# NME4 may enhance non-small cell lung cancer progression by overcoming cell cycle arrest and promoting cellular proliferation

WENQIAN WANG[1,2*], MING DONG[3*], JIE CUI[1,2], FEI XU[1,2], CHEN YAN[1,2], CHENG MA[1,2], LA YI[1,2], WEIFENG TANG[1,2], JINGCHENG DONG[1,2] and YING WEI[1,2]

[1]Department of Integrative Medicine, Huashan Hospital, Fudan University;
[2]The Institutes of Integrative Medicine of Fudan University, Shanghai 200040;
[3]Gumei Community Health Center of Minhang District of Shanghai, Shanghai 201102, P.R. China

**Abstract.** Nucleoside diphosphate kinase 4 (NME4) is abnormally expressed in a variety of cancer types. However, the function of the NME4 gene in non-small cell lung cancer (NSCLC) remains to be elucidated. In order to investigate the role of NME4 in NSCLC, the present study detected the expression of the NME4 gene in the Cancer Genome Atlas database, and in BEAS-2B, NCI-H1299 and A549 cell lines. NME4 was significantly overexpressed in NSCLC tissues and NSCLC cell lines. Furthermore, lentivirus-mediated knockdown vector infection, cell proliferation, cell cycle, apoptosis, colony formation and MTT assays were conducted to explore the effect of NME4 on NSCLC *in vitro*. After knockdown of NME4 with short hairpin RNA, the cell cycle was arrest at the G1 phase, and proliferation and colony formation were inhibited in the NCI-H1299 and A549 cell lines. The present results suggested that NME4 may serve as a novel tumor promoter, capable of enhancing NSCLC progression by overcoming cell cycle arrest and promoting proliferation.

## Introduction

Lung cancer is one of the most common types of cancer and the leading cause of cancer-associated mortality in America (1). Of all lung cancer cases, ~83% are of the non-small cell lung cancer (NSCLC) histological type (2). Despite advances in lung cancer treatment (3), NSCLC remains an aggressive type of lung cancer due to its metastatic potential (3). Considering the important contribution of NSCLC to the worldwide burden of cancer (4), it is important to further elucidate the mechanisms and to explore novel treatments.

Nucleoside diphosphate kinase 4 (NME4), also known as non-metastatic clone 23 human isoform 4 (NM23-H4), is one of the members of the NM23 family (5). The NME4 protein, which contains a mitochondrial target sequence (6), has been shown to be associated with mitophagy (7), cell apoptosis (8), invasive potential (9) and inflammatory reactions (10). Kagan *et al* (7) found that high expression of wild-type NME4 in HeLa cells increases cardiolipin externalization, thus regulating the elimination of mitochondria via autophagy. In most cancer models, the loss of autophagy reduces tumor growth, survival and proliferation (11). Genomic aberrations or altered gene expression have been observed with respect to NME4 in several types of cancer, such as gastric cancer (12,13), colorectal carcinoma (14), renal tumors (14), breast cancer (15), testicular germ cell tumors (16) and large cell anaplastic lymphoma (17). However, rarely has the relationship between NME4 and NSCLC been reported. A previous study demonstrated that a decreased n-6/n-3 fatty acid ratio reduces the invasive potential of human lung cancer cells by downregulating the expression of cell adhesion/invasion-associated molecules such as NME4 (9). In addition, autophagy in NSCLC preserves mitochondrial quality and regulates their abundance to promote tumorigenesis (18). The NME4 protein is involved in the function of the outer and inner mitochondrial membranes (19) and is critical for mitochondrial mitophagy (10). This indicates that the NME4 gene may be implicated in the mechanisms of NSCLC progression.

The present study was designed to further explore the effect of NME4 on NSCLC *in vitro*. Firstly, The Cancer Genome Atlas (TCGA) database and reverse transcription-quantitative PCR (RT-qPCR) were used to assess the expression of NME4 in NSCLC tissues and NSCLC cell lines. Then, short hairpin RNA (shRNA) was used to silence the expression of NME4 in NSCLC cell lines; cell proliferation, cell cycle, apoptosis, colony formation and MTT assays were performed to clarify the possible role of NME4.

*Correspondence to:* Dr Ying Wei or Dr Jingcheng Dong, Department of Integrative Medicine, Huashan Hospital, Fudan University, 12 Middle Urumqi Road, Shanghai 200040, P.R. China
E-mail: weiying_acup@126.com
E-mail: jcdong2004@126.com

*Contributed equally

## Materials and methods

*Analysis of NME4 in NSCLC from TCGA.* TCGA (http://cancergenome.nih.gov/) is a collection of microRNA-sequencing (seq), RNA-seq, single-nucleotide polymorphism array, DNA methylation and exome sequencing data, among other data types. TCGA can be used to analyze complex cancer genomics and clinical parameters. In the present study, data of RNA-Seq in lung adenocarcinoma for NME4 were extracted from TCGA, and the expression levels of NME4 in each case was calculated according to the distributions of the exon reads.

*Cell lines and cell culture.* The NCI-H1299 and A549 cell lines, which are commonly used in NSCLC research, were purchased from the Cell Bank of the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mmol/l L-glutamine at 37°C in humidified air containing 5% $CO_2$. BEAS-2B cells were cultured in DMEM/F-12 (Gibco; Thermo Fisher Scientific, Inc.), containing 5% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mmol/l L-glutamine at 37°C in humidified air containing 5% $CO_2$. The complete medium in the present study was changed at least once every 2 days.

