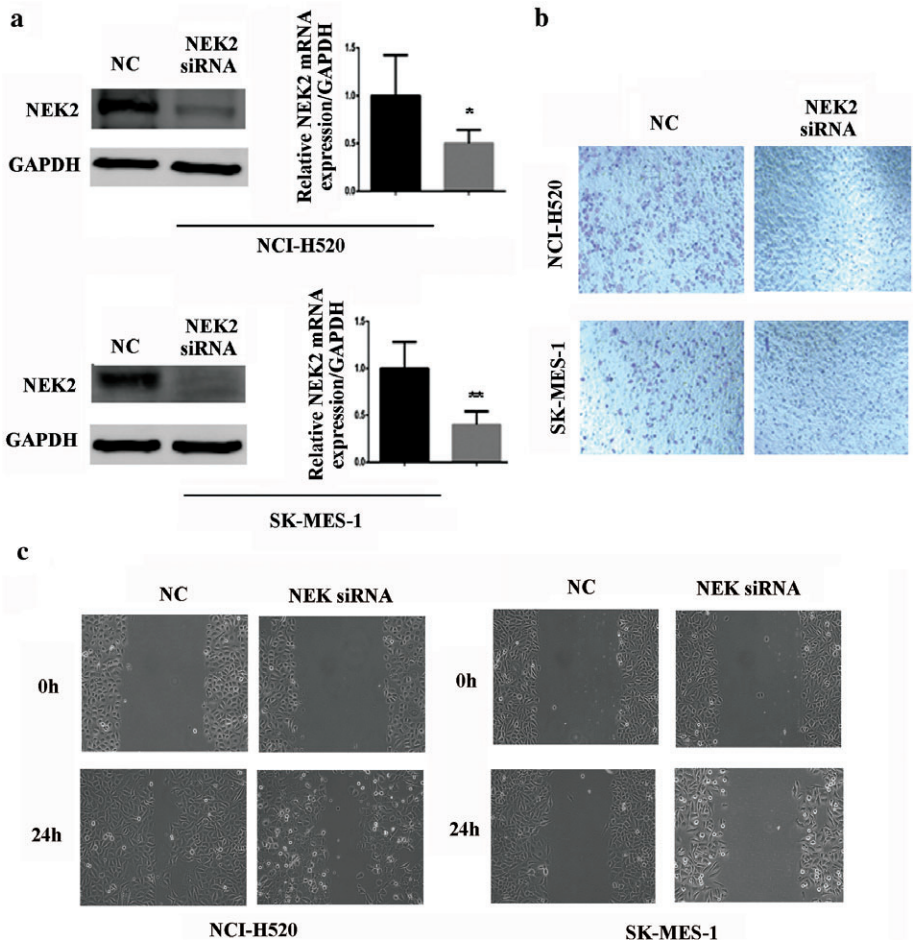

Figure 3 NIMA-related kinase 2 (NEK2) knockdown inhibited NSCLC cells metastasis. (**a**) Western blot analysis of NEK2 protein level after transfection with NEK2 small interfering (si)RNA or negative control (NC) siRNA. Real time-PCR analysis of NEK2 messenger (m)RNA expression in (**a**, upper) NCI-H520 and (**a**, lower) SK-MES-1 cells. (**b**) Invasion assay analysis of NCI-H520 and SK-MES-1 cells after transfection with NEK2 siRNA or negative control ones. Wound-healing assay for (**c**, left) NCI-H520 and (**c**, right) SK-MES-1 cell migration after cells were transfected with NEK2 siRNA or NC siRNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. All experiments were repeated in triplicate.

