Nucleophosmin promotes lung adenocarcinoma cell proliferation, migration and invasion by activating the EGFR/MAPK signaling pathway

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Abstract. Lung adenocarcinoma (LUAD) is the main cause of death globally. The present study investigated the prognostic value and functional verification of nucleophosmin (NPM1) in LUAD. LUAD and normal samples from The Cancer Genome Atlas were analyzed to identify whether NPM1 is associated with LUAD prognosis. NPM1 protein expression level was verified by western blotting. Cell proliferation, migration and invasion were detected by Cell Counting Kit-8, wound healing and Transwell assays, respectively. EGFR/MAPK pathway-related proteins [phosphorylated (p)-EGFR/EGFR, p-MEK/MEK, and p-ERK/ERK] expression was measured through western blotting. A xenograft tumor mice model was constructed to perform the *in vivo* verification. NPM1 was upregulated in LUAD cells, and high-level NPM1 indicated poor prognosis in patients with LUAD. *In vitro* experiments revealed that NPM1 knockdown inhibited LUAD cell proliferation, migration and invasion. Moreover, protein expression of p-EGFR/EGFR, p-MEK/MEK and p-ERK/ERK was reduced with the NPM1 silencing. Furthermore, EGF, an activator of the EGFR/MAPK pathway, reversed the effects of NPM1. *In vivo* experiments showed that NPM1 knockdown inhibited tumor growth and protein levels of p-EGFR/EGFR, p-MEK/MEK and p-ERK/ERK. NPM1 is related to the poor prognosis of LUAD and promotes the malignant progression of LUAD by activating the EGFR/MAPK pathway. This discovery provides a new potential therapeutic target for the diagnosis and treatment of LUAD.

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Abbreviations: NSCLC, non-small-cell lung carcinoma; LUAD, lung adenocarcinoma; NPM1, nucleophosmin; DEG, differentially expressed gene; PPI, protein-protein interaction network; NC, negative control; ROC, receiver operating characteristic; CCK-8, Cell Counting Kit-8

Key words: lung adenocarcinoma, nucleophosmin, EGFR/MAPK signaling pathway, tumorigenesis, prognosis

Introduction

Lung cancer is the leading cause of cancer-related deaths (18%) worldwide, characterized by fast growth, high mortality and poor prognosis (1). Lung cancer is generally classified into non-small-cell lung carcinoma (NSCLC) and small-cell lung carcinoma based on histological morphology. Lung adenocarcinoma (LUAD) is the major type of NSCLC, ~50% of all lung cancers (2). Advanced LUAD patients have a poor prognosis, and the average five-year survival rate is only 15% (3). Currently, with the development of biological therapy and immunotherapy, the clinical treatment of LUAD has broken the traditional treatment methods including surgery, radiotherapy and chemotherapy (4,5). For patients with LUAD, prompt surgical intervention can significantly lower death and postoperative recurrence rates (6). Therefore, more research into new LUAD therapeutic targets is required. With the advancement of bioinformatics, new cancer-related genes can be recognized. Previous studies have successfully found some LUAD-related genes, including C10rf74 (7), CLDN18 (8), and HMGB2 (9). The identification of potential targets is conducive to the development of LUAD therapy.

Nucleophosmin (NPM1) is a multifunctional phosphoprotein, expressed ubiquitously in all tissues (10). NPM1 serves as a molecular chaperone for both nucleic acids and proteins with shuttling activity between the cytoplasm and nucleus (11). NPM1 participated in multiple cellular functions, including cell cycle regulation and DNA repair (12). A previous study has revealed that NPM1 regulates cell cycle progression and centrosome replication, which is essential for cell growth and proliferation (13). NPM1 overexpression frequently predicts poor prognosis in patients with breast cancer (14), oral squamous

cell carcinomas (15), and other cancers. Liu *et al* (16) reported that NPM1 was overexpressed in LUAD. However, its specific function and mechanism were rarely explored.

The EGFR/MAPK signaling pathway, a conserved receptor tyrosine kinase pathway, regulates cell survival, growth, proliferation and differentiation (17). EGFR activation regulates the levels of proteins associated with cancer cell growth, differentiation and migration via the MAPK pathway (18). EGFR dimerizes and auto-phosphorylates after binding to its ligand, triggering downstream intracellular signaling through RAS/RAF/MEK/ERK phosphorylation (19). EGFR/MAPK signaling pathway is involved in multiple tumor progression; for instance, gastric (20), ovarian (21) and pancreatic cancer (22). A recent study has revealed that EGFR/MAPK signaling pathway was activated in LUAD to promote tumor growth and metastasis (19). Additionally, NPM1 could specifically activate EGFR/MAPK pathway in prostate cancer (23). Whether NPM1 promotes LUAD progression through the EGFR/MAPK signaling pathway remains unknown.

