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Down-regulation of long non-coding RNA DUXAP8 suppresses proliferation, metastasis and EMT by modulating miR-498 through TRIM44-mediated AKT/mTOR pathway in non-small-cell lung cancer

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Abstract. – OBJECTIVE: The long non-coding RNA double homeobox A pseudogene 8 (DUXAP8) was reported to be involved in the initiation and development of multiple cancers. However, the detailed biological role of DUXAP8 in non-small-cell lung cancer (NSCLC) remains unclear. Herein, we aimed to explore the biological function and molecular mechanism of DUXAP8 in NSCLC.

PATIENTS AND METHODS: The levels of DUXAP8, microRNA-498 (miR-498) and tripartite motif-44 (TRIM44) were detected by Quantitative Real-time polymerase chain reaction (qRT-PCR). The cell proliferation, migration and invasion were detected by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and transwell assays. Protein expression levels were detected by Western blot. The target relationships among DUXAP8, miR-498 and TRIM44 were predicted by starBase2.0 and confirmed using luciferase reporter and RNA pull-down assays. To detect the role of DUXAP8 in *vivo,* tumor xenografts were created.

RESULTS: DUXAP8 and TRIM44 were upregulated in NSCLC tissues and cell lines, while miR-498 was downregulated. Functionally, knockdown of DUXAP8 could repress proliferation, migration, invasion, Epithelial-Mesenchymal Transition (EMT) and phosphorylation of AKT/mTOR in NSCLC cells. This inhibition could be restored by inhibiting miR-498 or overexpressing TRIM44. Furthermore, we also observed a positive correlation between DUXAP8 and TRIM44 expression, while the expressions of miR-498 and DUXAP8 as well as miR-498 and TRIM44 were negatively correlated in NSCLC tissues. Importantly, DUXAP8, could regulate the expression of TRIM44, *via* miR-498. Moreover, knockdown of DUXAP8 notably decreased the xenograft tumor volume, weight and number of metastatic nodules *in vivo*.

CONCLUSIONS: Our results identified that LncRNA DUXAP8 could regulate cell proliferation, metastasis and EMT in NSCLC cells by inhibiting miR-498 through the activation of TRIM44-mediated AKT/mTOR pathway.

Key Words:
NSCLC, LncRNA DUXAP8, MiR-498, TRIM44, Progression.

Introduction

Non-small-cell lung cancer (NSCLC) is one of the most familiar cancers worldwide, accounting for 80% of all patients with lung cancer, including squamous cell carcinoma (SCC), adenocarcinoma, and large-cell carcinoma (LCC)[1]. Although progress has been made in the treatment of NSCLC, the patients are still in poor health after treatment, mainly due to advanced patients and metastatic cancer cells[2]. Early diagnostic testing and prognostic markers are important challenges for most cancers, and NSCLC is no exception[3]. Hence, elucidating the relevant molecular and cellular mechanisms of NSCLC is pivotal.

Long non-coding RNAs (lncRNAs) are a class of RNAs that do not encode proteins longer than 200 nucleotides in length[4]. They were reported to be related to many basic biological processes, such as cell development, proliferation, apoptosis, metastasis, and various immune responses[5,6]. The abnormal expression of lncRNAs has a significant effect on promoting or inhibiting tumorigenesis and progression in various types of cancer[7,8]. As for NSCLC, lncRNA associated with it has been identified and used as a therapeutic target or prognostic marker[9].

Pseudogene-derived lncRNA double homeobox A pseudogene 8 (DUXAP8) has been reported to be up-regulated in most cancers. DUXAP8 could markedly restrain the expres-

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sion of PLEKHO1 in gastric cancer[10] and enhance cell proliferation and migration. In gliomas[11], the inhibition of the expression of the pseudogene DUXAP8 significantly impeded tumor cell proliferation. Sun et al[12] showed that DUXAP8 promoted proliferation and invasion of NSCLC cells via EGR1 and RHOB. The specific molecular regulation mechanism of DUXAP8 in NSCLC progression remains unclear.

