

TASK-1 induces gefitinib resistance by promoting cancer initiating cell formation and epithelial-mesenchymal transition in lung cancer

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Abstract. Cancer initiating cell (CIC) formation and epithelial-mesenchymal transition (EMT) are pivotal events in lung cancer cell invasion and metastasis. They have been shown to occur in gefitinib resistance. Studying the molecular mechanisms of CICs, EMT and acquired gefitinib resistance will enhance the understanding of the pathogenesis and progression of the disease and offer novel targets for effective therapies. TWIK-related acid-sensitive K(+) (TASK-1) is expressed in a subset of non-small-cell lung cancer (NSCLC) cell lines, where it promotes cell proliferation while inhibiting apoptosis. In the present study, TASK-1 was demonstrated to induce gefitinib resistance in the A549 NSCLC cell line. Overexpression of TASK-1 promoted the acquisition of CIC-like traits by A549 cells. CD133, octamer-binding transcription factor 4 (OCT-4) and Nanog have been suggested to be markers of CICs in lung cancer. It was demonstrated that overexpression of TASK-1 promoted CD133, OCT-4 and Nanog protein expression in A549 cells. Increased formation of stem cell-like populations results in EMT of cancer cells. The present study found that overexpression of TASK-1 promoted EMT of A549 cells. Thus, downregulation of TASK-1 may represent a novel strategy for EMT reversal, decreasing CIC-like traits and increasing gefitinib sensitivity of NSCLCs.

Introduction

Lung cancer is a heterogeneous malignancy with aggressive phenotypes (1,2). Non-small-cell lung cancer (NSCLC) accounts for ~85% of lung cancer cases (2). Lung cancers harboring somatic mutations in exons encoding the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) exhibit a significant tumor regression when treated with the EGFR tyrosine kinase inhibitors (TKIs) gefitinib or erlotinib in ~70% of cases (3-5). However, acquired resistance inevitably develops in an overwhelming majority of these patients.

Epithelial to mesenchymal transition (EMT) is associated with the acquired resistance of NSCLC to gefitinib (6,7). It is a process by which cells undergo a morphological shift from the epithelial polarized to the mesenchymal fibroblastoid phenotype. EMT has been recognized to have pivotal roles in several diverse processes during embryonic development, chronic inflammation and fibrosis, as well as tumor progression (8-11). During EMT, epithelial cells lose their defined cell-cell/cell-substratum contacts and their structural/functional polarity, and become spindle-like.

Lung cancer stem cells (CSCs) or cancer-initiating cells (CICs) have been identified and demonstrated to constitute a primitive cell population capable of self-renewal and differentiation that have the unique capacity to give rise to new tumors upon serial transplantation (12-15). They represent a small population of undifferentiated tumorigenic cells responsible for tumor initiation, maintenance and spreading. Resistance to conventional chemotherapeutic drugs is a common characteristic of CICs (16). It has been reported that lung CICs were associated with gefitinib resistance (17).

TWIK-related acid-sensitive K(+) (TASK-1) is expressed in a subset of NSCLCs, where it is functional, and promoted the proliferation and inhibited apoptosis in a highly TASK-1-expressing lung cancer cell line (18). The present study demonstrated that TASK-1 induced gefitinib resistance in NSCLC A549 cells. Overexpression of TASK-1 promoted the formation of CICs in A549 cells. CD133, octamer-binding transcription factor 4 (OCT-4) and Nanog have been proposed as markers of CICs in lung cancer (13,19,20). The

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present study demonstrated that overexpression of TASK-1 promoted CD133, OCT-4 and Nanog protein expression in A549 cells. Increased formation of CSC-like populations may result in EMT of cancer cells (21-24). The present study found that overexpression of TASK-1 promotes EMT of A549 cells.

Materials and methods

Cell line and culture. The A549 NSCLC cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) within 3 months of performing the experiments. They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and antibiotics (100 mg/ml penicillin and 100 U/ml streptomycin) in a 5% CO₂ incubator at 37°C.

Cell transfection. The pGCMV/EGFP/TASK-1 plasmid and the pGCMV/EGFP/Neo plasmid were constructed (Tiangen Biotech Co., Ltd., Beijing, China). The two plasmids were transfected into A549 NSCLC cells separately using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). In order to detect the transfection efficiency of the plasmids, green fluorescent signal was measured by fluorescence microscopy. Subsequent experimentation was performed after 24 h.