*RNA extraction, reverse transcription (RT) and quantitative PCR (qPCR).* TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and M-MLV Reverse Transcriptase (Promega Corporation) were used to extract the total RNA from cells and to perform RT, respectively. RT was conducted at 73.5°C for 7 min, in an ice water mixture for 5 min, at 43.5°C for 1 h and finally at 73.5°C for 3 min. Next, qPCR was performed on a MX3000P qPCR System (Agilent Technologies, Inc.) using the Takara SYBR Master Mixture (Takara Biotechnology Co., Ltd.). NME4 expression was quantified in real time with SYBR Green and normalized to GAPDH expression, which was used as an internal control. The denaturing, annealing and extension conditions of each PCR cycle were 95°C for 30 sec, 95°C for 5 sec and 60°C for 30 sec, respectively. The gene-specific primer pairs were as follows: NME4 forward, 5'AGGGTACAATGTCGTCCGC3' and reverse, 5'GACGCT GAAGTCACCCCTTAT3'; and GAPDH forward, 5'TGACTT CAACAGCGACACCCA3' and reverse, 5'CACCCTGTTGCT GTAGCCAAA3'. Each experiment was repeated twice in triplicate. The expression of target genes was calculated using the $2^{-\Delta\Delta Cq}$ method (20).

*Lentivirus-mediated knockdown vector transduction.* Lentiviral vectors for NME4-shRNA, which were purchased from Shanghai GeneChem Co., Ltd., were used to examine the function of NME4 (human NME4 cDNA; National Center for Biotechnology Information accession no. NM_005009); the vector used was hU6-MCS-CMV-EGFP. A total of two experimental groups for each cell line were constructed. The shNME4 group was infected with NME4-shRNA lentivirus (5'TGATTGGACACACCGACTC3'), while control cells were infected with a lentivirus containing a scramble sequence (5'TTCTCCGAACGTGTCACGT3'). NCI-H1299, A549 and BEAS-2B cells in 6-well plates ($2x10^6$ cells/well) were infected with lentiviral particles at a multiplicity of infection of 10 ($5x10^6$ TU/ml) for 16 h using polybrene (Sigma-Aldrich; Merck KGaA), following which the medium was replaced with fresh culture medium and cells were cultured for a further 56 h; the knockdown efficiency was detected by RT-qPCR. Prior to transduction, the medium of A549 cells was replaced with Opti-Minimal Essential Medium (Gibco; Thermo Fisher Scientific, Inc.) + polybrene, whereas the H1299 cell medium was replaced with RPMI-1640 medium + polybrene; BEAS-2B cells were cultured with DMEM/F12 + polybrene for transduction.

*Cell proliferation.* NME4-shRNA-transfected cells and control cells were collected with 0.25% trypsin-EDTA and resuspended in RPMI-1640 standard medium once they had achieved logarithmic growth. Cells were then seeded at a density of 2,000 cells/well and further incubated at 37°C in humidified air containing 5% $CO_2$ for 5 days. GFP expression (from the hU6-MCS-CMV-EGFP vector) was measured in each well using a Cellomics ArrayScan VT1 (Thermo Fisher Scientific, Inc.) for a 5-day period. Data were mapped, and cell proliferation curves were generated for each group.

*Cell cycle analysis.* A total of $2x10^6$ cells were seeded in 6-well plates, incubated overnight and then starved in culture medium without serum for 12 h to synchronize their cell cycle. Next, cells were cultured in complete medium for 48 h, harvested, washed and fixed in 70% ethanol overnight at 4°C. Cells were then washed, and stained with propidium iodide (PI; 10 $\mu$g/ml) and RNase A (100 $\mu$g/ml) at room temperature for 30 min, followed by flow cytometry detection using a Guava easyCyte HT flow cytometer (EMD Millipore) and Shortcut 3.1 software (Incyte Corporation). Cells with sub-G1 DNA content were considered apoptotic cells. All experiments were performed in triplicate.

*Apoptosis analysis.* The cells in each group were harvested with 0.25% trypsin, washed once with ice-cold PBS and analyzed with the Annexin V-Allophycocyanin Apoptosis Detection kit with PI (eBioscience; Thermo Fisher Scientific, Inc.) to assess apoptosis. Cells were centrifuged at 200 x g and room temperature for 10-15 min, resuspended and incubated at room temperature for 10-15 min according to the manufacturers' instructions, followed by flow cytometry detection of the apoptotic cells using Shortcut software. Detection was performed at 72 h after NME4 knockdown.