Therefore, in the present study, it was assessed whether NPM1 is a valid target for LUAD prognosis via The Cancer Genome Atlas (TCGA) database. The effects of NPM1 and EGFR/MAPK signaling pathways on LUAD progression were explored *in vitro* and *in vivo*. The present study aimed to prove that NPM1 is a novel promising target in LUAD therapy through the EGFR/MAPK signaling pathway.

Materials and methods

Data download and pre-processing. LUAD-related gene expression was acquired from the TCGA database (https://tcga-data.nci.nih.gov/) via R software 3.6.5 (http://r-project.org). A total of 510 LUAD samples and 58 healthy samples were collected. The mRNA expression was obtained using the HUGO Gene Nomenclature Committee mRNA gene annotation file (24). To ensure high confidence in the results, the identification data were standardized by localization probability >0.75.

Differentially expressed genes (DEGs) and protein-protein interaction network (PPI) analysis. The GEO2R was used to identify DEGs between LUAD and normal lung tissues. Significant criteria of DEGs were a |log fold change (log_2FC)|>1 and P<0.05. DEGs were put into the STRING database (25) to get their PPI networks. The networks were then identified and visualized through Cytoscape software version 3.8.0 (26). Hub genes were obtained using the cytoHubba plugin.

Diagnostic analysis. cBioPortal for Cancer Genomics is a common resource for the interactive exploration of cancer genomics datasets (27). Hub genes were put into cBioPortal for mutation analysis with lung cancer. Next, the receiver operating characteristic (ROC) curve was performed to evaluate the diagnostic effect of hub genes. ROC curve was acquired using survival ROC package 1.0.3. The GEPIA2 tool (28) was further used to validate survival biomarkers, and Logrank P<0.01 was considered statistically significant.

Cell culture and treatment. A total of three LUAD cell lines (A549, PC9 and H1299) and human normal lung epithelial cell line BEAS-2B were provided by the Chinese Academy of Sciences (Beijing, China). These cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2.

A549 cells ($2x10^5$ ml/well) were inoculated in six-well plates and cultured to a confluence of 70-90% at 37˚C with 5% CO_2. Then, $1x10^8$ TU/ml short hairpin (sh)-negative control (NC; 40 μl/well) and sh-NPM1 (20 μl/well) were transfected to A549 cells using HighGene transfection agent (ABclonal Biotech Co., Ltd.). After incubation for 48 h, RPMI-1640 medium with 2.5 μg/ml puromycin was utilized to select stably transfected cells. Subsequently, part of sh-NPM1 transfected A549 cells received 100 ng/ml EGF treatment for 10 min, which has shown favorable efficiency in the activation of EGFR (29). The sh-NPM1 sequence (Human) was supplied by the Designer of Small Interfering RNA website (http://biodev.cea.fr/DSIR/DSIR.html), as follows: SS sequence, CCAAGA ATGTGTTGTCCAA; AS sequence, TTGGACAACACATTC TTGG.

Cell counting kit-8 (CCK-8) assay. A549 cell suspension was inoculated to a 96-well plate (100 μl/well) and cultured at 37˚C with 5% CO_2. After 10 μl CCK-8 solution (Beyotime Institute of Biotechnology) was added into the well at 24 and 48 h, the plate continued to be cultured for 2 h at 37˚C. Subsequently, absorbance at 450 nm was measured through a microplate reader (Molecular Devices, LLC).

Wound healing assay. Overall, A549 cells were treated with 0.25% trypsin and cultured in six-well plates ($5x10^5$ cells/well). A uniform scratch on the cell layer was created via a sterile pipette tip. Cell migration images were captured at 0 and 24 h through a light microscope (Olympus Corporation).

Transwell invasion assay. First, 50 mg/l Matrigel (Beijing Solarbio Science & Technology Co., Ltd.) was diluted at 1:4, and 50 μl diluent was then placed into the upper chamber for 4 h at 37˚C. A549 cells were diluted into $1x10^5$ cell/ml suspension. Then, 200 μl cell suspension was added into the upper chamber, with 600 μl RPMI-1640 medium containing 10% FBS into the lower one. After being cultured for 24 h and cleaned with phosphate-buffered saline (PBS; Beyotime Institute of Biotechnology), cells were fixed with methanol (Sinopharm Group Co., Ltd.) for 30 min and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology) for 20 min. A total of three fields were randomly chosen and images were captured, and the cell number was determined using ImageJ software 1.8.0 (National Institutes of Health).

Animal experiments. The animal experiments were approved (approval no. kmmu20230850) by the Animal Research Ethics Committee of The First Affiliated Hospital of Kunming Medical University (Kunming, China) and conformed with the guidelines for the use of laboratory animals. A total of 15 BALB/c male nude mice (age, 5 weeks-old; weight, 18-22 g) were purchased from GemPharmatech Co. Ltd. All mice were acclimatized in individually ventilated cages (specific-pathogen-free conditions) at 22˚C with 12/12 h light/dark cycle, fed and watered *ad libitum* for 1 week. Non-transfected or sh-NPM1/sh-NC-transfected A549 cells

($5x10^6$ cells/100 μl) were subcutaneously injected into mice, respectively, to establish the LUAD model.