Western blot analysis. Total proteins in cells were extracted using protein lysis solution (Tiangen Biotech Co., Ltd.). Protein concentration was measured using a bicinchoninic acid kit (Tiangen Biotech Co., Ltd.). Protein extracts (50 µg/lane) were resolved through 8% SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and blocked with 5% non-fat milk for 1 h at room temperature. Membranes were probed with antibodies against human TASK-1 (ab135883), vimentin (ab92547), E-cadherin (ab40772), CD133 (ab16518), OCT4 (ab109183), Nanog (ab109250), matrix metalloproteinase (MMP)9 (ab76003), MMP2 (ab92536) or β-actin (ab8227) (all 1:500; Abcam, Cambridge, MA, USA) at 4°C overnight. Membranes were then incubated with anti-rabbit secondary antibodies (1:500; ab218695; Abcam) for 1 h at room temperature. An enhanced chemiluminescence system (GE Healthcare, Chicago, IL, USA) was used to detect the antibody binding.

Sphere formation assay. Cells were seeded at 2.5x10⁴ cells/well on 0.5% agar pre-coated 6-well plates. After 1 week, half the medium was exchanged every third day. After a total of 14 days, single spheres were picked and counted (25). Sphere formation efficiency was calculated by dividing the total number of spheres formed by the total number of live cells seeded multiplied by hundred.

MTT assay. To monitor resistance to gefitinib, A549 cells were treated with gefitinib (purity >99%; AstraZeneca, Cambridge, UK) at different concentrations for 24 h. An MTT assay was performed as described previously (2). Data were analyzed with the software Origin 7.5 (OriginLab, Northampton, MA, USA) to fit a sigmoidal curve. The IC₅₀ was the gefitinib concentration that reduced the number of viable cells by 50%.

Immunofluorescence staining. Cells seeded on glass coverslips in 6-well plates were fixed in 4% formaldehyde solution for 30 min at room temperature and permeabilized with 0.5% Triton-X-100/PBS. Cells were blocked with 5% BSA-PBS (BSA from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1 h at room temperature and incubated with primary antibodies against E-cadherin (1:500; ab92547; Abcam) or vimentin (1:500; ab40772; Abcam) at 4°C overnight. Cells were then incubated with rhodamine- or fluorescein isothiocyanate-conjugated secondary antibodies (1:500; ab150077; Abcam) for 1 h at room temperature. The coverslips were counterstained with DAPI and imaged under a confocal microscope (TCS SP5; Leica Microsystems, Wetzlar, Germany).

Wound healing assay. Cells (5x10⁵) were seeded onto each 35-mm glass bottom dish (MatTek Co., Ashland, MA, USA) and cultured at 37°C with 5% CO₂ for 24 h. The confluent monolayer of cells was wounded with yellow 200 µl pipette tips (Tiangen Biotech Co., Ltd.). After washing with warm PBS, the cells were incubated in fresh serum-free culture medium. Images of the wounded areas were captured at different time-points with an inverted microscope (Eclipse TE-2000U; Nikon, Tokyo, Japan) equipped with a video camera (DS-U1; Nikon). Results were examined at five randomly selected fields in each field, at x20 magnification. The wound areas were calculated by ImageJ 1.43b software (NIH, Bethesda, MD, USA).

Statistical analysis. Values are expressed as the mean ± standard error of the mean. SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for the analysis of experimental data. Student's t-test (two-tailed) was used for comparison between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpression of TASK-1 promotes gefitinib resistance. In order to confirm the efficiency of plasmid-mediated TASK-1-expression, A549 cells transfected with TASK-1-expressing plasmid and empty vector were subjected to western blot analysis. The results showed that TASK-1 protein was significantly upregulated in A549 cells transfected with TASK-1-expressing plasmid (Fig. 1A). To further identify whether TASK-1 affected the efficacy of gefitinib in A549 cells, A549 cells transfected with TASK-1-expressing plasmids or empty vector were subjected to an MTT assay (Fig. 1B). The results demonstrated that TASK-1 transformed native, gefitinib-sensitive A549 cells into gefitinib-resistant A549 cells, suggesting that its overexpression induced gefitinib resistance (Fig. 1B).

