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Original Article

Neuroepithelial transforming gene 1 controls proliferation and migration of non-small cell lung cancer A549 cells via regulating RhoA

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Abstract: Non small-cell lung cancer (NSCLC), the main cause of cancer deaths worldwide, lacks effective treatments. Neuroepithelial Transforming Gene 1 (NET1) is a well identified oncogene and a proven regulator of cell proliferation and migration in multiple human cancers, including lung cancer. However, molecular mechanisms underlying its role in NSCLC are poorly elucidated. In this study, Net1 gene was removed by small interfering RNA, and then Real-time quantitative PCRs (qRT-PCRs) and western blot were used to detect the expression level of Net1 in A549 cells, while Cell Counting Kit 8 (CCK-8) and 5-Ethynyl-2'-deoxyuridine (EdU) assay were used to access the cell viability and proliferation. The effects on A549 cell migration were determined by wound healing assay. We demonstrated that down-regulation of Net1 resulted in a decrease of proliferation and migration in A549 cells, whereas co-transfection of NET1 siRNA and C3 exoenzyme (a RhoA inhibitor) further reduced cell proliferation and migration in comparison with the cells transfected with NET1 siRNA alone, validating that NET1 act as a driver of tumor cell proliferation and migration, a regulator mediated by RhoA activation.

Keywords: NET1, Non small-cell lung cancer, proliferation, migration, RhoA

Introduction

Lung cancer is one of the most rapidly fatal malignancies, and is the major cause of cancer-related mortality worldwide [1]. Non-small cell lung cancer (NSCLC) is the most common histological cancer sub-type, which accounts for approximately 80% of lung cancer cases [2]. Despite the therapeutic treatments have made great progress, including surgical resection, chemotherapy, radiation therapy or a combination of targeted therapy, however, the survival and prognosis of advanced NSCLC patients remain poor with 5-year survival rate less than 20% [3-6]. Therefore, to understand the mechanism on NSCLC formation and progression and identify novel therapeutic strategies are urgently needed.

Neuroepithelial transforming gene 1 (Net-1) is a 54-kDa oncoprotein and is a member of the guanine nucleotide exchange factors (GEFs) family, proteins in which are known to regulate Rho family members [7, 8]. Net1 has two distinct isoforms (Net-1 and Net-1A) and plays a crucial role in cell signal transduction, proliferation, migration and invasion; it is also a biomarker of poor prognosis of cancer patients [9, 10]. NET1 promotes OAC cell invasion and proliferation and it mediates LPA-induced OAC cell migration [11]. NET1 promotes lysophosphatidic acid-induced cell migration and invasion in gastric cancer [8]. Although NET1 was identified as an oncogene, its functional importance in other malignancies, especially in NSCLC, has not yet been demonstrated.

To date, the function of NET1 and the possible regulatory mechanism have not been performed in lung cancer cells. In this study, we identified the role of NET1 in proliferation and migration of human lung cancers and investigated the possible regulatory mechanism in lung cancer cells A549. Our work may provide a new way for the clinical diagnosis and therapy for human lung cancer patients.

Materials and methods

Cell culture and transfection

The human non-small cell lung cancer A549 cell line was obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium containing 10% fetal bovine serum with 100 U/ml of penicillin and 100 μg/ml of streptomycin. And it was maintained at 37°C in a humidified incubator with 5% CO_2. A549 cells were transfected and performed with commercial reagent Lipofectamine 2000 (Invitrogen, USA) following manufacturer's protocols. A549 cells were treated 4 mg ml^{-1} C3 exoenzyme (Sigma-Aldrich, USA) for 4 h.

Cell proliferative assay

The A549 cells were seeded in 24-well dishes at a density of 5 × 10^3 cells/well. After transfection with Net-1 sIRNA, cells were cultured with 5-Ethyny-2'-deoxyuridine (EdU) for 8 h before detection. The proliferative rate of A549 was then assessed using a Cell-Light™ EdU Cell Proliferation Detection kit (RiboBio, China) following the manufacturer's instructions.