The size of the tumor xenografts was measured weekly, and tumor volume was calculated by the following formula: Tumor volume (mm^3)=(1/2 x length) x $width^2$. After 5 weeks of model construction, tumor weight was measured. The mice were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital. At the end of the modeling, mice were then sacrificed with 200 mg/kg sodium pentobarbital, and their death was indicated by respiratory failure and cardiac arrest.

Western blotting. Using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.), total proteins were extracted from A549 cells and tumor tissues. Protein concentration was determined by a BCA kit (Beyotime Institute of Biotechnology). The proteins (25 μg) were separated in 10% SDS-PAGE gels and then transferred to PVDF membranes, which were blocked with 5% non-fat dry milk for 1 h at room temperature. After incubation with specific primary antibodies (all purchased from Abcam) at 4˚C overnight, the membranes were washed thrice with 1X 0.05% TBST for 10 min and incubated with goat-anti-rabbit IgG H&L secondary antibodies (1:2,000; cat no. ab6721; Abcam) at room temperature for 1 h. Protein bands were visualized by an ECL reagent (Applygen Technologies, Inc.). The primary antibodies were as follows: NPM1 (1:400; cat no. ab183340), phosphorylated (p)-EGFR (1:1,000; cat no. ab40815), EGFR (1:5,000; cat no. ab52894), p-MEK (1:2,500; cat no. ab96379), MEK (1:2,500; cat no. ab32091), p-ERK (1:1,000; cat no. ab201015), ERK (1:10,000; cat no. ab184699), GAPDH (1:2,500; cat no. ab9485). The protein bands were visualized using an ECL kit (Applygen Technologies, Inc.), and the relative protein levels were quantified using ImageJ software 1.8.0 (National Institutes of Health).

Statistical analyses. Each experiment was repeated at least thrice, and data were analyzed by GraphPad Prism 8.0 (Dotmatics). Survival analysis was performed using the 'survival' R package. The data were expressed as the mean values ± standard deviation. Data from multiple groups were analyzed by one-way ANOVA followed by Tukey's post hoc test, and those from two groups were analyzed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

NPM1 is upregulated and related to poor prognosis in LUAD. The PPI network of LUAD-related DEGs extracted by GEO2R was obtained from the STRING database (Fig. 1A). The cytohubba plug-in was used to obtain the top 20 hub genes, and NPM1 showed high relevance with LUAD (Fig. 1B). Hub genes were then inserted into GEPIA2 for survival analysis associated with LUAD. The results revealed that NPM1 demonstrated a favorable prognostic effect on overall survival with 2.4% of alteration rate, and the major alteration type of NPM1 was amplification (Fig. 1C). Next, single gene ROC analysis of hub genes was performed to evaluate the prognosis for patients with LUAD. It was found that NPM1, NOLC1 and NCL showed favorable diagnostic effects in LUAD (AUC >0.8, Fig. 1D). To investigate the effects of NPM1 on survival and prognosis, patients with LUAD in TCGA-LUAD were grouped into high- and low-NPM1 expression. The result revealed that the survival time of patients in the high-NPM1 group was shorter (Logrank $P<0.01$; Fig. 1E). Additionally, western blotting verified that NPM1 was upregulated in LUAD cells (A549, PC9 and H1299) compared with BEAS-2B, in which A549 cells showed the most obvious significance ($P<0.01$, Fig. 1F). Therefore, A549 cells were chosen for subsequent functional verification experiments.

NPM1 silencing inhibits the proliferation, migration and invasion of LUAD cells. A549 cells were transfected with sh-NC/sh-NPM1 to investigate the function of NPM1 in LUAD. NPM1 expression in A549 cells was detected. It was revealed that NPM1 protein expression was significantly reduced in sh-NPM1 cells compared with sh-NC cells ($P<0.01$, Fig. 2A), which revealed successful transfection. CCK-8 assay indicated that NPM1 silencing suppressed A549 cell viability ($P<0.01$, Fig. 2B). As demonstrated by wound healing and Transwell assays, A549 cell migration and invasion were restrained by NPM1 knockdown ($P<0.01$, Fig. 2C and D).