TASK-1 gives A549 cells CIC-like traits. In order to identify whether TASK-1 affects CIC-like traits in A549 cells, a sphere formation assay was performed to assess the capacity of A549 cells for self renewal, which is associated with CICs and CSCs. The results demonstrated that after 14 days of culture TASK-1-overexpressing cells formed bigger spheres than control cells, indicating markedly increased CIC-like traits provided by the TASK-1-expressing plasmids (Fig. 2A). To

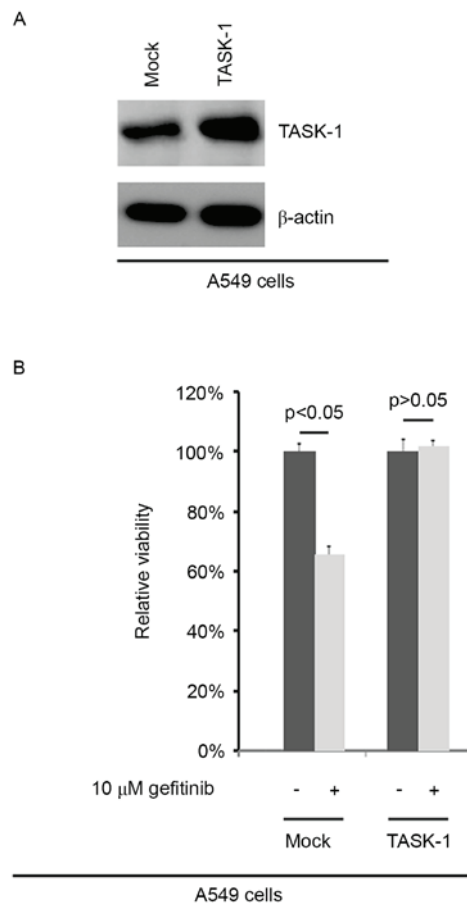


Figure 1. Overexpression of TASK-1 promotes gefitinib resistance. (A) Western blot analysis of TASK-1 in A549 cells. β -actin was used as a loading control. (B) MTT cell viability assay. A549 cells transfected with TASK-1 expressing plasmids or empty vector (mock) were untreated or treated with gefitinib. Values are expressed as the mean \pm standard error of the mean (n=3). TASK-1, TWIK-related acid-sensitive K(+).

identify whether TASK-1 regulates CD133, OCT-4 and Nanog protein expression, western blot analysis of A549 cells transfected with TASK-1-expressing plasmids and empty vector was performed. The results revealed that CD133, OCT-4 and Nanog protein were upregulated in A549 cells by TASK-1 (Fig. 2B).

TASK-1 induces EMT in A549 cells. Cell morphological observation revealed that overexpression of TASK-1 induced EMT phenotypes in A549 cells (transition from a cobblestone-like to a spindle-like morphology; Fig. 3A). In order to detect whether TASK-1 affects E-cadherin (epithelial marker) and vimentin (mesenchymal marker) protein, immunofluorescence analysis of A549 cells transfected with TASK-1 and empty vector was performed. It was found that overexpression of TASK-1 promoted vimentin expression and inhibited E-cadherin expression in A549 cells (Fig. 3B). Western blot analysis was also performed to detect E-cadherin and vimentin protein in A549 cells transfected with TASK-1 and empty vector. The results revealed that vimentin protein is upregulated, while E-cadherin is downregulated in A549 cells transfected with TASK-1 overexpression vector (Fig. 3C).

Overexpression of TASK-1 promotes migration of A549 cells. In an attempt to identify the role of TASK-1 in regulating the

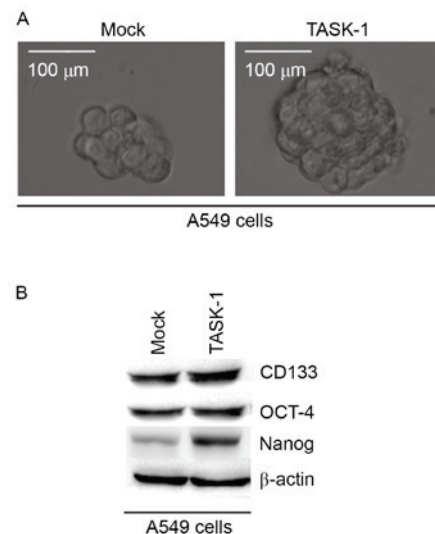


Figure 2. TASK-1 induces formation of CIC phenotypes in A549 cells. (A) Sphere growth of A549 cells transfected with TASK-1-expressing plasmids and empty vectors (mock) (scale bar, 100 μ m). (B) Western blot analysis of CD133, OCT-4 and Nanog in A549 cells transfected with TASK-1-expressing plasmids and empty vectors (mock). β -actin was used as a loading control. Representative images of triplicate experiments are shown. TASK-1, TWIK-related acid-sensitive K(+); OCT-4, octamer-binding transcription factor 4.