*Colony formation assay.* Cell colony formation was examined by a colony formation assay. Cells in each group were digested with 0.25% trypsin and seeded into 6-well plates at a density of 800 cells/well. After 2 weeks of incubation, colonies that included >50 cells were scored as surviving colonies. Colonies were visualized under a fluorescence microscope (IX71; Olympus Corporation). Cells were washed with PBS, fixed with 4% paraformaldehyde (Sangon Biotech Co., Ltd.) for 30 min at room temperature and stained with 500 $\mu$l Giemsa solution (ECM550; Chemicon International; Thermo Fisher
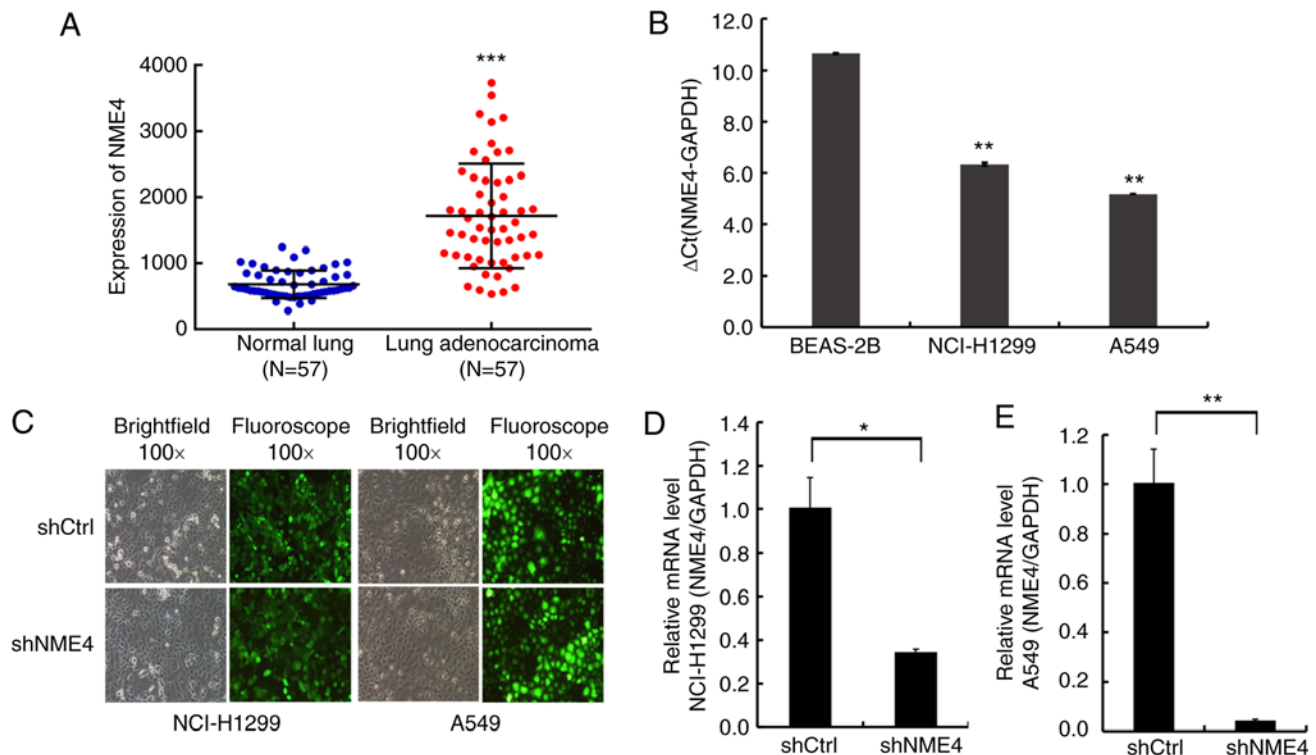
Figure 1. Results for NME4 expression in TCGA database and different cell lines. (A) NME4 was highly expressed in lung adenocarcinoma compared with non-cancerous lung tissues, based on TCGA database. ***P<0.001 vs. normal lung. (B) Expression levels of NME4 mRNA in BEAS-2B, NCI-H1299 and A549 cell lines (n=3). **P<0.01 vs. BEAS-2B. (C) After lentiviral transduction for 72 h, the infection rate of cells reached >80% and the status of the target cells was normal. (D) After lentiviral transduction, relative NME4 mRNA expression was significantly inhibited in the NCI-H1299 NME4-shRNA silenced cells (shNME4 group) compared with the negative control cells, as assessed by RT-qPCR. (E) After lentiviral transduction, relative NME4 mRNA expression was significantly inhibited in the A549 NME4-shRNA silenced cells compared with the negative control cells, as assessed by RT-qPCR. GAPDH was used as an internal control. Comparisons between two groups were performed by Student's t-test and between multiple groups by ANOVA. Error bars indicate SD. *P<0.05, **P<0.01. RT-qPCR, reverse transcription-quantitative PCR; NME4, nucleoside diphosphate kinase 4; sh, short hairpin; TCGA, The Cancer Genome Atlas.

Scientific, Inc.) for 20 min at room temperature. Following several washes with deionized distilled water, the cells were allowed to air dry at room temperature. Colonies were counted and images were captured with a digital camera under light microscopy (magnification, x100; XDS-100; Shanghai CaiKang Optical Instrument Co., Ltd.). The assay was repeated three times.

*MTT assay.* Cell proliferation was examined by MTT assay. Cells (2,000 cells/ml) were seeded into 96-well plates and incubated at 37˚C for 24, 48, 72, 96 or 120 h. At 4 h prior to each time point, 0.5% MTT solution (Thermo Fisher Scientific, Inc.) was added, followed by incubation for 4 h at 37˚C. The cell supernatants were discarded, and the formazan crystals were dissolved in 100 $\mu$l dimethyl sulfoxide. The optical density (OD) of each group was measured using a microplate reader (M2009PR; Tecan Group, Ltd.) at a wavelength of 490/570 nm.

*Statistical analysis.* Data were analyzed using SPSS 19.0 statistical software (IBM Corp.). Data are expressed as the mean ± SD of three experimental repeats. Comparisons between two groups were performed by Student's t-test and between multiple groups were performed by one-way ANOVA followed by Student-Newman-Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Information from TCGA database.* To further elucidate the relationship between NME4 and NSCLC, a clinical study was performed using original data from TCGA. It was found that NME4 was highly expressed in lung adenocarcinoma compared to non-cancerous lung tissues (P<0.001; Fig. 1A).