Cell viability

Cell viability was confirmed with Cell Counting Kit 8 (CCK-8, Dojindo, Japan). In brief, A549 cells were planted into 96-well plate (5 × 10^3/well). After transfection, 10 μL CCK-8 solution was added into each well, and cells were incubated for 1 hour at 37°C. CCK-8 solution was added to each well and incubated for 1 h at 37°C. Absorbance was then measured at 450 nm using a Microplate Absorbance Reader (Bio-rad).

Wound healing assay

Cell migration of A549 cells was determined by wound healing assay. In brief, the A549 cells were seeded in a 24-well plate with the same numbers in complete medium, respectively, and incubated until the cells grown to 80% confluence. After transfection, a sterile pipette tip was used to scratch wounds on each monolayer with the same width, then the plates were washed with PBS to remove the detached cells and the remaining cells were cultured in serum-free DMEM medium. Pictures were subsequently taken at 0, 24, and 48 h. The closure of the wounds was determined by the width of the scratched area.

Real-time quantitative PCR

Total RNA was isolated from A549 cells after transfection for 24 h using RISO reagent (Biomics), and then was submitted to a 25 μl PCR reaction in the presence of 12.5 μl of 2 × Master Mix, 0.5 μl of each primer mix (10 μM), 0.5 μl of 50 × SYBRGreen I and 5 μl RNA. The PCR mixtures were first subjected to 30 min at 42°C for reverse transcription and initially denatured for 10 min at 94°C and then to 40 cycles of amplification with the following cycling parameters: 20 s at 95°C, 30 s at 55°C and 30 s at 72°C. GAPDH was used as a house-keeping gene for mRNA analysis. The sequence of NET-1 Primers: 5'-GTGGCTTCACCAACTATACG-3' (Forward), 5'-GACTGCATTAGTTCGGATGT-3' (Reverse). The sequence of GAPDH primer: 5'-GAAGGTGAAGGTCGGAGTC-3' (Forward), REV ACACCATGTATTCCGGGT CAAT (5'-3'). 5'-GAAGATGGTGATGGGATTTC-3' (Reverse).

Western blot

After siRNA transfection for 48 h, A549 cells were lysed with RIPA buffer (Beyotime Biotechnology, China). Extracted proteins were separated with SDSPAGE gels and were transferred onto PVDF membranes (Millipore, Bedford, MA, USA) for western blotting analysis. The following primary antibodies were used: NET-1 polyclonal antibody (Santa Cruz, USA), RhoA mouse monoclonal antibody (Santa Cruz, USA), Vimentin rabbit monoclonal antibody (Epitomics, USA), Mouse monoclonal [NH3] to F-actin (abcam, USA), Rabbit polyclonal to E-Cadherin (abcam, USA), β-actin (Bioworld, USA), then were detected using HRP-linked secondary Antibodies and the ECL System.

Statistical analysis

All data are based on the mean ± SEM and performed in triplicate, and a student's t-test was used to calculate all data via SPSS version 20. P value < 0.05 was considered as statistically significant.

Results

Net-1 is significantly decreased by siRNAs in A549 cells

To test whether Net-1 could be down-regulated, we first transfected Net-1 siRNAs, Net-1 siRNA-01 and Net-1 siRNA-02 to human NSCLC cell

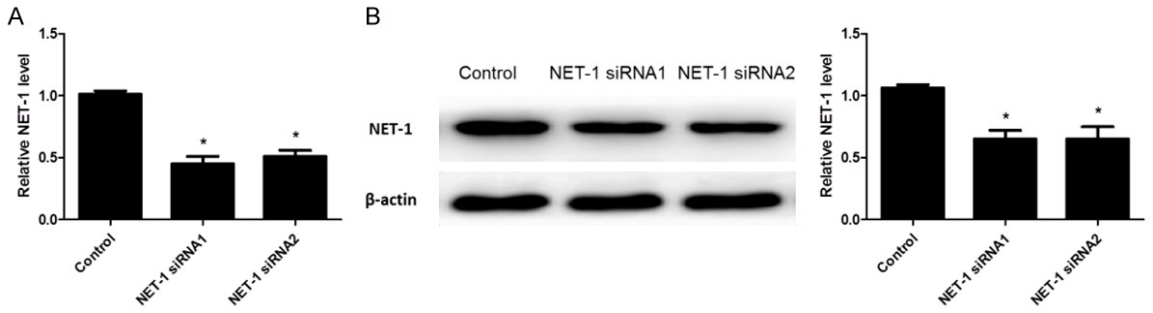

Figure 1. Transfection of small interfering RNA successfully took effects in decreasing Net-1 levels in A549 cells. The transfection of either Net-1 si-01 or Net-1 si-02 led to efficient knockdown of Net-1 as detected by both qRT-PCR (A) and western bolt (B). (n = 3). *P < 0.05.