migration of A549 cells, a wound healing assay was performed to detect the migration of A549 cells transfected with TASK-1-expressing plasmid and empty vector. Overexpression of TASK-1 was found to promote the migration in the cells (Fig. 4A). In order to detect whether TASK-1 affects MMP-2 and MMP-9 protein expression, western blot analysis of A549 cells transfected with TASK-1 and empty vector was performed. It was revealed that expression of TASK-1 promotes MMP-2 and MMP-9 expression in A549 cells (Fig. 4B).

Discussion

NSCLC patients with early-stage disease are treated by surgery, and 30-60% develop recurrent tumors, which results in mortality (26,27). Chemotherapeutic agents, including gemcitabine, platinum compounds and taxanes, improve survival to a limited extent, but overall survival rates remain low due to recurrence of more aggressive, drug-resistant tumors (28,29). NSCLC in non-smokers predominantly has mutations in EGFR (30); such patients respond well to EGFR inhibitors such as gefitinib, but eventually develop resistance and succumb to the disease (31). The recurrence may be local or metastatic, and commonly occurs after a period of clinical dormancy. Resistance to EGFR inhibitors occurs through various mechanisms, including the appearance of a T790M gatekeeper mutation, expression of the c-Met gene or activation of alternate signaling pathways (32,33). Development of strategies to combat resistance to EGFR inhibitors in NSCLC will provide an immense benefit to a large number of patients (34).

A recent study reported that knockdown of TASK-1 by small interfering RNA significantly enhanced apoptosis and reduced proliferation in A549 cells (18). For the first time, to the best of our knowledge, the present study demonstrated that overexpression of TASK-1 induced gefitinib resistance in

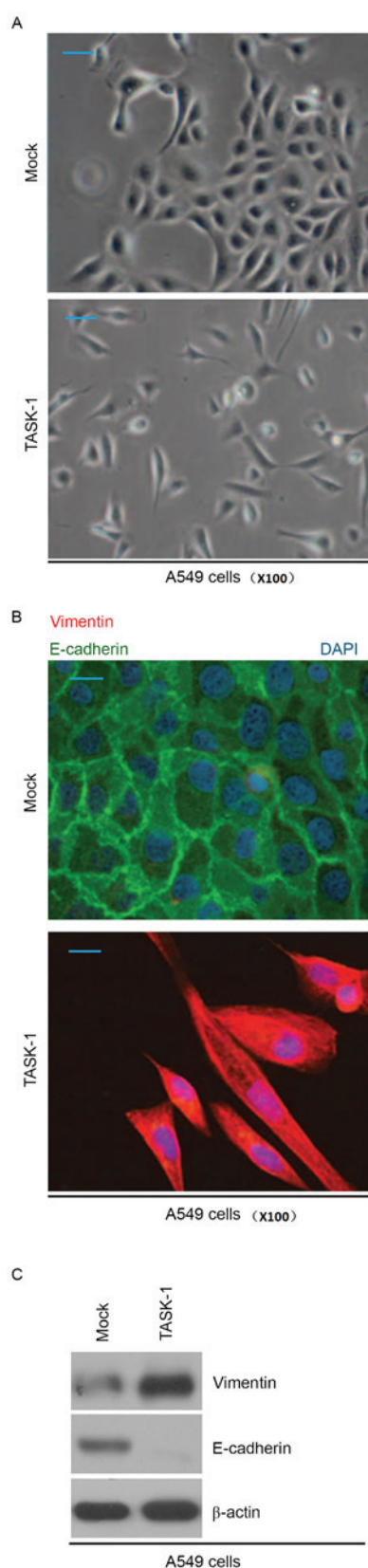


Figure 3. TASK-1 induces epithelial to mesenchymal transition of A549 cells. (A) Phase-contrast microscopy images of A549 cells transfected with TASK-1-expressing plasmids and empty vectors (mock). Scale bar=100 μm. (B) Immunofluorescence analyses for vimentin and E-cadherin in A549 cells transfected with TASK-1-expressing plasmids and empty vectors (mock). The nuclei were counterstained with DAPI. Scale bar=100 μm. (C) Western blot analysis of vimentin and E-cadherin in A549 cells transfected with TASK-1 expressing plasmids and empty vectors (mock). β-actin was used as a loading control. Representative images of triplicate experiments are shown. TASK-1, TWIK-related acid-sensitive K(+).