NPM1 silencing suppresses malignant characteristics by inhibiting the EGFR/MAPK signaling pathway. The EGFR/MAPK signaling pathway is abnormally activated in LUAD, and NPM1 was related to signaling by EGFR in cancer. To investigate whether NPM1 functions on LUAD malignant characteristics via the EGFR/MAPK pathway, the protein expression of p-EGFR, EGFR, p-MEK, MEK, p-ERK and ERK, which are the EGFR/MAPK pathway-related proteins, were determined using western blot analysis. The results demonstrated that protein levels of p-EGFR/EGFR, p-MEK/MEK and p-ERK/ERK were all decreased in A549 cells after NPM1 knockdown ($P<0.05$, Fig. 3A). This result indicated that the inhibitory effect of NPM1 silencing on LUAD malignancy may be involved in suppressing the EGFR/MAPK pathway. Subsequently, EGF, an activator of EGFR, was used to perform feedback experiments. It was found that cell proliferation, migration and invasion were increased in sh-NPM1 + EGF cells compared with sh-NPM1 cells ($P<0.01$, Fig. 3B-D). Furthermore, in comparison with sh-NPM1 cells, levels of p-EGFR/EGFR, p-MEK/MEK, and p-ERK/ERK were successfully increased in sh-NPM1 + EGF cells ($P<0.05$, Fig. 3E).

NPM1 silencing inhibits LUAD tumor growth by restraining the EGFR/MAPK signaling pathway. A xenograft tumor mice model was conducted to deeply explore the NPM1 effect on LUAD. Compared with model mice, the tumor weight and volume were significantly smaller in NPM1-knockdown mice ($P<0.01$, Fig. 4A-C). Additionally, protein levels of NPM1, p-EGFR/EGFR, p-MEK/MEK and p-ERK/ERK were found to be significantly reduced in NPM1-knockdown mice ($P<0.01$, Fig. 4D).

Discussion

LUAD with a poor prognosis is one of the leading causes of global cancer-related mortalities (30). Clinical LUAD outcomes