*NME4 is overexpressed in NSCLC cell lines and silenced upon lentivirus transduction.* To investigate the NME4 mRNA expression levels in NSCLC, RT-qPCR was performed in BEAS-2B, NCI-H1299, and A549 cells. NME4 mRNA expression levels were high in NCI-H1299 and A549 cells compared to BEAS-2B (Fig. 1B). Then, NCI-H1299 and A549 cells were transfected with a NME4-shRNA lentivirus or a scramble vector lentivirus. Upon lentiviral infection for 72 h, the infection rate was >80% (Fig. 1C), making these cells suitable for subsequent experiments. Upon NME4-shRNA lentiviral transduction, RT-qPCR analysis revealed that NME4-shRNA decreased the mRNA expression of endogenous NME4 significantly (P<0.05; Fig. 1D and E).

*NME4 silencing inhibits NCI-H1299 and A549 cell proliferation.* Following lentiviral transduction, the proliferation of NCI-H1299 and A549 cells was obviously inhibited in NME4-shRNA-silenced cells compared with control cells, as shown by GFP-based Cellomics ArrayScan
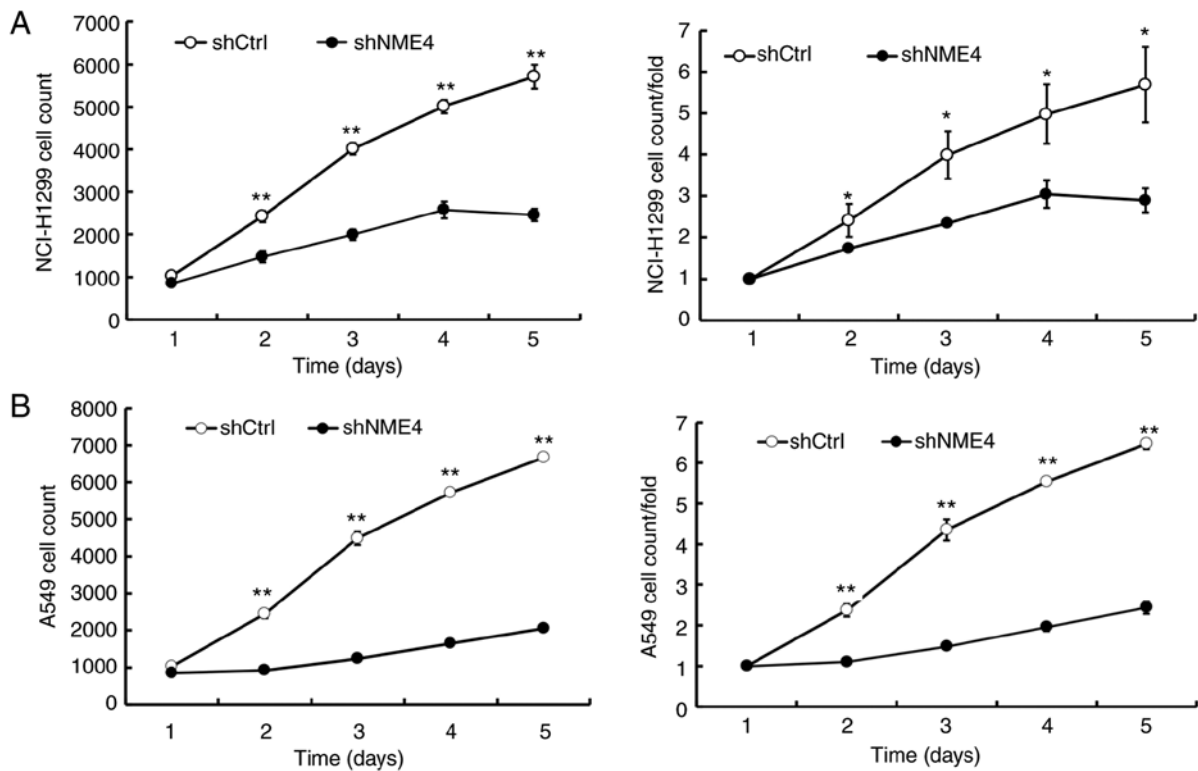
Figure 2. Cell proliferation analysis by green fluorescent protein-based imaging. (A) After lentiviral transduction of NCI-H1299 cells, cell counts and fold-changes were significantly inhibited in NME4-shRNA-silenced cells compared with control cells. (B) After lentiviral transduction of A549 cells, cell counts and fold-changes were significantly inhibited in NME4-shRNA-silenced cells compared with control cells. Statistical significance was assessed by ANOVA for the comparison of multiple groups. Error bars indicate SD. $^{*}P<0.05$, $^{**}P<0.01$ vs. shCtrl. NME4, nucleoside diphosphate kinase 4; sh, short hairpin; Ctrl, control.

VTI imaging (Fig. 2). The cell numbers in each group were monitored for 5 days. Cell numbers were significantly reduced in the NME4-shRNA-silenced cells (Fig. 2). These results suggested that NME4 was associated with NCI-H1299 and A549 cell proliferation.

