

# HnRNPAB is an independent prognostic factor in non-small cell lung cancer and is involved in cell proliferation and metastasis

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**Abstract.** Heterogeneous nuclear ribonucleoprotein A/B (hnRNPAB) is an RNA binding protein that is closely associated with the biological function and metabolism of RNA, which is involved in the malignant transformation of various tumor cells. However, the role and mechanisms of hnRNPAB in non-small cell lung cancer (NSCLC) are still unclear. In the present study, the expression levels of hnRNPAB in NSCLC and normal tissues were analyzed using the human protein atlas database and UALCAN database. The clinical significance of hnRNPAB was assayed using the data of NSCLC cases from The Cancer Genome Atlas database. Subsequently, two stable NSCLC cell lines with hnRNPAB knockdown were constructed and the effects of hnRNPAB silencing on cell viability, migration, invasion and epithelial-mesenchymal transition (EMT) were identified. Genes associated with hnRNPAB expression in NSCLC were screened using the Linked Omics database and verified by quantitative real-time PCR (RT-qPCR). The database analysis indicated that hnRNPAB was mainly expressed in the nucleus of NSCLC cells. Compared with the normal tissues, hnRNPAB expression was overexpressed in NSCLC tissues and was closely associated with the overall survival, sex, tumor-node-metastases classification, and poor prognosis of patients with lung adenocarcinoma. Functionally, knockdown of hnRNPAB inhibited

the proliferation, migration, invasion and EMT of NSCLC cells and arrested the cell cycle at G<sub>1</sub> phase. Mechanistically, the bioinformatics analysis and RT-qPCR verification demonstrated that hnRNPAB knockdown led to a significant expression change of genes associated with tumorigenesis. In conclusion, the present study indicated that hnRNPAB played an important role in the malignant transformation of NSCLC, supporting the significance of hnRNPAB as a novel potential therapeutic target for the early diagnosis and prognosis of NSCLC.

## Introduction

Lung cancer is one of the most common malignancies (1). For more than a decade, lung cancer has remained the leading cause of cancer death worldwide (1). Lung cancer is divided into small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), of which NSCLC accounts for about 85-90% and mainly includes lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) (2). Although the overall treatment effect of patients has been improved with the continuous progress of diagnosis and treatment technology in recent years, the prognosis of lung cancer is still poor (2,3). Therefore, exploring the molecular mechanism of lung cancer pathogenesis and progression is necessary for improving the survival rate of patients with lung cancer.

Tumor invasion and metastasis are important reasons for the poor prognosis of patients with NSCLC (4). A critical intrinsic factor for epithelial tumors to infiltrate and metastasize is the epithelial-mesenchymal transition (EMT) of tumor cells, which can be abnormally activated at various stages of cancer progression and plays critical roles in cell growth, invasion, metastasis, and resistance to the therapy of NSCLC (5-7). Consequently, it is of great significance to find the key factors that activate EMT for the treatment of NSCLC.

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are a kind of RNA binding proteins (RBPs) closely related to the function and metabolism of RNA, such as transcription, alternative splicing, nucleocytoplasmic transport, stability, and translation (8). Studies have shown that the dysregulation of hnRNPs expression is closely related to the EMT of

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various malignant tumor cells (9). HnRNPAB is a member of the hnRNPs family. Zhou *et al* (10) and Yang *et al* (11) reported that hnRNPAB was overexpressed in hepatocellular carcinoma (HCC) and promoted the EMT of tumor cells by regulating the transcriptions of snail family transcriptional repressor 1 (SNAIL, SNAI1) and long-noncoding RNA (lncRNA) ELF209. In addition, the expression of hnRNPAB is increased in breast cancer, colon cancer, and gastric cancer, in which hnRNPAB could serve as a prognostic biomarker and therapeutic target (12-14). However, the function of hnRNPAB in NSCLC has not yet been reported.

In this study, the expression and localization of hnRNPAB in NSCLC tissues and its correlation with clinicopathological parameters of patients with NSCLC were analyzed through public databases. Then the role of hnRNPAB silencing in the malignant transformation of NSCLC cells was investigated *in vitro*, and finally the molecular mechanism of hnRNPAB promoting the proliferation and metastasis of NSCLC cells was investigated by bioinformatics and RT-qPCR. This study may contribute to discovering a new prognostic biomarker and therapeutic target for NSCLC patients.

## Materials and methods

**HnRNPAB expression analysis.** The expression and localization of hnRNPAB in lung cancer and normal tissues were analyzed by the human protein atlas database (<http://www.proteinatlas.org/>) and UALCAN database (<http://ualcan.path.uab.edu/>). The data of 585 LUAD and 550 LUSC cases were downloaded from the TCGA database (<https://tcga-data.nci.nih.gov/tcga/>), including mRNA expression profile, sex, age, tumor-node-metastases (TNM) classification, and overall survival. The median expression level of hnRNPAB mRNA in 585 LUAD or 550 LUSC cases was used as the cutoff. Then the survival curve was drawn by the Kaplan-Meier method and compared by the log-rank test. The relationships between hnRNPAB expression and clinicopathological parameters were analyzed by the Chi-square ( $\chi^2$ ) test. Univariate and multivariate analyses of potential prognostic factors for overall survival were performed using the Cox proportional hazard model. Genes associated with hnRNPAB expression in NSCLC were analyzed using the Linked Omics database (<http://linkedomics.org/>) based on the Pearson correlation coefficient.

**Cell Culture.** The human NSCLC cell line NCI-H292 and the human renal epithelial cell line 293T were purchased from Kunming Cell Bank of Chinese Academy of sciences. The human NSCLC cell line PC-9 was obtained from FuHeng Biology Company (Shanghai, China). NCI-H292 and PC-9 were maintained in RPMI-1640 medium (C11875500BT, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (10270-106, FBS, Gibco). 293T was cultured in Dulbecco's Modified Eagle Medium (DMEM, C11995500BT, Gibco) with 10% FBS. All cell lines were cultured at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

**Plasmid construction.** To construct hnRNPAB knockdown recombinants, hnRNPAB and negative control (NC) shRNA sequences (sense and antisense) were designed

(shown in Table I) and synthesized, which were dissolved in Tris-EDTA buffer to a final concentration of 100  $\mu$ M. For each shRNA, the sense and antisense strands were mixed with a 1:1 ratio and then annealed through a PCR program (95°C for 2 min, 72°C for 2 min, 37°C for 2 min, 25°C for 2 min). The paired shRNA was cloned into the vector psi-LVRU6GP (GeneCopoeia, Germantown, MD, USA) digested with *Bam*H I and *Eco*R I (1010S and 1040S, Takara, Beijing, China). All constructs were confirmed by DNA sequencing (Sangon Biotech, Shanghai, China).