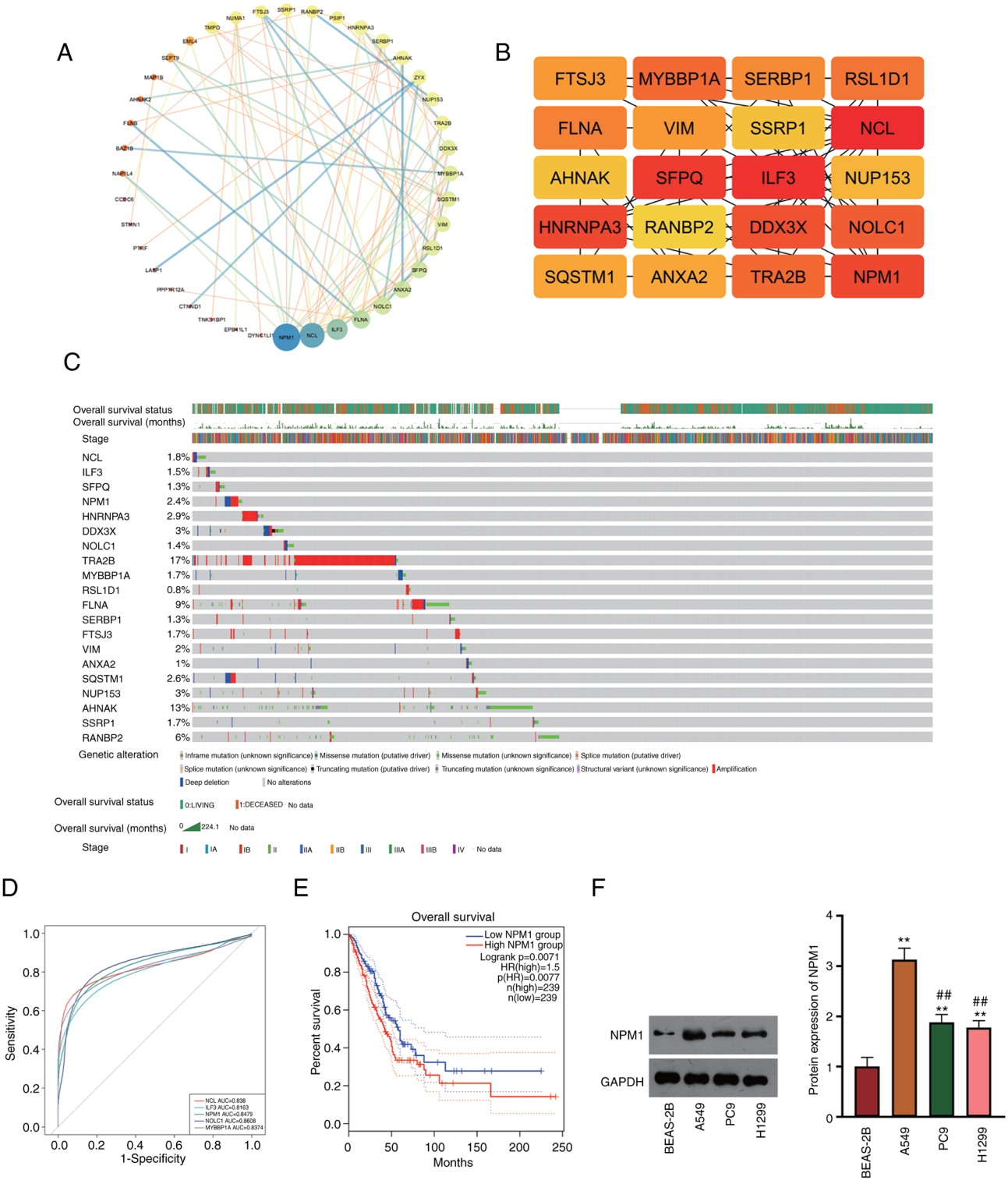

Figure 1. NPM1 is upregulated and related to poor prognosis in LUAD. (A) Protein to protein interaction network of differential expression genes from TCGA-LUAD. (B) Top 20 hub genes related to LUAD. (C) Mutation oncoPrint of hub genes in LUAD. (D) Single gene receiver operating characteristic analysis of LUAD. (E) Survival curve of NPM1 in TCGA-LUAD. (F) NPM1 expression in LUAD cell lines (A549, PC9 and H1299) and normal cells (BEAS-2B) was determined using western blotting. **P<0.01 vs. BEAS-2B and $^{\#\#}$P<0.01 vs. A549. NPM1, nucleophosmin; LUAD, lung adenocarcinoma; TCGA, The Cancer Genome Atlas.

using current treatments are not satisfactory (31). Identification of potential biomarkers is important for predicting prognosis and guiding individualized treatments. NPM1 is a highly abundant protein crucial for multiple cellular functions. The present study revealed that NPM1 is a potential target and is upregulated in LUAD. NPM1 prompted LUAD progression by activating the EGFR/MAPK signaling pathway.

NPM1 is a well-known nucleocytoplasmic shuttling protein required for normal cellular function. NPM1 is involved in the maintenance of genomic stability, chromatin