*NME4 silencing induces cell cycle progression.* Cell cycle distribution was assessed to further elucidate the growth-suppressing effect of NME4-shRNA on NCI-H1299 and A549 cells. Compared with the control group, NME4-shRNA significantly increased the fraction of G1-phase cells in the NME4-shRNA group (Fig. 3). The results suggested that NME4 silencing may induce cell cycle arrest at the G1 phase, and the effect of NME4 on the cell cycle was time-dependent. G1 arrest was more pronounced in A549 cells compared with NCI-H1299 cells (Fig. 3), which may be due to the less efficient knockdown of NME4 (Fig. 1C and D). Whether the silencing of NME4 was associated with apoptosis in NCI-H1299 and A549 cells was further investigated. The proportions of apoptotic cells were increased by only 3-5% in NME4-silenced groups of NCI-H1299 and A549 cells when compared with control cells, comparable to the effects observed in shRNA-transduced BEAS-2B cells (Fig. 4). These data do not suggest that NME4 had an effect on the apoptosis of NCI-H1299 and A549 cells. This level of increase in apoptosis may be due to the state of the cells under the conditions of the assay. These results may indicate that NME4 silencing interrupted cell cycle progression and thus affected the progression of NSCLC, rather than increasing cell apoptosis.

*NME4 silencing reduces NCI-H1299 and A549 cell colony formation.* Silencing of NME4 suppressed the anchorage-independent growth of NCI-H1299 and A549 cells in soft agar (Fig. 5). Infection with NME4-shRNA in NCI-H1299 and A549 cells significantly reduced the number of cell clones (P<0.01), which confirmed that the silencing of NME4 suppressed the proliferative potential of NCI-H1299 and A549 cells, and revealed the critical role of NME4 in the survival of NSCLC cells.

*MTT assay.* An MTT assay was performed to detect the proliferation of NCI-H1299 and A549 cells upon transfection with NME4-shRNA for 24, 48, 72, 96 or 120 h. The OD value at 490 nm indicated the viability of the cells. The results of MTT assay demonstrated that the silencing of NME4 significantly decreased the proliferation of NCI-H1299 and A549 cells (Fig. 6), which indicates that NME4 contributes to NSCLC proliferation.

## Discussion

As one of the most common cancer types, NSCLC also has the highest mortality rate. Although the mainstays of lung cancer treatment may slow down tumor growth, the genetic versatility of tumor cells may induce resistance to the currently available therapies. Thus, it is critical to develop novel, effective and safe approaches for NSCLC treatment.

NME4 is one of the members of the NM23 family (5), which have been postulated to be involved in cell adhesion
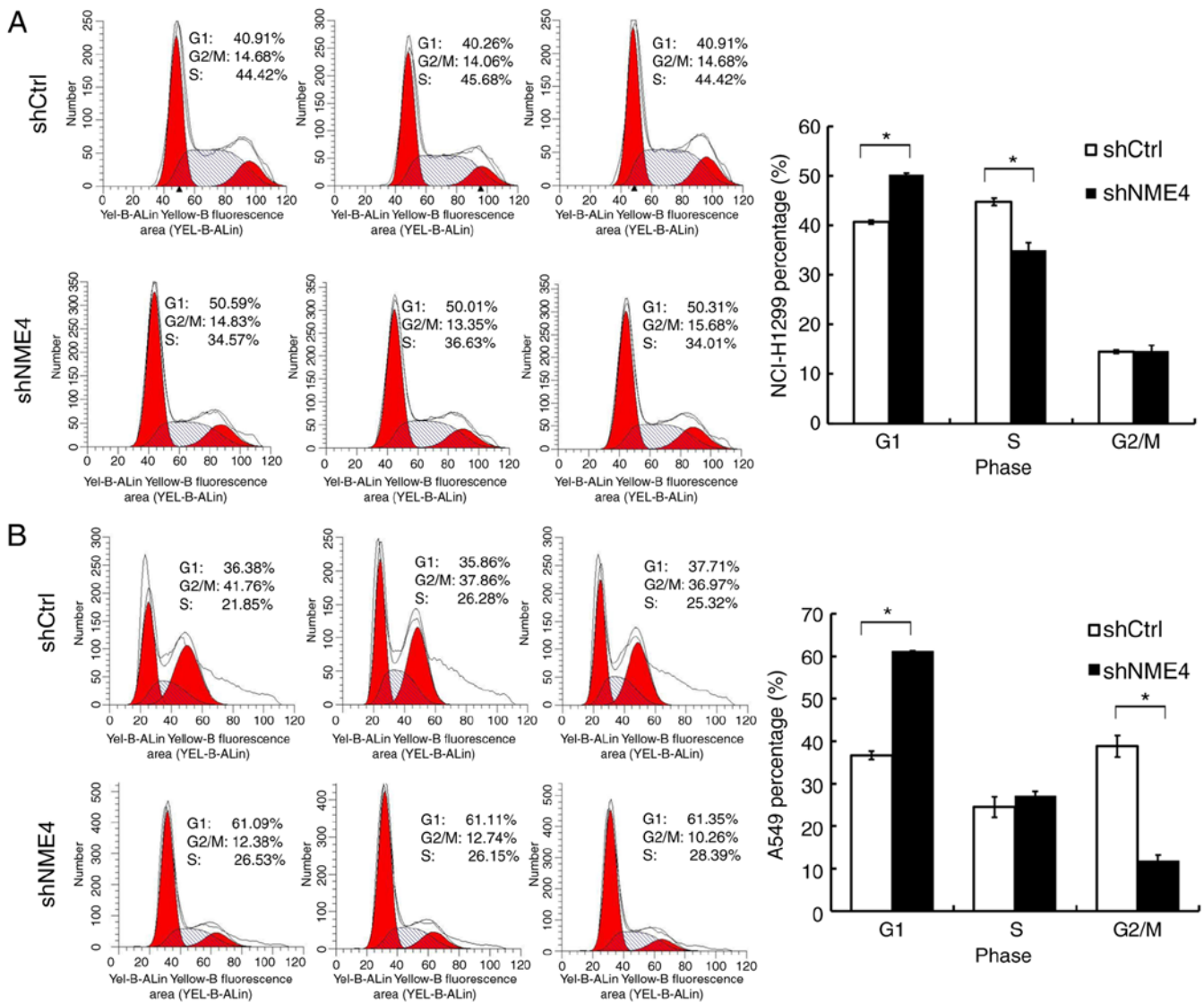
Figure 3. Effects of the silencing of NME4 on cell cycle distribution. (A) After lentiviral transduction of NCI-H1299 cells, cell cycle assessment showed that knockdown of NME4 in NCI-H1299 cells induced accumulation in the G1 phase, but a decreased number of cells in the S phase. (B) After lentiviral transduction of A549 cells, cell cycle assessment showed that knockdown of NME4 in A549 cells induced accumulation in the G1 phase, but a decreased number of cells in the G2/M phase. The percentages of cells in different phases are shown as the mean ± SD of three independent experiments. Statistical significance was assessed by two-tailed Student's t-test. Error bars indicate SD. *P<0.05. NME4, nucleoside diphosphate kinase 4; sh, short hairpin; Ctrl, control.