MicroRNAs (miRNAs) are small highly conserved non-coding RNAs of roughly 22 nucleotides in length[13]. MiRNAs could regulate the target gene expression via binding to the 3'untranslated regions (3'UTR) of target gene at post-transcriptional level and promote degradation or inhibit translation[14]. MiR-498 has been reported to be down-regulated in most cancers, such as ovarian[15], colorectal[16] and esophageal squamous cell carcinoma[17]. Wang et al[18] also showed that miR-498 was declined and inhibited cell proliferation in NSCLC[18]. More than that, Wang et al[19] reported that up-regulation of miR-498 inhibited the behavior of epithelial-mesenchymal transition (EMT) in esophageal cancer, a process by which epithelial cells gradually acquired mesenchymal (fibroblast-like) cell phenotype[20]. EMT has been proved to be associated with the progression of many types of tumors and is involved in tumor cell migration and invasion[21]. Nevertheless, the molecular mechanism of miR-498 interacting with lncRNAs to regulate NSCLC progression has been rarely reported. In addition, some members of the tripartite motif (TRIM) protein family are carcinogenic factors with important regulatory roles[22]. Among them, TRIM44 was reported to be abnormally expressed in several malignancies[23] and could be used as a potential therapeutic target. Wei et al[24] showed that TRIM44 promoted melanoma progression via the AKT/mTOR pathway by stabilizing TLR4. And TRIM44-mediated mTOR signaling was critical for cell proliferation and metastasis in NSCLC[25].

To date, the molecular mechanism of DUXAP8 regulation of NSCLC is not sufficient. Therefore, the study aims to elucidate the interactions among DUXAP8, miR-498 and TRIM44, affording a new regulatory molecular mechanisms and therapeutic target for NSCLC.

Patients and Methods

Tissue Samples and Cells Culture

NSCLC tissues and adjacent normal tissues were harvested from 54 patients in Xiangyang Central Hospital. The patients involved in this study signed the informed consent and didn't receive any treatment before the surgery. Then the NSCLC tissues were resected and kept in liquid nitrogen until RNA extraction. This research was approved by the Ethics Committee of Xiangyang Central Hospital.

Human NSCLC cell lines (H460, H520, A549 and H1975) and normal cell line (BEAS-2B) were acquired from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific), and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO_2.

Plasmids and Transfection

Overexpression vectors (DUXAP8 and TRIM44) and corresponding negative control (pcDNA) vector were synthesized by SyngenTech (Beijing, China). The miR-498 mimics (miR-498) or anti-miR-498 and the corresponding control mimics (miR-NC) or anti-miR-NC were obtained from GenePharma (Shanghai, China). The Lipofectamine 2000 (Invitrogen) reagent was used for transfection.

Lentivirus Production and Infection

The pLVX vector containing shRNA DUXAP8 or the control sequence (GenePharma) was co-transfected with Lenti-Pac HIV Expression Packaging Mix (GenePharma) into 293T cells using Lipofectamine 2000. Lentiviral particles in the supernatant 48 h post transfection were harvested and filtered. Then, the A549 and H1975 cells were infected with LV-sh-DUXAP8 or LV-sh-NC. The cells were disposed with puromycin (2 μg/mL) for 2 weeks, and then sh-DUXAP8 and sh-NC were picked.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted by TRIzol® reagent (Thermo Fisher Scientific). Prime Script TM RT Master Mix (TaKaRa Biotechnology, Dalian, China) was used to reverse transcribe RNA into cDNA. QRT-PCR for mRNA was carried out with SYBR PremixExTaq II reagent by 7500 RT-PCR System (Applied Biosystems, Foster City, CA, USA). The miR-498 relative expression was analyzed by $2^{-\Delta\Delta Ct}$ method normalized to U6 snRNA, while DUXAP8 and TRIM44

expressions were with respect to GAPDH. The primer sequences used were listed as below: DUXAP8 (Forward: 5'-AGGATGGAGTCTC-GCTGTATTGC-3'; Reverse: 5'-GGAG-GTTTGTTTTCTTCTTTTTT-3'); TRIM44 (Forward: 5'-AGGCAGCTCATCTGTGTCCT-3'; Reverse: 5'-GCCTTCAGTCCAC CTGAGTC-3'); miR-498 (Forward: 5'-TTTCAAGCCAGGGGG-CGTTTTTC-3'; Reverse: 5'-GCTTCAAGCTCT-GGAGGTGCTTTTC-3'); GAPDH (Forward: 5'-CTCACCGGATGCACCAATGTT-3'; Reverse: 5'-CGCGTTGCTCACAATGTTCAT-3'). Primers for U6 (Forward: 5'-AACGCTTCAC-GAATTTGCGT-3'; Reverse: 5'-CTCGCTTCG-GCAGCACA-3') were obtained from GenePharma (Shanghai, China).