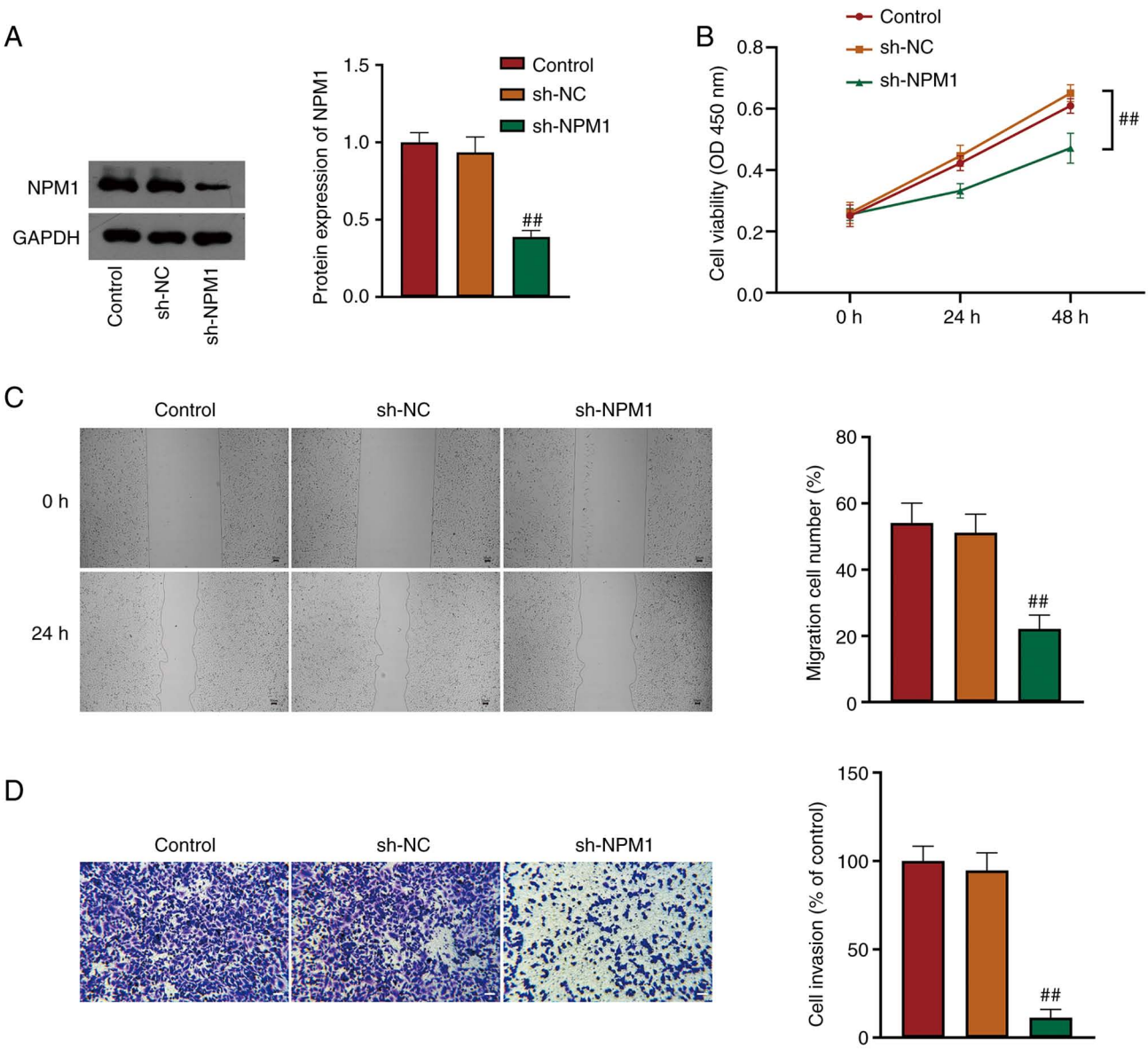

Figure 2. NPM1 silencing inhibits proliferation, migration and invasion of lung adenocarcinoma cells. (A) The expression level of NPM1 was detected by western blotting. (B) Cell viability was determined using Cell Counting Kit-8 assay. (C) Cell migration at 0 and 24 h was evaluated using wound healing assay. Scale bar, 50 μm. (D) Cell invasion was detected by Transwell assay. Scale bar, 50 μm. A549 cells were transfected with sh-NPM1 or sh-NC. $^{\#\#}$P<0.01 vs. sh-NC. NPM1, nucleophosmin; sh-, short hairpin; NC, negative control.

remodeling, ribosome biogenesis, cell cycle progression and apoptosis (32). Overexpression, mutation, translocation, function loss, or sporadic deletion of NPM1 would result in cancer and tumorigenesis (33,34). NPM1 overexpression is associated with high-grade malignancies and poor prognosis. In those with pancreatic cancer, NPM1 promotes aerobic glycolysis and tumor progression (35), and NPM1 promotes cell proliferation by targeting PRDX6 in colorectal cancer (36). Previous studies indicated that NPM1 expression could predict the prognosis of prostate (37) and gastric cancers (38). The present bioinformatics analysis showed that NPM1 was the hub gene in LUAD, and high expression of NPM1 may indicate poor prognosis of LUAD patients. The current data demonstrated that NPM1 was upregulated in LUAD cells. NPM1 overexpression could enhance the growth and proliferation of various tumors (36,39,40). Same as in previous studies, NPM1 silencing inhibited the proliferation, migration and invasion of LUAD cells and suppressed tumor growth in the present study.

Accumulating evidence revealed that the EGFR/MAPK pathway is involved in NSCLC progression (19,41). EGFR overexpression results in dimerization, auto-phosphorylation and downstream activation of the PI3K, STAT and MAPK pathways. These pathways mediate the transcription of genes that are associated with transformation, proliferation, migration and invasion (42). Phosphorylation and activation of EGFR/MAPK signaling cascade are regarded as key pathways in various cancers, for example, pancreatic cancer (43), clear cell renal cell carcinoma (44) and glioma (45). EGFR is expressed in 85% of NSCLC cells and is related to a poor prognosis (42). Activation of the EGFR/MAPK pathway promotes cyclin D1 expression in NSCLC (42). The mechanism of NPM1 on LUAD malignancy was further explored. The results indicated that EGFR/MAPK pathway-related proteins were