and migration (21), as well as possessing NDPK activity and being involved in DNA repair mechanisms (12,22). The NME4 protein contains a mitochondrial target sequence (19,23,24), and is involved in the function of the outer and inner mitochondrial membranes (19), which is critical for mitochondrial mitophagy (7). Mitophagy can maintain the normal metabolism of cells and prevent cellular stress responses and genomic damage, thereby inhibiting tumor development. Furthermore, tumor cells enhance their tolerance to hypoxia and low nutrition by enhancing mitophagy so as to survive; mitophagy serves a significant role in promoting tumor development (25,26). Cancer cells require altered mitochondrial functions, including organelle dynamics, to resist bioenergetic/biosynthetic reprogramming for supporting proliferation, migration and invasion (27). Since NME4 plays a role in all of these, it is tempting to speculate that its altered expression or function may affect the fate of a cancer cell. At the same time, it has been reported that NME4 may constitute

an important link between energy metabolism and cellular regulation. Abnormal expression of the NME4 gene may induce an imbalance of nucleotide pools in the mitochondria, resulting in the failure of checkpoint controls and the accumulation of subsequent genetic alterations, thus contributing to tumorigenesis (12).

Therefore, it was hypothesized that NME4 may participate in an important mechanism in NSCLC. NME4 was significantly overexpressed in NSCLC tissues, based on TCGA data. Furthermore, the present study demonstrated that NME4 mRNA expression levels were high in human NSCLC cell lines via qPCR. The present study also established low expression of NME4 in A549 and NCI-H1299 cells using lentivirus-mediated technology. When NME4 was expressed at reduced levels, the cell proliferation rate and colony formation of A549 and NCI-H1299 cells markedly decreased. Low expression of NME4 also resulted in cell cycle arrest at the G1 phase in these NSCLC cell lines. Based on these observations,
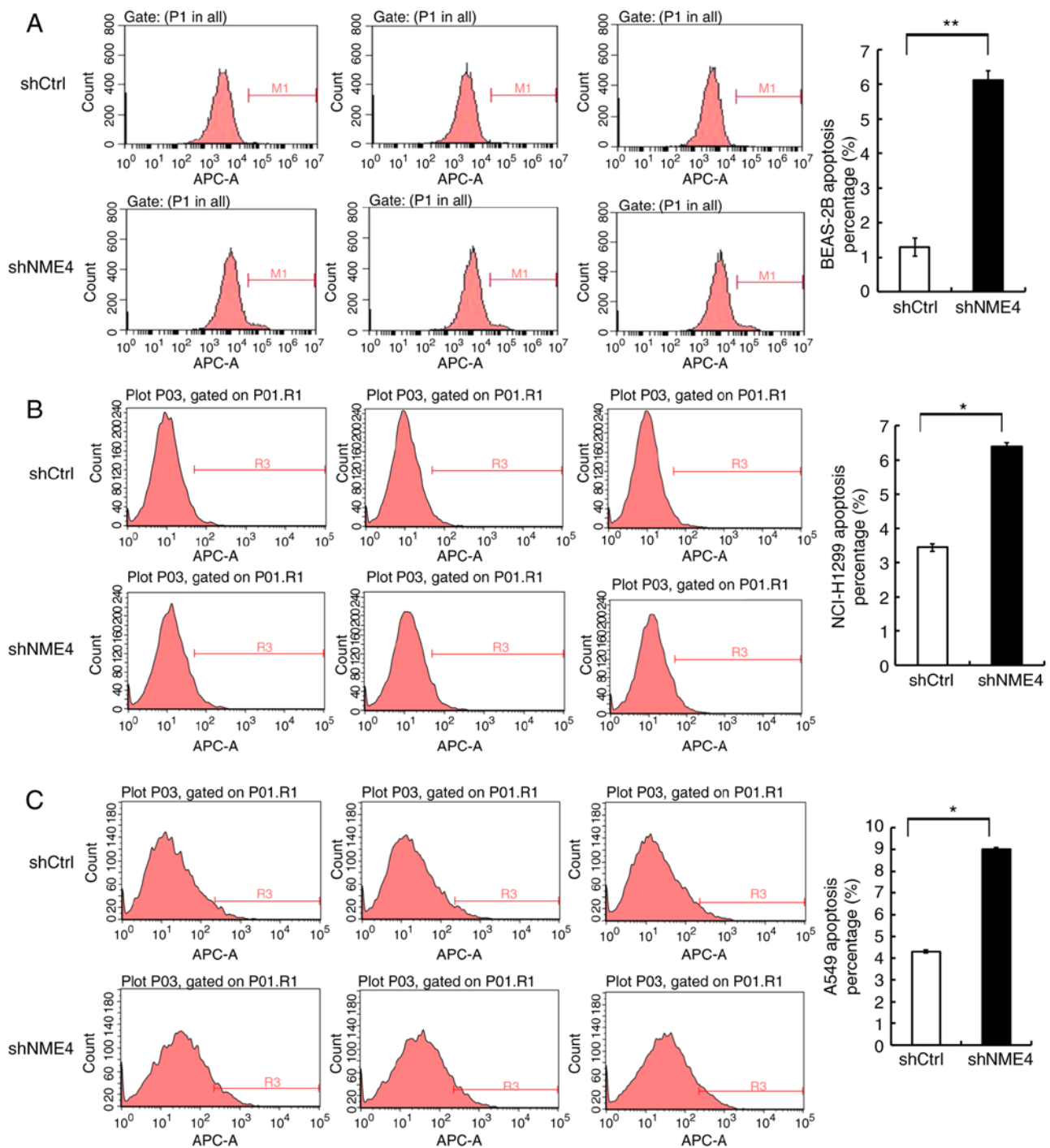
Figure 4. Effects of the silencing of NME4 on cell apoptosis. (A) After lentiviral transduction of BEAS-2B cells, apoptosis was increased in NME4-shRNA-silenced cells compared with control cells. (B) After lentiviral transduction of NCI-H1299 cells, apoptosis was increased in NME4-shRNA-silenced cells compared with control cells. (C) After lentiviral transduction of A549 cells, apoptosis was increased in NME4-shRNA-silenced cells compared with control cells. Apoptotic rates were analyzed by Annexin V-APC/propidium iodide assay. The percentages of cells in different phases are shown as the mean ± SD of three independent experiments. Statistical significance was assessed by two-tailed Student's t-test. Error bars indicate SD. *P<0.05, **P<0.01. NME4, nucleoside diphosphate kinase 4; sh, short hairpin; Ctrl, control; APC, allophycocyanin.

it was concluded that NME4 may serve as a novel tumor promoter capable of enhancing NSCLC progression. To the best of our knowledge, this was the first study to identify that NME4 expression may exhibit tumor-promoting potential in NSCLC. However, some limitations exist in the present analysis. First, only two types of NSCLC cell lines were used, which may result in a loss of comprehensiveness. Second, the less efficient knockdown of NME4 in the NCI-H1299 cell line may weaken the interpretation of the role of this gene from the results of the present study.