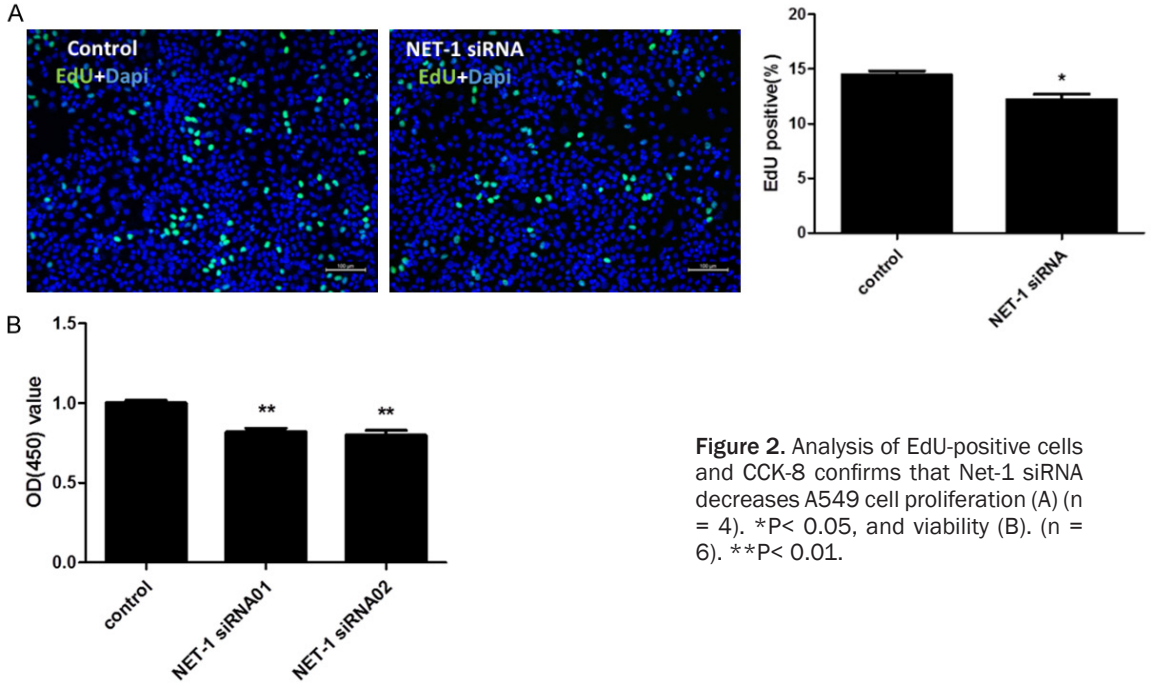

Figure 2. Analysis of EdU-positive cells and CCK-8 confirms that Net-1 siRNA decreases A549 cell proliferation (A) (n = 4). *P< 0.05, and viability (B). (n = 6). **P< 0.01.

line A549. As indicated, the transfection of Net-1 siRNAs led to efficient knockdown of Net-1 as detected by both qRT-PCR and western blot (**Figure 1A**, **1B**).

Knockdown of Net-1 reduces cell's proliferation

To check the effects of Net-1 in regulating A549 cell' proliferation, in this study we used EdU and Cell Counting Kit 8 assays. We showed that down-regulation of Net-1 significantly increased the percentage of EdU positive cells (**Figure 2A**) and cell viability (**Figure 2B**), indicating that Inhibition of Net-1 induces A549 cells' proliferation viability.

Inhibition of Net-1 attenuates migration potential of A549 cells

Migration potential of A549 cells was explored by wound healing assay. The results demonstrated that the healing ability of A549 cells in NET-1 siRNA group was significantly weaker than that of A549 cells in control group at 24 and 48 h after wound scratched (**Figure 3A**). As shown in (**Figure 3B**), the expression of migration suppressor gene E-cadherin increased, and the expression of migration promoting genes vimentin and F-actin decreased, indicating that down-regulation of Net-1 induces a weaker migration of A549 cells than control group.