**HnRNPAB knockdown.** 293T cells were plated in a 6-well plate and the cell confluence reached 90% after 24 h. The hnRNPAB shRNA or NC shRNA recombinants, lentivirus packaging plasmid psPAX2 and PMD2G were mixed in a 4:3:1 ratio and then transfected into 293T cells using an Entranster™-R4000 reagent (4000-4, Engreen Biosystem, Beijing, China) following the manufacturer's instructions. At 48 h after transfection, the viral supernatant was collected and filtered with 0.22  $\mu$ m filters. The virus solution was mixed with lentivirus infection reagent (30001-2-0.5, Engreen Biosystem) according to the manufacturer's instructions and then added into the NCI-H292 or PC-9 cells with a 40-60% cell confluence. After 8 h of lentivirus infection, the infection solution was replaced by fresh RPMI-1640 medium (10% FBS, no antibiotics). After 72 h of infection, puromycin (final concentration 2.0  $\mu$ g/ml) was added to screen the stable cell lines.

**Western blotting.** Cells were lysed in RIPA buffer (P0013B, Beyotime, Shanghai, China) for 20 min on ice and then centrifuged at 12,000 x g for 10 min at 4°C. The proteins in harvested supernatants were separated on 10% SDS-PAGE gels and then transferred onto polyvinylidene difluoride membranes (IPVH00010, Millipore, Merck, Billerica, MA, USA), which were successively probed with indicated antibodies and detected with an Enhanced Chemiluminescence Reagent (WBKLS0100, Millipore). Rabbit polyclonal antibodies against hnRNPAB (A17497, 1:2,000, ABclonal, Wuhan, China), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 60004-1-Ig, 1:5,000, Proteintech, Wuhan, China), ZEB1 (21544-1-AP, 1:1,000, Proteintech), N-cadherin (22018-1-AP, 1:5,000, Proteintech), E-cadherin (20874-1-AP, 1:5,000, Proteintech), Vimentin (10366-1-AP, 1:10,000, Proteintech) and SNAI1 (13099-1-AP, 1:2,000, Proteintech), and a horseradish peroxidase-conjugated secondary antibody (SA00001-2, 1:5,000, Proteintech) were used.

**Real-time quantitative PCR (RT-qPCR) analysis.** Total RNA was extracted using a TRIzol reagent (15596-026, Invitrogen, Thermo Fisher Scientific) and reverse transcribed into cDNA by a PrimeScript™ RT reagent Kit with gDNA Eraser (RR047A, Takara). Fluorescence quantitative PCR was performed using a 2x SYBR Green quantitative PCR Master Mix (B21203, Bimake, Shanghai, China) according to the manufacturer's protocol in an Applied Biosystems StepOne Plus Real-Time PCR System (Applied Biosystems, Foster, CA, USA). The primer sequences of each gene were shown in Table I. GAPDH was used as a reference gene. The relative mRNA expression was calculated by the 2<sup>- $\Delta\Delta$ Ct</sup> method. All reactions were performed in triplicate.

Table I. Primer sequences.

| Gene                           | Direction | Primer sequences (5'-3')   |
|--------------------------------|-----------|--|
| hnRNPAB (NM_004499) shRNA-1    | Sense     | GATCCGTGGAAGCAAGTGTGAGATCAATTCAA<br>GAGATTGATCTCACACTTGCTTCCATTTTTTG |
|                                | Antisense | AATTCAAAAAATGGAAGCAAGTGTGAGATCAA<br>TCTCTTGAATTGATCTCACACTTGCTTCCACG |
| hnRNPAB (NM_004499) shRNA-2    | Sense     | GATCCGGTTTGGGTTTATCCTGTTCATTCAAGA<br>GATGAACAGGATAAACCCAAACCTTTTTTG  |
|                                | Antisense | AATTCAAAAAAGGTTTGGGTTTATCCTGTTC<br>TCTCTTGAATGAACAGGATAAACCCAAACCG   |
| NC shRNA                       | Sense     | GATCCGCTTCGCGCCGTAGTCTTATTCAAG<br>AGATAAGACTACGGCGCAAGCTTTTTTG       |
|                                | Antisense | AATTCAAAAAAGCTTCGCGCCGTAGTCTTA<br>TCTCTTGAATAAGACTACGGCGCAAGCG       |
| hnRNPAB (NM_004499) qPCR       | Sense     | TTTGGCGAGTTTGGGGAGATT  |
|                                | Antisense | GCCATACTGCTGCTGATAGAC  |
| NOP16 (NM_001256539) qPCR      | Sense     | AAAGGAAATACTCTGTCTCGGG   |
|                                | Antisense | CTTCTGCAAAGAATCGAGGAAG   |
| DDX41 (NM_001321732) qPCR      | Sense     | CCACTACCTTCATCAACAAAGC   |
|                                | Antisense | ACCTTCTGCTTGGCTTCTAG   |
| NPM1 (NM_001355007) qPCR       | Sense     | AGTATATCTGGAAAGCGGTCGTG  |
|                                | Antisense | CATTTTTGGCTGGAGTATCTCG   |
| TCOF1 (NM_001135243) qPCR      | Sense     | TTTGTCGACCCTAATCGTAGTC   |
|                                | Antisense | CAGGAGTCAAGCACTGTGTAG  |
| TMED9 (NM_017510) qPCR         | Sense     | GAGACCATGGTCATAGGAAACT   |
|                                | Antisense | TATGGGAAGTGAAAGTGAACCT   |
| CENPH (NM_022909) qPCR         | Sense     | TTCCAGAACCTTATTTTTGGGGA  |
|                                | Antisense | CTTCTCAAGCTGCAGAACAATT   |
| CCNB1 (NM_001354844) qPCR      | Sense     | CTTGCAGTAAATGATGTGGATG   |
|                                | Antisense | GTGACTTCCCGACCCAGTAG   |
| PPM1G (NM_177983) qPCR         | Sense     | AACTCCTTCACAAGAAAATGGC   |
|                                | Antisense | TGTCCTCAAAGAACTTGGACTT   |
| SFXN1 (NM_001322977) qPCR      | Sense     | TGGGATCAAAGCACTTTCATTG   |
|                                | Antisense | TTTCTGTAAGACCAGGAGGAAC   |
| HSPA9 (NM_004134) qPCR         | Sense     | GTCAGATTGGAGCATTTGTGTT   |
|                                | Antisense | CGAGTCATTGAAATAAGCTGGG   |
| GAPDH (NM_001357943) qPCR      | Sense     | GGAGCGAGATCCCTCCAAAAT  |
|                                | Antisense | GGCTGTTGTCATACTTCTCATGG  |
| ZEB1 (NM_001128128) qPCR       | Sense     | TTACACCTTTGCATACAGAACCC  |
|                                | Antisense | TTTACGATTACACCCAGACTGC   |
| E-cadherin (NM_001317185) qPCR | Sense     | CGAGAGCTACACGTTACCGG   |
|                                | Antisense | GGGTGTGCGAGGGAAAAATAGG   |
| N-cadherin (NM_001308176) qPCR | Sense     | AGCCAACCTTAACTGAGGAGT  |
|                                | Antisense | GGCAAGTTGATTGGAGGGATG  |
| Vimentin (NM_003380) qPCR      | Sense     | TGCCGTTGAAGCTGCTAACTA  |
|                                | Antisense | CCAGAGGGAGTGAATCCAGATTA  |
| SNAI1 (NM_005985) qPCR         | Sense     | ACTGCAACAAGGAATACCTCAG   |
|                                | Antisense | GCACTGGTACTTCTTGACATCTG  |