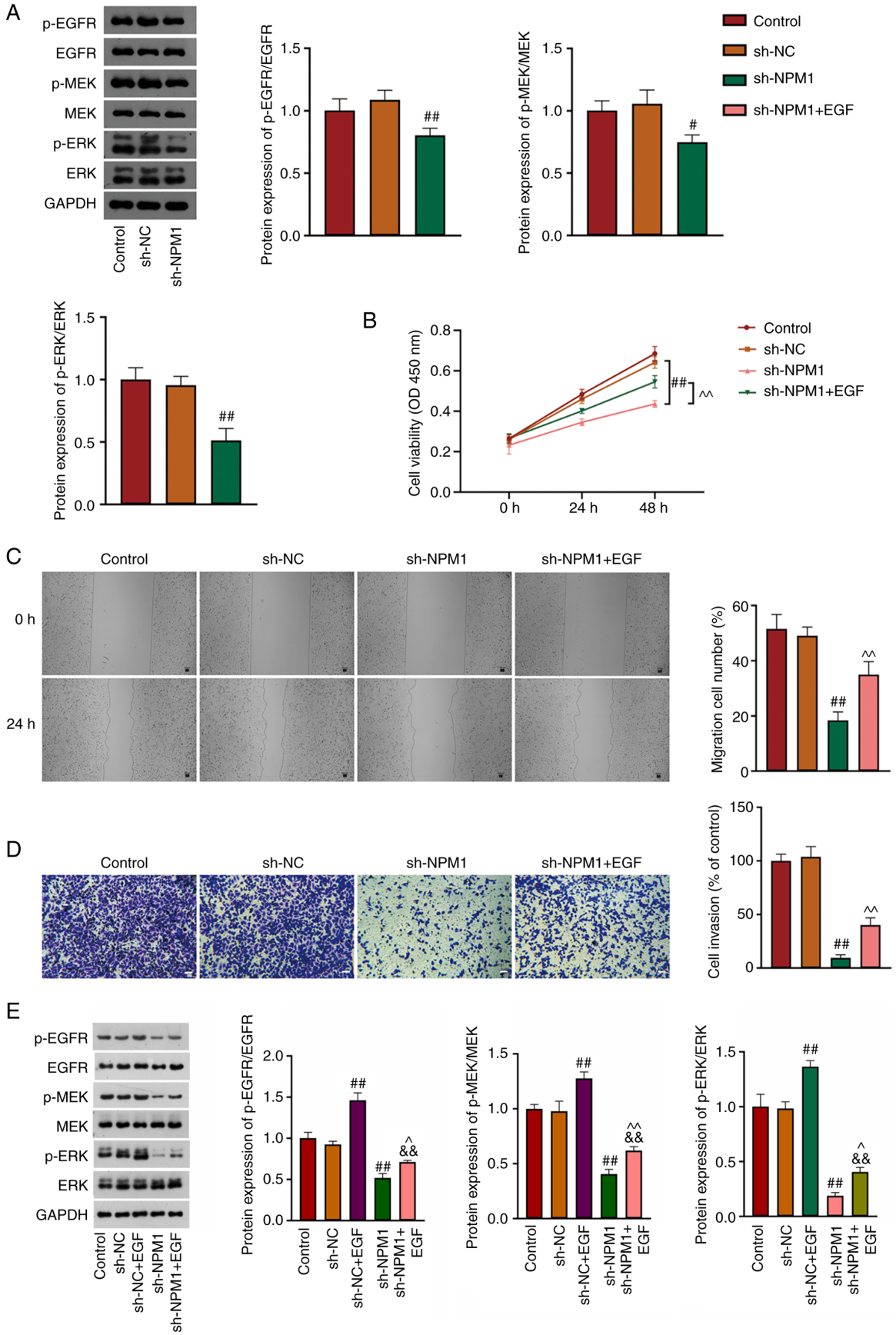

Figure 3. NPM1 silencing suppresses malignant characteristics by inhibiting the EGFR/MAPK signaling pathway. (A) The expression of p-EGFR/EGFR, p-MEK/MEK and p-ERK/ERK was detected using western blotting. (B) Cell viability was evaluated by Cell Counting Kit-8 assay. (C) Cell migration at 0 and 24 h was determined using wound healing assay. Scale bar, 50 μm. (D) Cell invasion was detected by Transwell assay. Scale bar, 50 μm. (E) The expression of p-EGFR/EGFR, p-MEK/MEK and p-ERK/ERK was evaluated using western blotting. sh-NPM1 transfected A549 cells were treated with EGF. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. sh-NC, $^{\&\&}$P<0.01 vs. sh-NC + EGF, $^{\wedge}$P<0.05 and $^{\wedge\wedge}$P<0.01 vs. sh-NPM1. NPM1, nucleophosmin; p-, phosphorylated; sh-, short hairpin; NC, negative control.