MTT Assay

Cells were plated at the density of 1×10^5 cells/well in 96-well plates (Costar, Corning, NY, USA). At designated times (0 h, 24 h, 48 h, and 72 h) post-transfection, 10 μL MTT regent (KyeGEN BioTECH, Nanjing, China) (5 mg/mL) was added to each well, and then cells were maintained for 4 h at 37°C. After discarding the supernatant, the 200 μL dimethyl sulfoxide (DMSO; Gibco, Rockville, MD, USA) was added to each well to solubilize the formazan salt. Then, the cell proliferation was detected by measuring the absorbance at 490 nm on a UV microplate reader (Tecan Austria GmbH, Grödig, Austria).

Transwell Migration and Invasion Assay

The 24-well transwell chamber was used to detect cell migratory (without Matrigel) and invasive rates (with Matrigel) (Corning Life Sciences, Corning, NY, USA). Cells were fixed with 100 mL of serum-free medium and seeded into the upper chamber, and 600 mL of DMEM containing 10% FBS was added to the lower chamber. After 24-h incubation, the cells attached to the lower surface of the upper chamber were fixed with ethanol and stained with 0.1% crystal violet and were analyzed under a microscope.

Western Blot Assay

Cell and tissue lysates were prepared with RIPA Reagent (Sigma-Aldrich, St. Louis, MO, USA), and the protein concentration was detected by BCA reagent (Thermo Fisher Scientific, Waltham, MA, USA). Proteins were loaded on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were then transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific). The membranes were blocked with 5% non-fat milk for 1 h at 37°C and then incubated with primary antibodies at 4°C overnight. Membranes were then washed and probed with the secondary antibody for 1 h at 37°C. Membranes were detected via the ECL assay (Amersham Biosciences, Little Chalfont, UK). Antibodies were as follows: TRIM44 (1:300, Novus Biologicals, Centennial, CO, USA), mTOR (1:1000;Cell Signaling Technology, Danvers, MA, USA), AKT (1:1000; Cell Signaling Technology), E-cadherin (1:1000; Cell Signaling Technology), Vimentin (1:1000, Abcam, Cambridge, UK), N-cadherin (1:100 dilution, Abcam), GAPDH (1:4000; Cell Signaling Technology) and horseradish peroxidase (HRP)-labeled secondary antibody (Abcam).

Luciferase Reporter Gene Assay

DUXAP8 wild type (WT-DUXAP8) and mutant type (MUT-DUXAP8) of containing miR-498 interacting site or not were transfected into cells with miR-NC and miR-498 mimics using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 36 h transfection, the luciferase activity was measured. TRIM44 wild type (TRIM44 3'UTR-WT) and mutant type (TRIM44 3'UTR-MUT) including miR-498 targeting sites or not were also transfected into cells. The luciferase activity was checked with Dual-luciferase reporter system (Promega, Madison, WI, USA).

RNA Pull-Down Assay

The experiment process was carried out as described previously[26]. Briefly, cells were split by lysis buffer supplemented with protease inhibitor (Solarbio, Beijing, China). Cell lysates were mixed with biotin-labeled DUXAP8 probe sequences, and incubated with streptavidin coated magnetic beads (Thermo Fisher Scientific) for 0.5 h, and the beads were washed by salt buffer. Finally, the RNA was quantified by qRT-PCR assay.

Tumor Xenograft Assay

Sh-DUXAP8 (right) or sh-NC (left) transfected H1975 cells (5×10^6) were subcutaneously injected into 4-week-old nude mice (n = 5). In a week, tumor volumes were estimated every week for 5 weeks. 5 weeks later, mice were euthanized, and the weight of tumors was weighted. DUXAP8, miR-498 and TRIM44 expression in excised tumors were measured by RT-qPCR. All animal protocols were approved by the Animal Care and Use Committee of Xiangyang Central Hospital.