*CCK8 assay.* Cells were seeded into 96-well plates at a concentration of  $2 \times 10^3$  cells per well and then cultured for 24, 48, and 72 h, respectively. At the indicated time, 10  $\mu$ l CCK-8 solution

was added to each well and mixed. The cells were incubated for 1-4 h at 37°C and the absorbance at 450 nm was measured with a Biotek ELx800 (Biotek Winooski, Vermont, USA).

**Adherent colony formation.** Cells were seeded in 6-well plates at a concentration of  $1 \times 10^3$  cells per well and cultured for 2 weeks. The cells were washed twice with 1x phosphate buffered solution (PBS), fixed with 4% paraformaldehyde for 30 min, and stained with 0.01% crystal violet for 15 min. The number of colonies was counted using ImageJ.

**Wound healing assay.** Cells were plated in 6-well plates and reached confluence after 24 h. A straight wound was scratched on the cell layer with a 200  $\mu$ l pipette. Then the cells were washed twice with 1x PBS and cultured in the medium with 4% FBS. The photographs were taken at 0 and 24 h for the same visual fields. Cell migration was expressed as the percentage of scratch healing, which was calculated by [(scratch area at 0 h - scratch area at 24 h) / scratch area at 0 h]  $\times 100\%$ .

**Invasion assay.** The upper Transwell chamber (CLS3464, Corning Costar, Tewksbury, MA, USA) was coated with 100  $\mu$ l 10% BD Matrigel matrix (356234, BD Biosciences, San Jose, CA, USA) diluted with the serum-free medium, and then incubated in a cell incubator for 2 h.  $2 \times 10^4$  cells per well suspended in the serum-free medium were plated into the upper chamber. The bottom chamber was filled with 700  $\mu$ l medium containing 10% FBS. After incubation for 48 h, cells in the upper membrane of chamber were removed and cells adhering to the lower membrane were fixed with 4% paraformaldehyde for 30 min and stained with crystal violet for 15 min. Five random areas for each chamber were selected to count cell numbers.

**Cell cycle analysis.** Cells were collected, washed twice with precooled 1x PBS, and fixed overnight at 4°C with 75% alcohol (diluted with 1x PBS). Then the cells were harvested again, washed twice with precooled PBS, and incubated with 500  $\mu$ l staining solution (50  $\mu$ g/ml propidium iodide, 100  $\mu$ g/ml RNase A, 0.2% Triton X-100, 1x PBS) for 30 min. Cell-cycle was detected on a BD Accuri C6 flow cytometry (BD Biosciences, San Jose, CA, USA) and the results were analyzed with the software Modifit (Verity Software House, Topsham, ME, USA).

**Cell apoptosis analysis.** Cells were harvested and washed twice with precooled 1x PBS. After staining with Annexin V-APC and 7-AAD (API05, MULTISCIENCES, Hangzhou, China), cells were analyzed on a BD Accuri C6 flow cytometry (BD Biosciences). All samples were assayed in triplicate.

**Statistical analysis.** Software SPSS 21.0 (IBM, Armonk, NY, USA) was used for the statistical analysis. Differences between two groups were determined by Student's t-test, and analysis of one-way ANOVA followed by the post hoc Dunnett's test was used for comparisons of more than two. Survival curves were analyzed by the Kaplan-Meier log-rank test. Genes expression correlation was analyzed using the Pearson correlation coefficient. Experiments were performed in triplicate. All experimental data are presented as the mean  $\pm$  SD.  $P < 0.05$  indicates a statistically significant difference.

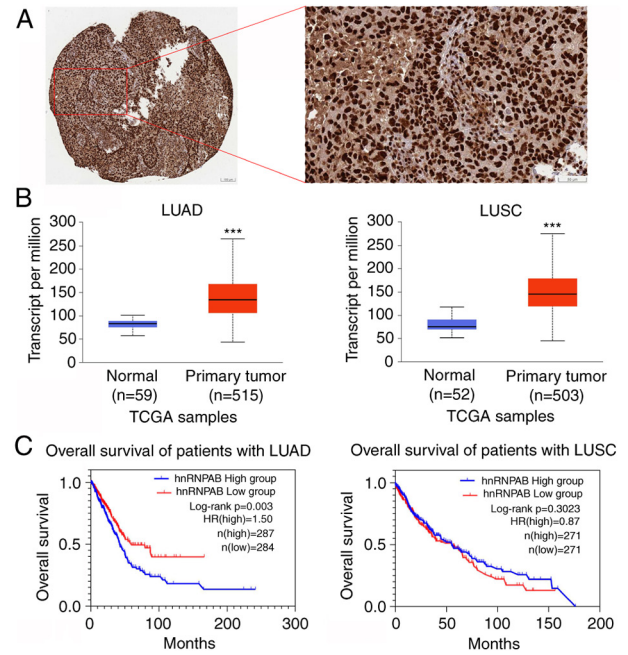


Figure 1. hnRNPAB expression is upregulated in NSCLC tissues and correlates with poor prognosis of patients with LUAD. (A) Localization of hnRNPAB protein in NSCLC tissues was analyzed using the human protein atlas database (left scale bar, 100  $\mu$ m; right scale bar, 50  $\mu$ m). (B) Expression levels of hnRNPAB in NSCLC and normal tissues were analyzed using the UALCAN database. (C) Prognostic effect of hnRNPAB expression in NSCLC was analyzed using the Kaplan-Meier method and compared using the log-rank test. \*\*\* $P < 0.001$  compared with normal group. hnRNPAB, heterogeneous nuclear ribonucleoprotein A/B; NSCLC, non-small cell lung cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