In conclusion, the present study determined that NME4 serves critical roles in NSCLC development. Although the detailed mechanisms remain to be elucidated, the critical role of NME4 in NSCLC development may provide evidence for the development of novel therapeutics against NME4 for the treatment of NSCLC.
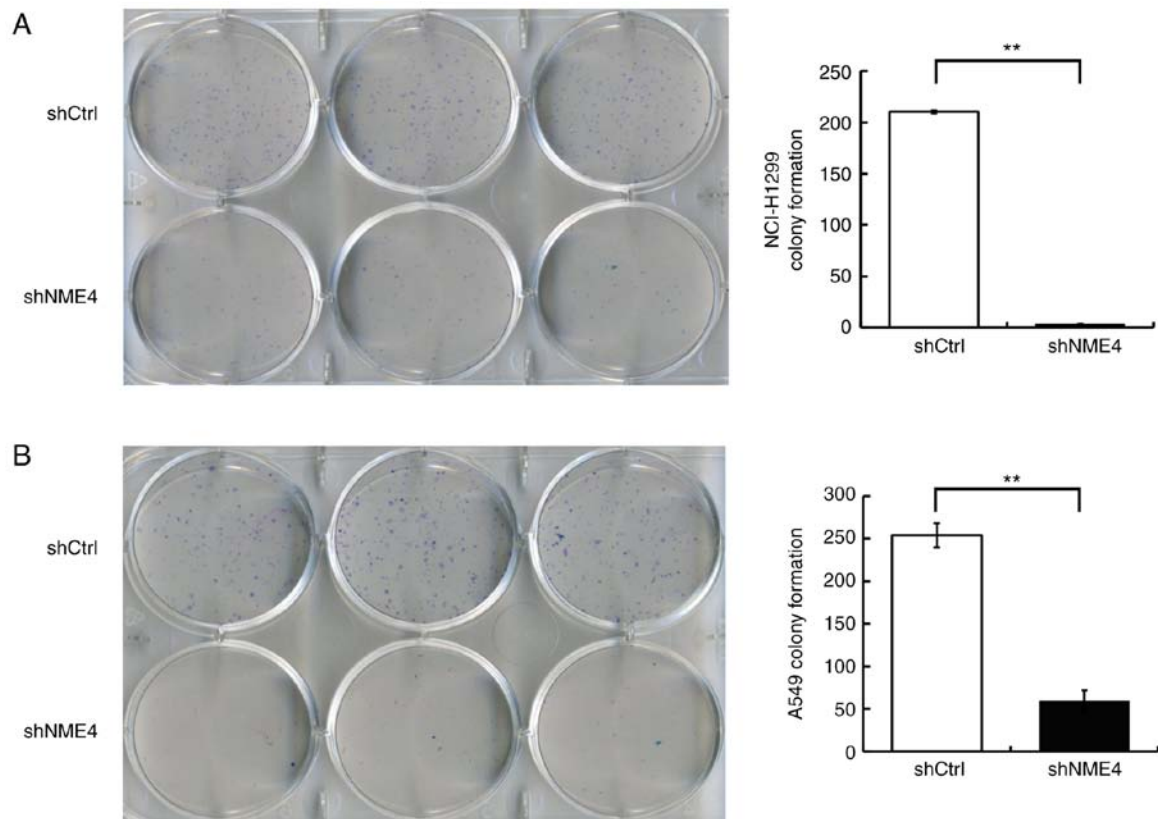
Figure 5. Effects of the silencing of NME4 on cell colony formation. (A) After lentiviral transduction of NCI-H1299 cells, the NME4-shRNA-silenced cells displayed a significantly reduced number of cell colonies compared with control cells. (B) After lentiviral transduction of A549 cells, the NME4-shRNA-silenced cells displayed a significantly reduced number of cell colonies compared to the control cells. Whole-plate fields were photographed and are presented. The number of cell colonies from triplicate counts in a representative experiment are presented. Statistical significance was assessed by two-tailed Student's t-test. Error bars indicate SD. **$P<0.01$. NME4, nucleoside diphosphate kinase 4; sh, short hairpin; Ctrl, control.
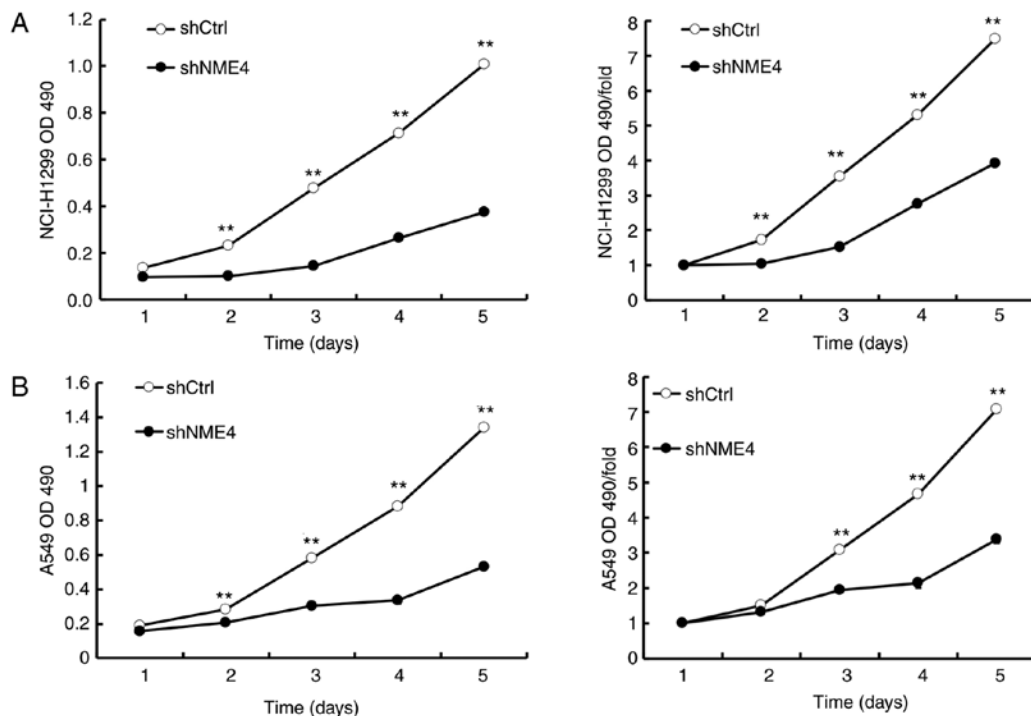


Figure 6. Cell proliferation analysis by MTT assay. (A) After lentiviral transduction of NCI-H1299 cells, MTT assays were performed on the indicated days to show the proliferation of NCI-H1299 cells. The OD values and fold-changes were significantly reduced in the NME4-shRNA-silenced cells as compared to the control cells. (B) After lentiviral transduction of A549 cells, the OD values and fold-changes were significantly reduced in the NME4-shRNA-silenced cells as compared to the control cells. Statistical significance was assessed by ANOVA for the comparison of multiple groups. Error bars indicate SD. **$P<0.01$ vs. shCtrl. NME4, nucleoside diphosphate kinase 4; sh, short hairpin; Ctrl, control; OD, optical density.

## 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

YW and JD conceived and designed the study. WW and MD performed the majority of the experiments, performed preliminary analysis and drafted the manuscript. YW reviewed the data analysis and revised the manuscript. JC, FX, CY, CM, LY and WT participated in the completion of the experiments. All the authors read and approved the final manuscript and agree to be accountable for all aspects of the work.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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