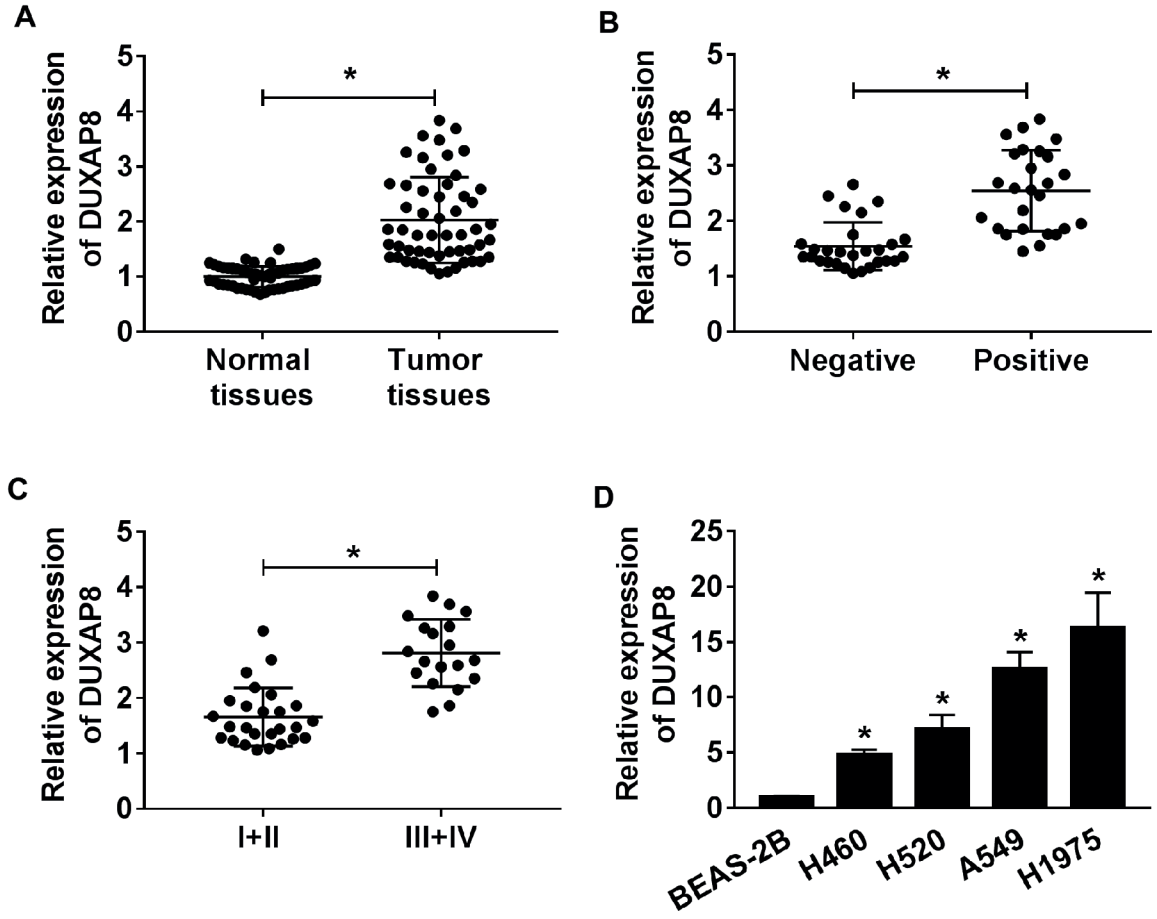

Figure 1. LncRNA DUXAP8 expression in NSCLC tissue samples and cells and its correlation with clinical parameters. **(A)** Relative expression of DUXAP8 in NSCLC tissues was measured by qRT-PCR in comparison with corresponding non-tumor normal tissues (n=54). **(B)** DUXAP8 expression level in lymph node metastasis positive and negative tissue samples was detected by qRT-PCR. **(C)** DUXAP8 expression in clinical stage I+II and III+IV of NSCLC tissues was measured by qRT-PCR. **(D)** DUXAP8 expression in human normal lung epithelial cell line BEAS-2B and NSCLC cell lines H460, H520, A549 and H1975 was detected by qRT-PCR. *p<0.05.