## Results

**hnRNPAB expression is up-regulated in NSCLC tissues and associated with the poor prognosis of patients.** Firstly, the expression and role of hnRNPAB in NSCLC tissues were examined. Immunohistochemical staining (IHC) analysis from the human protein atlas database indicated that hnRNPAB protein was mainly expressed in the nucleus of NSCLC cells (Fig. 1A). Compared with normal tissues, hnRNPAB mRNA was highly expressed in both LUAD and LUSC tissues (Fig. 1B). Overall survival analysis showed that high hnRNPAB mRNA expression was significantly associated with a poor prognosis in the patients with LUAD (Fig. 1C). Moreover, hnRNPAB mRNA expression was strongly associated with the sex and TNM classification of patients with LUAD (Table II). Univariate and multivariate analyses indicated that hnRNPAB expression was an independent prognostic factor for patients with LUAD (Table III). Therefore, the analysis suggested that hnRNPAB may be a useful biomarker in the prognosis of patients with LUAD.

**hnRNPAB silencing inhibits the proliferation of NSCLC cells.** The role of hnRNPAB expression dysregulation in NSCLC cells was further explored by constructing two stable hnRNPAB knockdown cell lines. Compared with the control group (NC shRNA), hnRNPAB protein and mRNA expression levels in NCI-H292 and PC-9 cells with hnRNPAB knockdown (hnRNPAB shRNA-1 and shRNA-2) were

Table II. Correlations between hnRNPAB mRNA expression level and clinicopathological parameters in NSCLC.

| Variable         | hnRNPAB expression in LUAD |                 |          |                    | hnRNPAB expression in LUSC |                 |          |         |
|------------------|----------------------------|-----------------|----------|--------------------|----------------------------|-----------------|----------|---------|
|                  | Low<br>(n=292)             | High<br>(n=293) | $\chi^2$ | P-value            | Low<br>(n=275)             | High<br>(n=275) | $\chi^2$ | P-value |
| Sex              |                            |                 | 6.419    | 0.011 <sup>a</sup> |                            |                 | 1.133    | 0.287   |
| Female           | 119                        | 150             |          |                    | 208                        | 197             |          |         |
| Male             | 173                        | 143             |          |                    | 67                         | 78              |          |         |
| Age              |                            |                 | 1.410    | 0.235              |                            |                 | 1.029    | 0.310   |
| ≤60              | 94                         | 108             |          |                    | 58                         | 68              |          |         |
| >60              | 198                        | 185             |          |                    | 217                        | 207             |          |         |
| T classification |                            |                 | 10.487   | 0.033 <sup>a</sup> |                            |                 | 4.077    | 0.253   |
| T1               | 112                        | 79              |          |                    | 55                         | 71              |          |         |
| T2               | 150                        | 171             |          |                    | 172                        | 151             |          |         |
| T3               | 21                         | 29              |          |                    | 38                         | 39              |          |         |
| T4               | 7                          | 13              |          |                    | 10                         | 14              |          |         |
| T <sub>x</sub>   | 2                          | 1               |          |                    | 0                          | 0               |          |         |
| N classification |                            |                 | 13.298   | 0.010 <sup>a</sup> |                            |                 | 2.258    | 0.688   |
| N0               | 194                        | 178             |          |                    | 181                        | 170             |          |         |
| N1               | 46                         | 61              |          |                    | 65                         | 79              |          |         |
| N2               | 38                         | 49              |          |                    | 23                         | 20              |          |         |
| N3               | 0                          | 2               |          |                    | 2                          | 3               |          |         |
| N <sub>x</sub>   | 14                         | 3               |          |                    | 4                          | 3               |          |         |
| M classification |                            |                 | 6.050    | 0.049 <sup>a</sup> |                            |                 | 3.144    | 0.208   |
| M0               | 188                        | 212             |          |                    | 227                        | 221             |          |         |
| M1               | 12                         | 15              |          |                    | 6                          | 2               |          |         |
| M <sub>x</sub>   | 92                         | 66              |          |                    | 42                         | 52              |          |         |

<sup>a</sup>P<0.05 analyzed by  $\chi^2$  test. hnRNPAB, heterogeneous nuclear ribonucleoprotein A/B; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

significantly reduced (Fig. 2A). CCK-8 and adherent colony formation assays indicated that hnRNPAB knockdown decreased cell viability and proliferation (Fig. 2B and C). In addition, the flow cytometry showed that silencing of hnRNPAB arrested the cell cycle at G<sub>0</sub>/G<sub>1</sub> phase but did not promote cell apoptosis, which indicated that hnRNPAB silencing suppressed cell proliferation by arresting the cell cycle at G<sub>0</sub>/G<sub>1</sub> phase (Figs. 2D and S1).

*HnRNPAB knockdown inhibits cell migration, invasion, and EMT of NSCLC.* Next, the effects of hnRNPAB knockdown on cell migration, invasion, and EMT were detected. The scratch test showed that the wound healing degree of hnRNPAB shRNA group was significantly lower than that of NC shRNA group (Fig. 3A). The invasion assay showed that the cell number passing through the Matrigel matrix in hnRNPAB shRNA group was obviously less than that in NC shRNA group (Fig. 3B). Western blotting and RT-qPCR showed that the protein and mRNA expression levels of EMT-related markers ZEB1, SNAIL, N-cadherin and Vimentin in hnRNPAB shRNA group were decreased, while the protein and mRNA expression levels of E-cadherin were increased (Fig. 3C). Collectively, the results suggested that hnRNPAB

knockdown significantly inhibited the migration, invasion, and EMT of NSCLC cells.