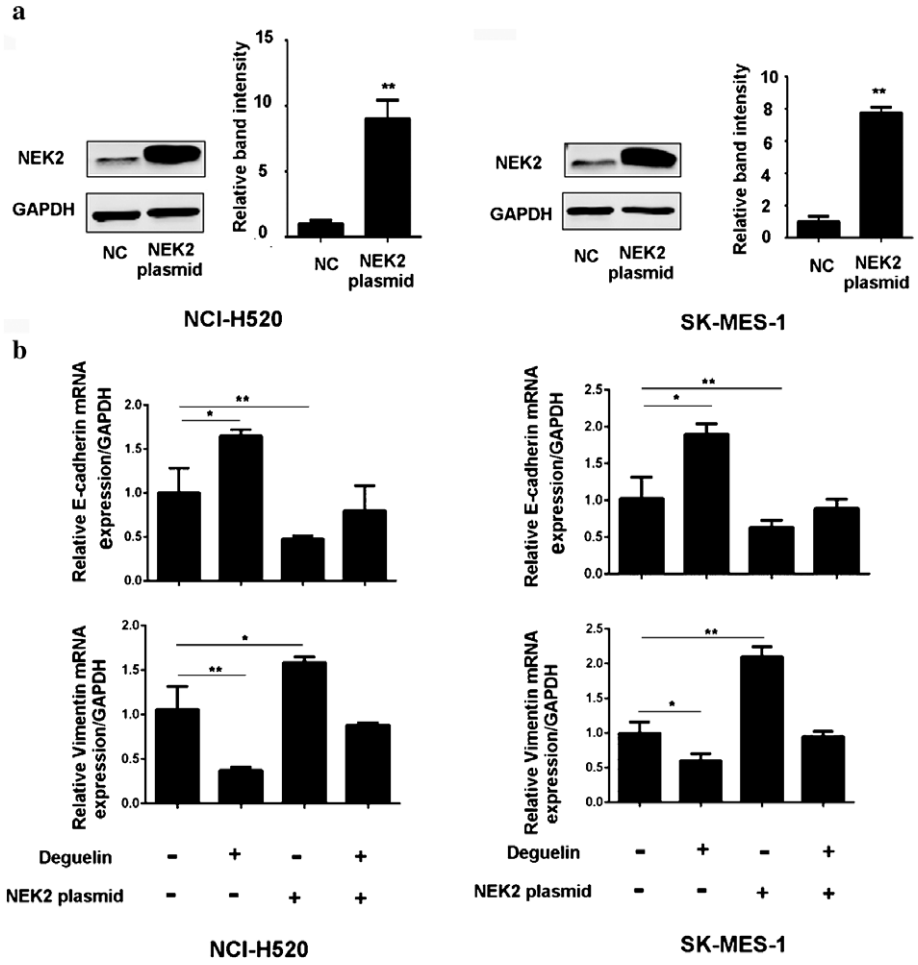

Figure 4 NIMA-related kinase 2 (NEK2) overexpression restored deguelin-induced epithelial-to-mesenchymal transition (EMT). Western blot analysis of NEK2 overexpression in (**a**, left) NCI-H520 and (**a**, right) SK-MES-1 cells. Real time-PCR analysis of epithelial marker E-cadherin and mesenchymal marker Vimentin messenger (m)RNA expression levels in (**b**, left) NCI-H520 and (**b**, right) SK-MES-1 cells in indicated groups treated with deguelin (50 μM) or transfection of NEK2 plasmid. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. All experiments were repeated in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$.

inhibitory effect on heat shock protein 90 expression.[26] Previous studies had shown that deguelin could intervene in different tumor biological processes, including apoptosis,[27] proliferation,[8] invasion, and migration,[3] and inhibit signaling pathways, including hedgehog,[8] epidermal growth factor receptor/insulin-like growth factor receptor 1-protein kinase B (Akt),[22] and mitogen-activated protein kinase signaling pathways.[28] Tumor EMT and metastasis are pivotal steps to induce tumorigenesis and drug resistance. Deguelin, as a natural herbal extract, suppressed tumor metastasis by restraining EMT in pancreatic cancer and inhibited human osteosarcoma cell migration and invasion by downregulating MMP-2/-9. In our study, we showed that migration/invasion and EMT were inhibited by deguelin in NSCLC lines.

Increasing evidences had demonstrated that aberrant NEK2 expression could serve as a biological marker for cancer diagnosis and prognosis.[17,29] However, the exact mechanisms of tumorigenesis induced by aberrant NEK2 expression remain poorly understood. It has been reported that NEK2 played an important role in inducing metastasis and drug resistance in hepatoma cells by activating the phosphorylated protein kinase B/nuclear factor-κB signaling pathway and MMPs. Other reports have suggested that NEK2 overexpression induced the activation of the phosphoinositide 3-kinase/protein kinase B signaling pathway[30] and increased β-catenin protein expression[31] and β-catenin relocalization in colorectal cancer.[14] In our study, we investigated the role of NEK2 in NSCLC and found that NEK2 silencing could inhibit the migration and invasion of NSCLC cells. This evidence showed that NEK2 plays a pivotal role in inducing NSCLC cell metastasis.

In conclusion, our study demonstrated that deguelin could inhibit migration, invasion, and EMT in NSCLC cells. We further determined that deguelin could decrease NEK2 expression in a concentration-dependent manner, and NEK2 overexpression in NSCLC cells could restore the inhibitory effect of deguelin on metastasis and EMT. These findings indicated that downregulation of NEK2 through the administration of deguelin suggests a promising approach for NSCLC pharmaceutical therapy.

Disclosure

No authors report any conflict of interest.

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