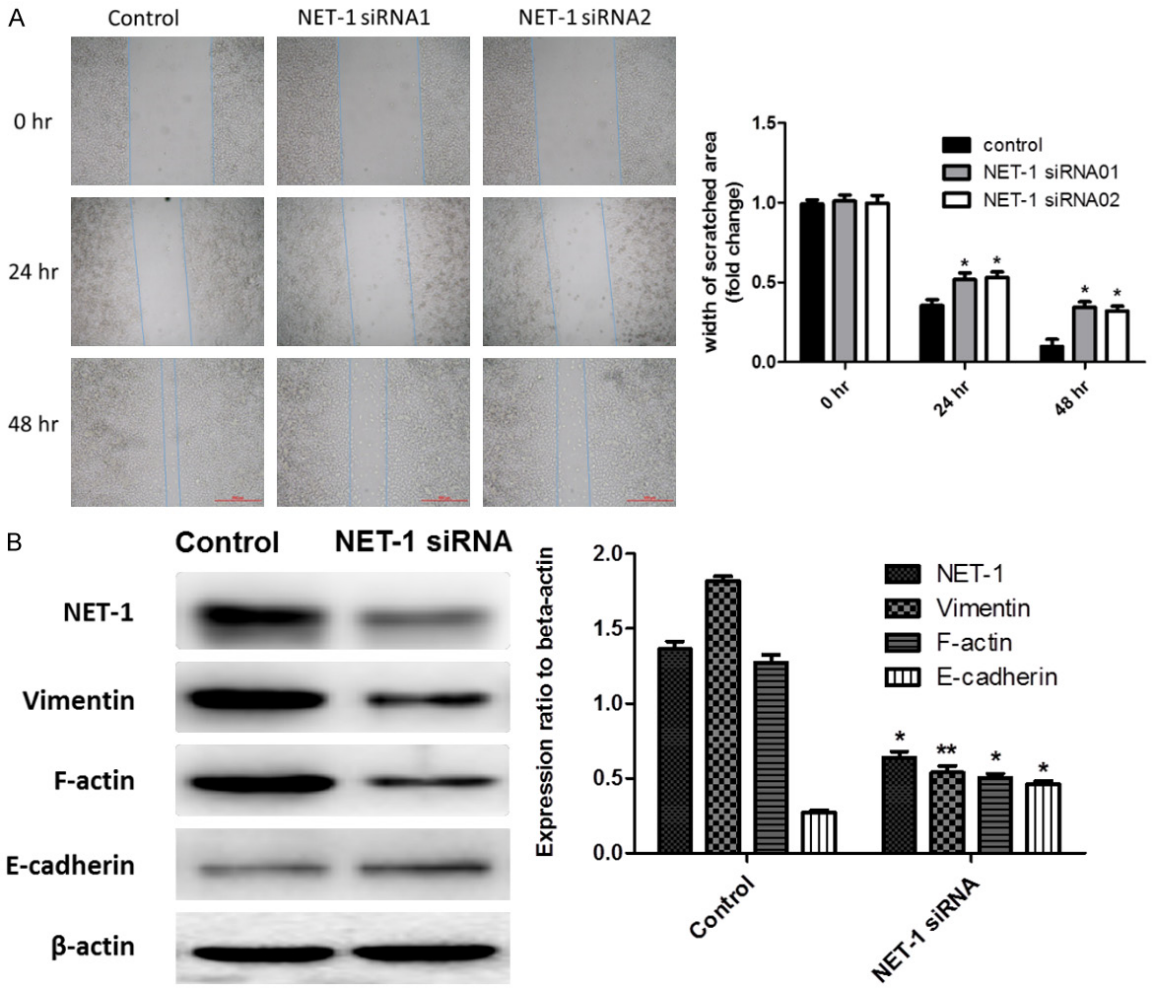

Figure 3. Up-regulation of Net-1 attenuates migration of A549 cells. The healing ability of A549 cells in NET-1 siRNA group was significantly weaker than that of A549 cells in control group at 24 and 48 h after wound scratched (A). As shown in (B), the expression of migration suppressor gene E-cadherin increased, and the expression of migration promoting genes vimentin and F-actin decreased. (n = 3). *P < 0.05.