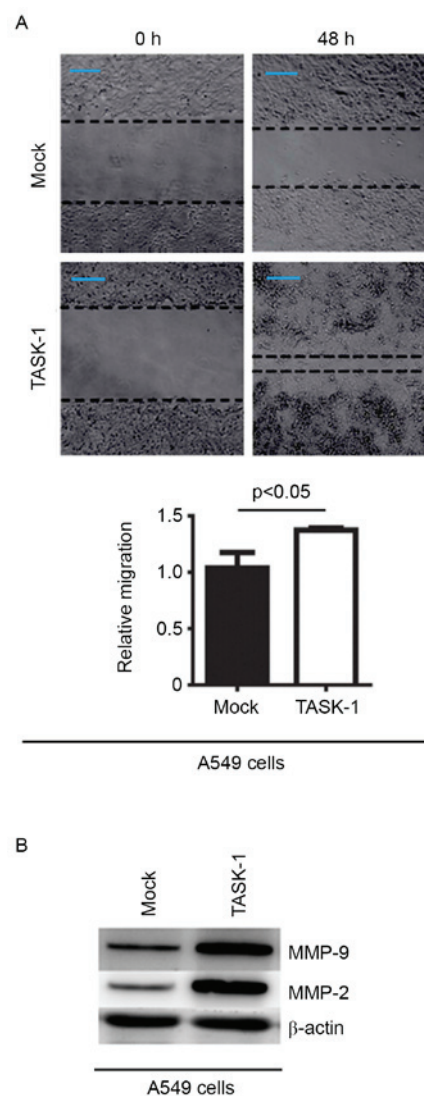


Figure 4. Overexpression of TASK-1 promotes migration of A549 cells. (A) Wound-healing assay of A549 cells transfected with TASK-1-expressing plasmids and empty vectors (mock). Images of the cell monolayers were captured directly after scraping and subsequent to 48 h of incubation. The wound healing rate was quantified by detecting the percentage of wound closure vs. that of the original wound. Values are expressed as the mean ± standard error of the mean (n=3). Scale bar=100 μm. (B) Western blot analysis of MMP-2 and MMP-9 expression in A549 cells transfected with TASK-1-expressing plasmids and empty vectors (mock). β-actin was used as a loading control. Representative images of triplicate experiments are shown. MMP, matrix metalloproteinase; TASK-1, TWIK-related acid-sensitive K(+).

A549 cells, implying that TASK-1 may represent a novel target to reverse gefitinib resistance.

Resistance to radiation therapies and conventional chemotherapeutic drugs is a common characteristic of CSCs (35-37). It has been reported that aldehyde dehydrogenase 1 family member A1 (ALDH1A1)-positive CSCs promote gefitinib resistance in lung cancer (17). The present study found that overexpression of TASK-1 promoted A549 cells to adopt CIC-like properties. In addition, overexpression promoted CD133, OCT-4 and Nanog protein expression in A549 cells, indicating that TASK-1 induces the development of CIC-like traits. However, it remains elusive whether TASK-1 promotes the formation of ALDH1A1-positive lung CICs.

Increased formation of a CIC population may result in EMT of cancer cells and EMT has been shown to contribute to the formation of CIC-like characteristics (38). It has been suggested that the reversal of the EMT phenotype potentially enhances the sensitivity of lung cancer cells to gefitinib (39). Consistent with these results, the present study demonstrated that overexpression of TASK-1 induced EMT of A549 cells. The degradation of ECM and the destruction of basement membrane are prerequisites for tumor infiltration and metastasis (40). Tumor cells must have the ability to degrade ECM and basement membranes, and this degradation process depends on proteolytic enzymes, mainly serine-degrading enzymes, cysteine proteases and MMPs. MMPs are considered to be the most important. MMP2 and MMP9 are important members of the MMP family, encoding 72 KDa gelatinase A and 92 KDa gelatinase B, respectively, which have a partial co-acting substrate that degrades the major constituents of the basement membrane IV type collagen. This is conducive to the migration of tumor cells (41,42).

In conclusion, the results of the present study provided strong molecular evidence demonstrating that TASK-1 promotes gefitinib resistance, EMT and CIC-like properties of NSCLCs. Thus, downregulation of TASK-1 appears to be a novel strategy for the reversal of the EMT, decreasing CIC-like traits and increasing gefitinib sensitivity of NSCLCs.

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