*Analysis of genes related to hnRNPAB expression in NSCLC.*

To explore the molecular mechanism of hnRNPAB promoting the malignant transformation of NSCLC cells, the genes related to hnRNPAB expression in NSCLC were analyzed using the LinkedOmics database. As shown in Fig. 4A and B, the expressions of 9764 genes were positively correlated with the expression of hnRNPAB, and 10214 genes were negatively correlated with hnRNPAB. Several hnRNPAB-positive correlated genes with high Pearson Correlations that have been reported to be associated with tumorigenesis were screened to perform the co-expression analysis with hnRNPAB using the mRNA expression data of 585 LUAD and 550 LUSC samples obtained from the TCGA database, including NOPI6 nucleolar protein (NOPI6), nucleophosmin 1 (NPM1), treacle ribosome biogenesis factor 1 (TCOF1), centromere protein H (CENPH), cyclin B1 (CCNB1), heat shock protein family A (Hsp70) member 9 (HSPA9), sideroflexin 1 (SFXN1), protein phosphatase, Mg<sup>2+</sup>/Mn<sup>2+</sup> dependent 1G (PPM1G), transmembrane p24 trafficking protein 9 (TMED9), and DEAD-box helicase 41 (DDX41) (15-26). As shown in Fig. 4C, the screened 10 genes

Table III. Univariate and multivariate analyses of various prognostic parameters in patients with LUAD.

| Variable         | Number of patients | Univariate analysis |             |                    | Multivariate analysis |              |                    |
|------------------|--------------------|---------------------|-------------|--------------------|-----------------------|--------------|--------------------|
|                  |                    | HR                  | 95% CI      | P-value            | HR                    | 95% CI       | P-value            |
| Sex              |                    | 0.905               | 0.690-1.186 | 0.468              | 0.935                 | 0.706-1.239  | 0.640              |
| Female           | 262                |                     |             |                    |                       |              |                    |
| Male             | 309                |                     |             |                    |                       |              |                    |
| Age              |                    | 1.236               | 0.925-1.653 | 0.151              | 1.191                 | 0.889-1.1594 | 0.242              |
| ≤60              | 192                |                     |             |                    |                       |              |                    |
| >60              | 379                |                     |             |                    |                       |              |                    |
| T classification |                    | 1.511               | 1.285-1.776 | 0.000 <sup>a</sup> | 1.340                 | 1.136-1.581  | 0.001 <sup>b</sup> |
| T1               | 189                |                     |             |                    |                       |              |                    |
| T2               | 312                |                     |             |                    |                       |              |                    |
| T3               | 48                 |                     |             |                    |                       |              |                    |
| T4               | 19                 |                     |             |                    |                       |              |                    |
| T <sub>x</sub>   | 3                  |                     |             |                    |                       |              |                    |
| N classification |                    | 1.338               | 1.192-1.503 | 0.000 <sup>a</sup> | 1.298                 | 1.142-1.474  | 0.000 <sup>a</sup> |
| N0               | 365                |                     |             |                    |                       |              |                    |
| N1               | 106                |                     |             |                    |                       |              |                    |
| N2               | 82                 |                     |             |                    |                       |              |                    |
| N3               | 2                  |                     |             |                    |                       |              |                    |
| N <sub>x</sub>   | 16                 |                     |             |                    |                       |              |                    |
| M classification |                    | 0.943               | 0.803-1.107 | 0.471              | 1.0121                | 0.857-1.194  | 0.890              |
| M0               | 387                |                     |             |                    |                       |              |                    |
| M1               | 26                 |                     |             |                    |                       |              |                    |
| M <sub>x</sub>   | 158                |                     |             |                    |                       |              |                    |
| hnRNPAB          |                    | 1.509               | 1.147-1.984 | 0.003 <sup>b</sup> | 1.505                 | 1.138-1.989  | 0.004 <sup>b</sup> |
| Low              | 287                |                     |             |                    |                       |              |                    |
| High             | 284                |                     |             |                    |                       |              |                    |

<sup>a</sup>P<0.001 and <sup>b</sup>P<0.01 by Cox proportional hazard model. HR, hazard ratio; CI, confidence interval; hnRNPAB, heterogeneous nuclear ribonucleoprotein A/B; LUAD, lung adenocarcinoma.

were positively co-expressed with hnRNPAB in both LUAD and LUSC samples. Moreover, the expressions of these genes were verified in hnRNPAB knockdown cells using RT-qPCR technology and the results showed that 5 genes except for HSPA9, SFXN1, PPM1G, TMED9, and DDX41 were down-regulated (Figs. 5 and S2), suggesting that hnRNPAB may promote the proliferation, migration, invasion, and EMT of NSCLC cells partially by up-regulating the expressions of these 5 genes.

## Discussion

NSCLC has seriously threatened human health. Once NSCLC was diagnosed, most patients usually were in the late stage or their tumors have metastasized, thus losing the opportunity for surgery (27). Therefore, finding new diagnostic and therapeutic targets is of great importance to improve the survival rate of NSCLC patients.

HnRNPs are a kind of RNA binding proteins. So far, more than 20 hnRNP members have been identified, including hnRNPA ~ U (28). With the deepening of

the research, it is found that hnRNP members have been involved in the development and progression of tumors by regulating cell proliferation, metastasis, self-renewal of cancer stem cells and so on (9). For example, hnRNPL promotes the progression of prostate cancer by regulating circCSPP1/microRNA-520h/early growth response factor 1 (EGR1) axis (29). HnRNPA1, A2/B1, and K binding to the promoter of the tumor suppressor Annexin A7 (ANXA7) alter the splicing pattern of ANXA7, leading to the progression of prostate cancer (30).