The function of RhoA in NET1-mediated A549 cells proliferation and migration

Although having demonstrated the effect of NET-1 on mediating cell proliferation and migration, the roles of NET-1 needed to be further investigated. Knockdown of NET1 was shown to inhibit activation of RhoA (**Figure 4A**). Transfection of either small interfering RNA (siRNA) for NET-1 decreased A549 cell migration, whereas co-transfection of NET-1 siRNA and C3 exoenzyme (a RhoA inhibitor) further enhance A549 cell migration in comparison with the control group (**Figure 4B**), validating that NET1 is critical for regulation of RhoA, as well as suggesting that NET1 may mediate proliferation and migration of A549 cells through RhoA activation.

Discussion

We have previously reported NET1 as being up-regulated in NSCLC A549 cells [12]. Moreover, over expression of Net1 may contribute to the development of NSCLC, and it may be a novel prognostic predictor in lung cancer [13]. However, the role of Net1 in lung carcinoma has not been described. To understand the function of Net1 to A549 cells, we have generated a conditional *Net1* knockout model in vitro. In this study, we show that the *Net1* deletion decreases A549 cells proliferation and migration through regulating RhoA. Moreover, NET1 siRNA decreased A549 cell migration rate, whereas co-transfection of NET1 siRNA and C3 exoenzyme (a RhoA inhibitor) further