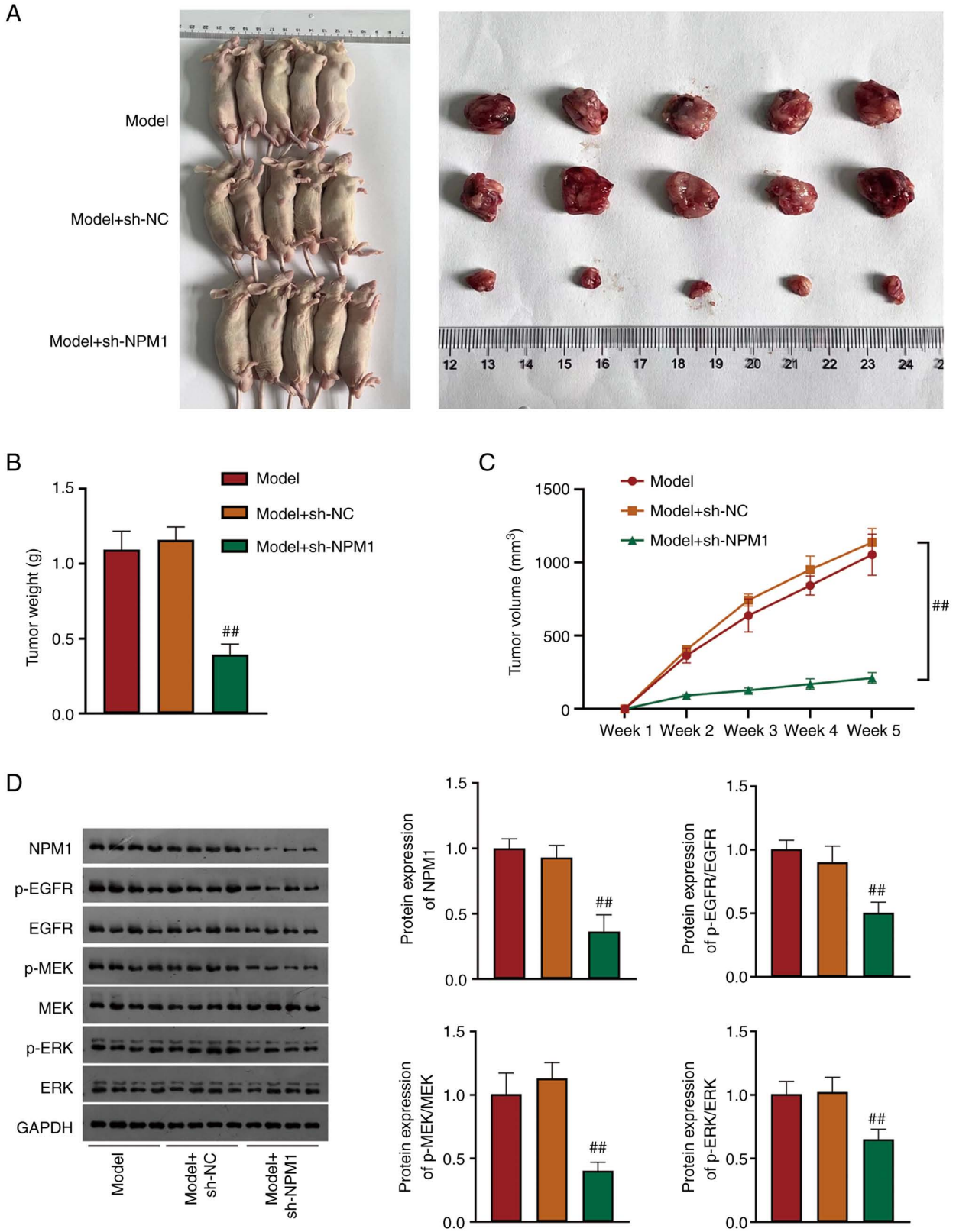

Figure 4. NPM1 silencing inhibits lung adenocarcinoma tumor growth by restraining the EGFR/MAPK signaling pathway. (A) Nude mice and tumors. (B) Tumor weight. (C) Tumor volume. (D) The expression of p-EGFR/EGFR, p-MEK/MEK and p-ERK/ERK was determined using western blotting. Non-transfected or sh-NPM1/sh-NC-transfected A549 cells ($5x10^6$ cells/100 μl) were injected subcutaneously into mice. $^{##}$P<0.01 vs. model + sh-NC. NPM1, nucleophosmin; p-, phosphorylated; sh-, short hairpin; NC, negative control.

downregulated in LUAD after NPM1 knockdown. A recent study revealed that NPM1 was related to signaling by EGFR in cancer via GSEA analysis (16). In the present study, NPM1 knockdown inhibited the malignant progression of LUAD by restraining the EGFR/MAPK pathway. However, EGF (an activator of the EGFR/MAPK pathway) reversed the effect

of NPM1 silencing. These indicated that NPM1 promotes the progression of LUAD by activating the EGFR/MAPK signaling pathway.

In summary, the results of the present study suggested that NPM1 is upregulated and associated with poor prognosis in LUAD. Furthermore, it was identified that NPM1 promoted cell proliferation, migration, invasion and tumor growth via the EGFR/MAPK pathway in LUAD. NPM1 is a potential therapeutic target in LUAD. Although the current study improved our understanding of NPM1 in LUAD, there were certain limitations. Firstly, the clinical significance of NPM1 in the LUAD progression requires further investigation. Secondly, the specific mechanism of NPM1 on the EGFR/MAPK pathway needs to be explored.

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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

ML and ZL conceptualized the present study. LW and RW curated data and conducted investigation. RW, DZ, MC, SL, RL, CD and MM conducted formal analysis and developed methodology. ZL supervise the study and acquired funding. RW, ML and ZL conducted project administration. RW, CD and ZL provided resources. SL, RL and MM performed software analysis. RW, HZ, CD, SZ and ZL validated data. RW and ML performed visualization. RW, CD and HZ wrote the original draft. ML, SZ, CD and ZL wrote, reviewed and edited the manuscript. RW, HZ, CD, SZ and ZL confirm the authenticity of all the raw data. All authors reviewed the results, read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal experiments were approved (approval no. kmmu20230850) by the Animal Research Ethics Committee of The First Affiliated Hospital of Kunming Medical University (Kunming, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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