HnRNPAB is a member of the hnRNP family. Previous studies have shown that hnRNPAB is dysregulated in multiple tumors, which is associated with various malignant phenotypes of tumors (10-12,31). However, the expression and role of hnRNPAB in NSCLC remain unclear. According to our results, hnRNPAB exhibited a relatively high expression in NSCLC samples, showing significant correlation with the overall survival, sex and TNM classification of patients with LUAD. Multivariate Cox regression analysis pointed toward hnRNPAB as an independent prognostic marker. These

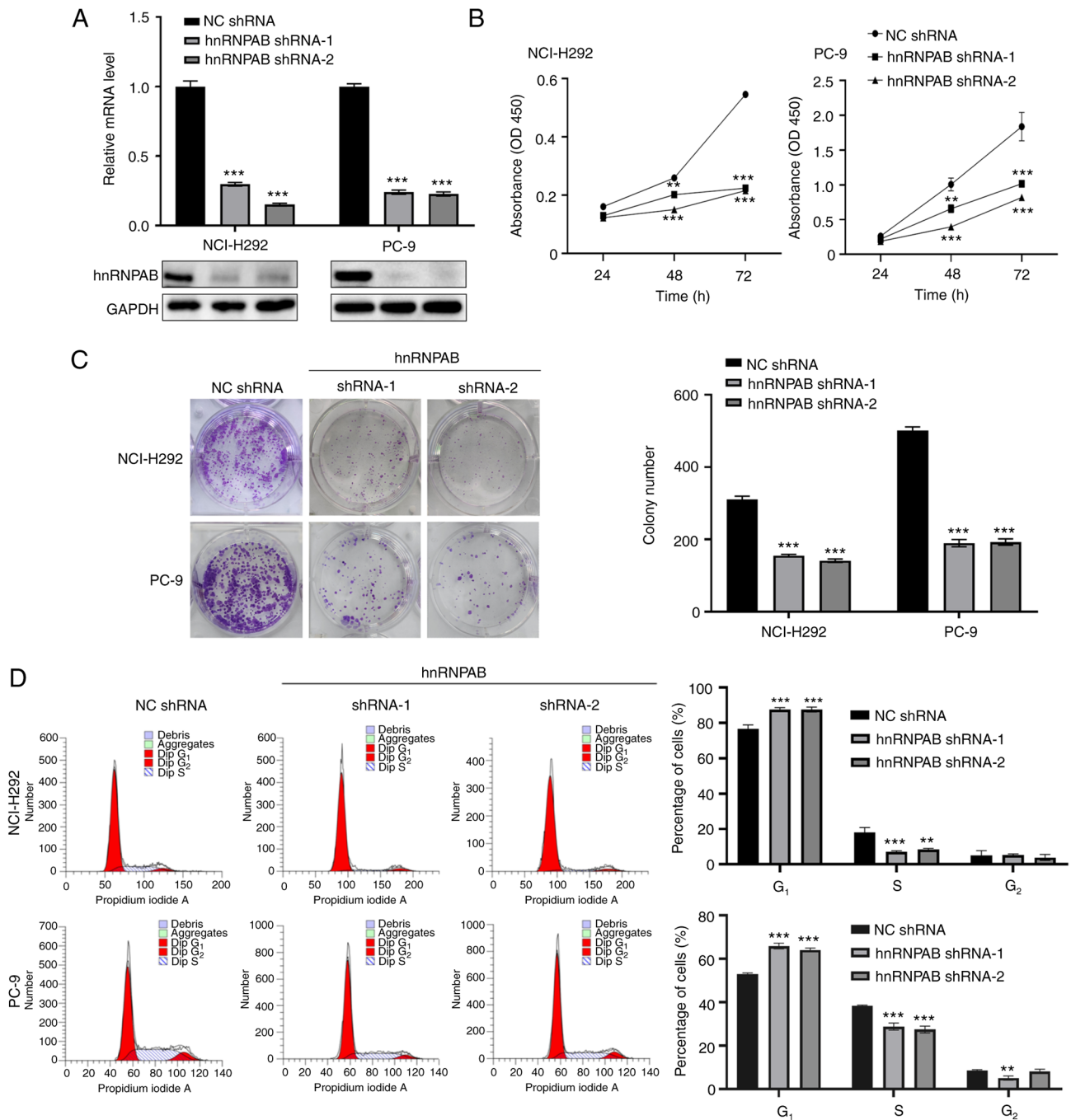


Figure 2. HnRNPAB knockdown inhibits the proliferation of non-small cell lung cancer cells. (A) Protein and mRNA expression levels of hnRNPAB in stable hnRNPAB knockdown cells were examined using western blotting and reverse transcription-quantitative PCR. GAPDH was used as an internal control. (B) Cell viability and (C) proliferation were tested by Cell Counting Kit-8 and adherent colony formation assays. (D) Cell cycle was detected using flow cytometry. Results are representative of three independent experiments. \*\*P<0.01 and \*\*\*P<0.001 compared with NC shRNA. HnRNPAB, heterogeneous nuclear ribonucleoprotein A/B; NC, negative control; shRNA, short hairpin RNA; OD, optical density.

results suggested that hnRNPAB may play an important role in NSCLC progression.

Metastasis is a main cause of mortality in cancer patients (32). EMT is often activated during tumor metastasis (32). EMT is a reversible biological cellular process in which cells transform from an epithelial form to a mesenchymal form (7). In the process of EMT, the expressions of many mesenchymal related proteins are often increased, especially Vimentin (33). Cells in EMT acquire stem cell-like properties, in which the protein expression changed from

E-cadherin to N-cadherin, ZEB1 and SNAIL, of which ZEB1 and SNAIL directly suppressed the expression of E-cadherin by binding to its promoter (34-36). Our results showed that hnRNPAB knockdown increased the expression of E-cadherin and reduced the expressions of ZEB1, N-cadherin, Vimentin, and SNAIL, indicating EMT was reversed. In general, cancer cells obtain stronger proliferation, migration, and invasion abilities after EMT (37,38). Consistently, hnRNPAB silencing inhibited the proliferation, migration, and invasion of NSCLC cells. These observations suggested that hnRNPAB contributes