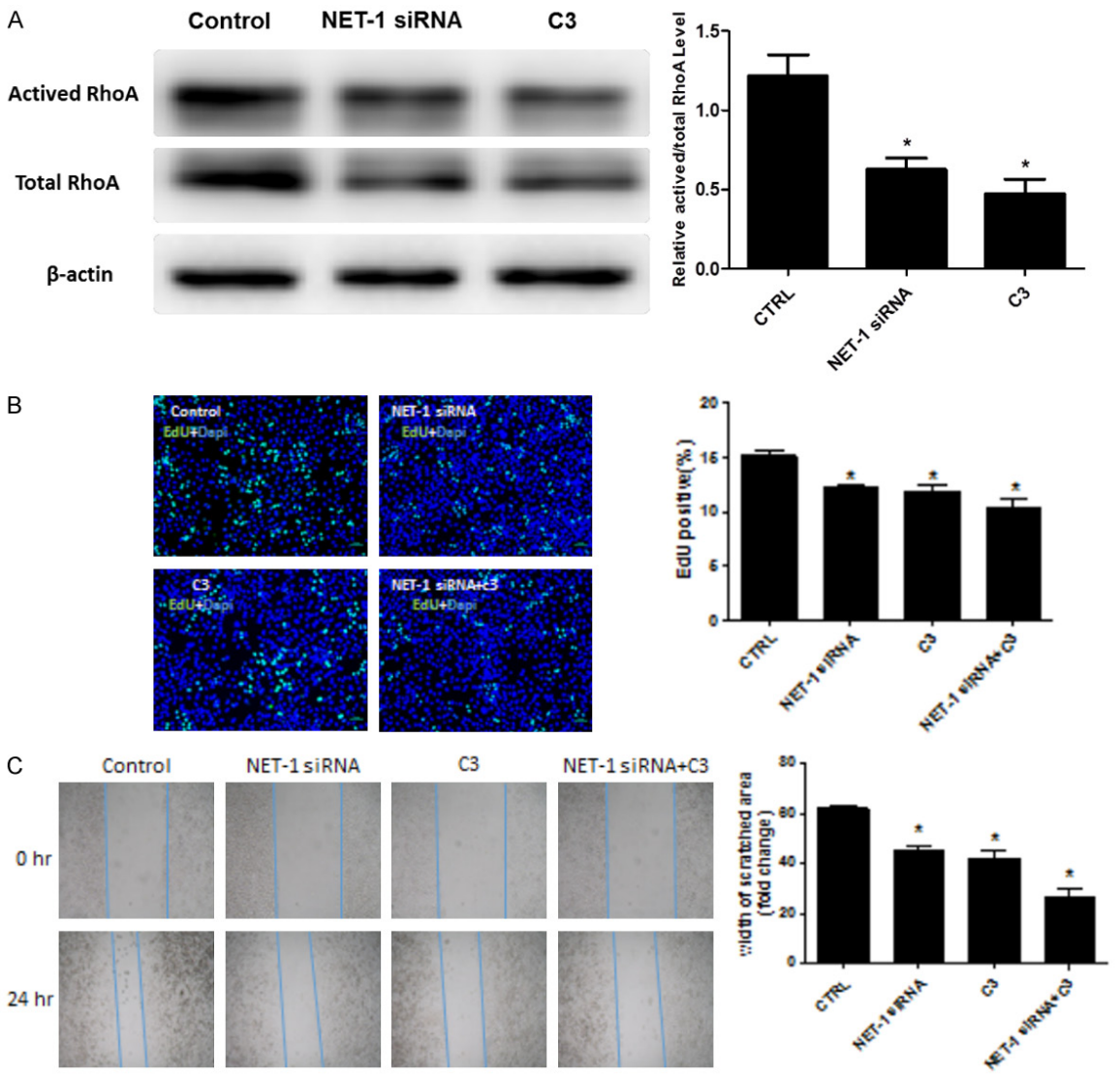

Figure 4. NET1 may mediate proliferation and migration of A549 cells through regulating RhoA. Knockdown of NET1 was shown to inhibit activation of RhoA (A). Transfection of either small interfering RNA (siRNA) for NET-1 decreased A549 cell proliferation and migration, whereas co-transfection of NET-1 siRNA and C3 further weaken A549 cell proliferation and migration in comparison with the control group, (B, C). (n = 3). *P < 0.05.

induces cell proliferation in comparison with the cells transfected with NET1 siRNA alone.

Previous studies indicate that Net-1 is frequently up-regulated in various cancer types, including hepatocellular carcinoma, gastric cancer, gliomas, rhabdomyosarcoma, cervical carcinomas and breast cancer [9, 10, 14-17]. Lahiff et al found that Net1 is markedly increased in invasive and metastatic adenocarcinoma of the oesophagogastric junction [18]. Murray et al demonstrated that the migration and invasion of AGS gastric cancer cells are inhibited via Net1-siRNA [8]. Besides, the data found that deletion of Net-1 resulted in the formation of round cells and a loss of definition in the actin cytoskeleton [8]. GEFs such as Tiam1 have been shown to increase migration and invasion of cancer cells, such as breast and colon cancer [19]. Studies also have shown that GEFs, such as GEFT, promote cellular proliferation [20]. In our work, we use Cell Counting Kit 8 (CCK-8) and 5-Ethynyl-2'-deoxyuridine (EdU) assay to access the cell viability and proliferation. We show that reduction of NET1 attenuates proliferation and migration of A549 cells. Meanwhile, migration was assessed using wound-healing assays. Significantly fewer

NET1 knockdown cells migrated across the scratch when compared to the control group ($P<0.05$). Moreover, NET1 act as a regulator mediated by RhoA activation. These results suggest a central role for NET1 in NSCLC A549 cell proliferation and migration. Inhibition of RhoA activation may function as a novel therapeutic strategy for NSCLC.

Our findings showed that the expression level of RhoA is positively correlated with NET1, further confirmed the notion that RhoA has the potential to function as an Oncogene. However, it is not yet clear what the downstream events of the interaction between NET1 and RhoA are. Also, which region of each protein is responsible for their interaction remains unclear. Therefore, further research will be necessary to elucidate the detailed mechanism for the tumor promoting gene function of RhoA.

In conclusion, this study provides a critical role for NET1 in NSCLC. We have shown that NET1 plays fundamental roles in A549 cell progression by regulating cancer cell migration and proliferation. Furthermore, we also have demonstrated that NET1 is a key player in activation of RhoA and the subsequent proliferation and migration of lung cancer cells. NET1 inhibition was as effective at reducing cell migration as treatment with the RhoA inhibitor, C3 exoenzyme, highlighting its importance in this setting. As NET1 is important to the growth and migration, we therefore propose that NET1 is an ideal potential therapeutic target in lung carcinoma.

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Disclosure of conflict of interest

None.

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