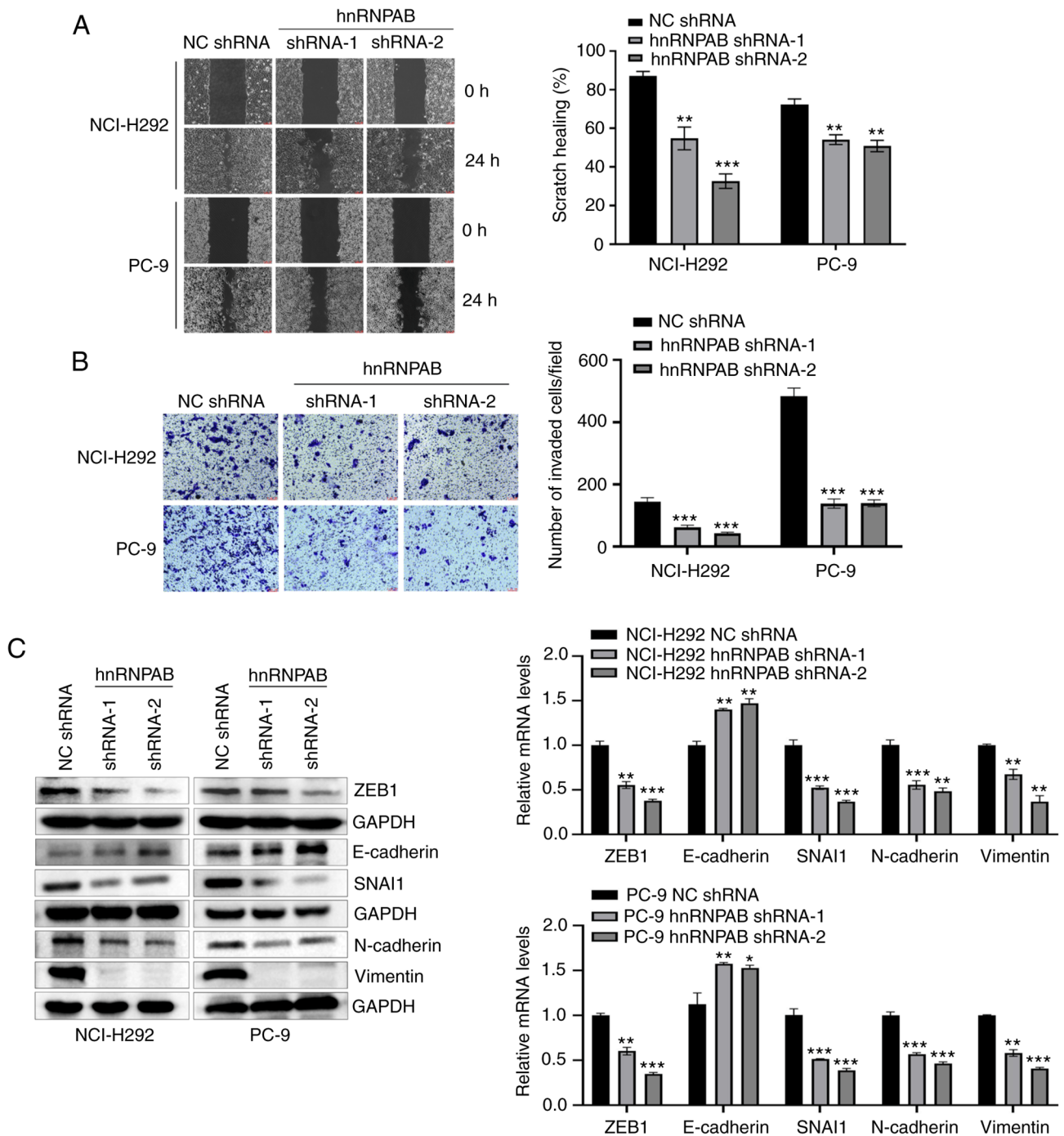


Figure 3. HnRNPAB-silencing inhibits the migration, invasion and EMT of NSCLC cells. (A) Cell migration was evaluated using wound healing assay (magnification, x100). (B) Cell invasion was evaluated using Matrigel matrix-coated Transwell assay (magnification, x100). (C) Protein and mRNA expression levels of EMT-related markers ZEB1, E-cadherin, SNAI1, N-cadherin and Vimentin were determined using western blotting and reverse transcription-quantitative-PCR. GAPDH was used as a loading control. For western blotting, multiple membranes from the same sample at the same time were used to expose different target proteins and so >1 GAPDH were provided. Results are representative of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared with respective NC shRNA group. HnRNPAB, heterogeneous nuclear ribonucleoprotein A/B; NC, negative control; shRNA, short hairpin RNA; EMT, epithelial-mesenchymal transition; ZEB1, zinc finger E-box binding homeobox 1.

to tumor progression, probably via the process of tumor proliferation and metastasis.

To explore the molecular mechanism of HnRNPAB regulating the proliferation and metastasis of NSCLC cells, 10 genes positively correlated with HnRNPAB expression were selected for verification. The results showed that the expressions of NOP16, NPM1, TCOF1, CENPH and CENBL were not only positively correlated with the expression of

HnRNPAB in LUAD and LUSC cases but also down-regulated in HnRNPAB silenced cells. Previous studies have shown that these 5 genes were highly expressed in a variety of tumor tissues, which promoted the malignant transformation of tumor cells (15-18,21). Increased rRNA production is a hallmark of cancer (15,39). It is verified that NOP16 and TCOF1 could coordinate the production of rRNA to promote HCC and breast cancer tumorigenesis (15,39). NPM1 is a well-known



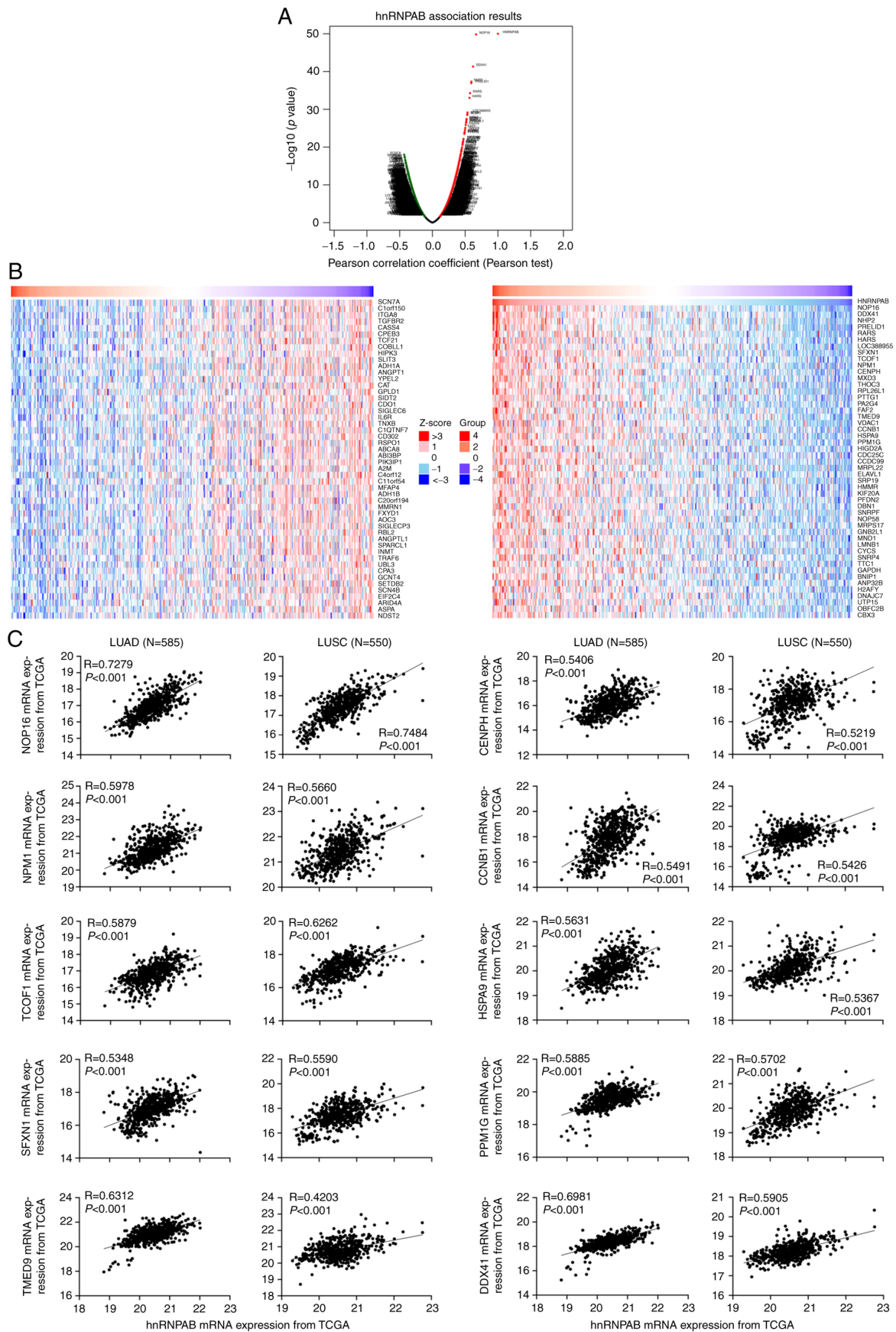


Figure 4. Analysis of genes related to hnRNPAB expression in NSCLC. (A) Analysis of hnRNPAB-related genes using LinkedOmics database based on Pearson correlation coefficient. (B) Analysis of positive and negative correlated genes with hnRNPAB. (C) Co-expression analysis between hnRNPAB and the screened genes using the mRNA expression data of NSCLC samples from The Cancer Genome Atlas database. HnRNPAB, heterogeneous nuclear ribonucleoprotein A/B; NSCLC, non-small cell lung cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NOPI6, NOPI6 nucleolar protein; NPM1, nucleophosmin 1; TCOF1, treacle ribosome biogenesis factor 1; CENPH, centromere protein H; CCNB1, cyclin B1; HSP, heat shock protein family; SFXN1, sideroflexin 1; PPM1G, protein phosphatase, Mg<sup>2+</sup>/Mn<sup>2+</sup> dependent 1G; TMED9, transmembrane p24 trafficking protein 9; DDX41, DEAD-box helicase 41.

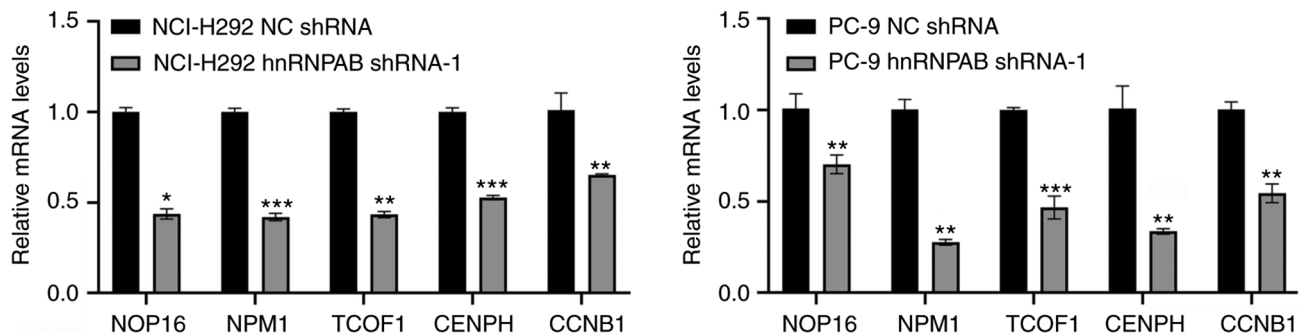


Figure 5. Expression levels of the screened genes in stable hnRNPAB-silenced cells were verified using RT-qPCR. The total RNA was extracted and the mRNA expression levels of NOP16, NPM1, TCOF1, CENPH and CCNB1 in stable non-small cell lung cancer cell lines were determined using RT-qPCR. GAPDH was used as an internal control. Results are representative of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared with respective NC shRNA group. HnRNPAB, heterogeneous nuclear ribonucleoprotein A/B; RT-qPCR, reverse transcription-quantitative PCR; NOP16, NOP16 nucleolar protein; NPM1, nucleophosmin 1; TCOF1, treacle ribosome biogenesis factor 1; CENPH, centromere protein H; CCNB1, cyclin B1; NC, negative control; shRNA, short hairpin RNA.

nucleocytoplasmic shuttling protein and its overexpression, translocation, mutation, or loss of function can lead to cancer and tumorigenesis (16). CENPH is highly expressed in NSCLC and may be used as a prognostic biomarker for patients with NSCLC (40). Likewise, CCNB1 is overexpressed in LUAD and involved in the proliferation and metastasis of tumor cells (21). These analyses indicate that hnRNPAB may promote the cell proliferation and metastasis of NSCLC by up-regulating the expressions of NOP16, NPM1, TCOF1, CENPH and CCNB1.

In conclusion, this study identified hnRNPAB as a useful biomarker in the prognosis of patients with LUAD. The overexpression of hnRNPAB promoted the proliferation and metastasis of NSCLC cells partially by up-regulating the expressions of NOP16, NPM1, TCOF1, CENPH and CCNB1. Our results may offer a potential novel target for the treatment of NSCLC.

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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

QW and KS designed the research and confirmed the authenticity of all the raw data. XG performed experiments. XG, LL and HY performed statistical analysis. QW wrote the

manuscript. QW and XG prepared figures. TZ, YZ, YX and JZ interpreted the data. All authors read and approved the final manuscript.

#### 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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