Statistical Analysis

Data were showed as mean ± standard deviation (SD) and obtained from at least three independent experiments. Statistics were analyzed with SPSS 22.0 software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA). Pearson's coefficient correlation was used for expression correlation assay. *p<0.05 was considered statistically significant.

Results

DUXAP8 Was Highly Expressed in NSCLC Tissues and Cell Lines

To explore the potential role of DUXAP8 in NSCLC, the DUXAP8 expression level in 54 pairs of NSCLC samples and adjacent normal tissues was investigated. The result showed that NSCLC expression was notably elevated in NSCLC tissues compared with adjacent healthy tissues (Figure 1A). Meanwhile, DUXAP8 was highly expressed in positive tissue samples compared to lymph node metastasis of negative tissues (Figure 1B). And DUXAP8 was significantly augmented in clinical late stages (III+IV) than in early clinical stages (I+II) (Figure 1C). The clinicopathological data was shown in Table I. Then, the expression of DUXAP8 was detected in human normal lung epithelial cell line BEAS-2B and human NSCLC cell lines H460, H520, A549 and H1975. The results indicated that DUXAP8 expression was drastically augmented in NSCLC cells (Figure 1D). These data demonstrated that DUXAP8 was likely to play a role in the progression of NSCLC.

Knockdown of DUXAP8 Inhibited Cell Proliferation, Migration, Invasion and EMT in NSCLC Cells

Next, we explored whether DUXAP8 could modulate biologic activity in NSCLC cells. DUXAP8 was silenced in A549 and H1975 cells transfected with sh-DUXAP8. Then, the cell proliferation, migration, invasion and EMT-related proteins were detected in A549 and H1975 cells. The result showed that DUXAP8 expression in sh-DUXAP8 groups was drastically declined relative to that in sh-NC groups (Figure 2A). In addition, the cell proliferation, migration and invasion in sh-DUXAP8 groups were aberrantly repressed in contrast to that in sh-NC groups (Figure 2B-E). Meanwhile, knockdown of DUXAP8 significantly decreased EMT-related protein Vimentin and N-cadherin, while E-cadherin protein expression was notably increased (Figure 2F-G). These data revealed that DUXAP8 inhibition could impede proliferation, metastasis and EMT of A549 and H1975 cells.

DUXAP8 as a Molecular Sponge Antagonized MiR-498 Availability

According to previous studies, lncRNA can participate in post-transcriptional regulation by interfering with miRNAs[27]. We found that DUXAP8 and miR-498 had potential binding site which searched by StarBase v.2.0 (Figure 3A). Then, the interaction was also confirmed by dual-luciferase assay and RNA pull-down. The result showed that the luciferase activity of WT-DUXAP8 in A549 and H1975 cells was decreased by the miR-498 mimic, but the WT-DUXAP8 activity was not significantly changed (Figure 3B-C). The RNA pull-down assay further confirmed the direct interaction between DUXAP8 and miR-498 because a significant amount of miR-498 was detected in DUXAP8 probe groups than that in control groups (Figure 3D). More than that, the expression of miR-498 was found to be down-regulated in NSCLC tissues (Figure 3E). Simultaneously, a significant inverse correlation between DUXAP8 and miR-498 was also observed (Figure 3F). Furthermore, the miR-498 expression in NSCLC cell lines A549 and H1975 was also exceptionally constrained *vs.* that in normal cell line BEAS-2B (R=-0.6441, $p<0.001$) (Figure 3G), and the miR-498 level was found to be up-regulated in cells transfected with sh-DUXAP8 (Figure 3H). In order to further confirm the regulatory mechanism between DUXAP8 and miR-498, the recovery experiments were adopted. The data displayed that the inhibitory effect of sh-DUXAP8 on cell proliferation was rescued by the addition of anti-miR-498 in A549 and H1975 cells (Figure 3I-J). As for the cell migration and invasion, the inhibition of sh-DUXAP8 was also attenuated by anti-miR-498 (Figure 3K-L).

TRIM44 Directly Interacted with miR-498

TRIM44 has been reported to be an oncogene in NSCLC (28). Meanwhile, starBase2.0 predicted that there were binding sites for miR-498 and

Table I. Association of DUXAP8 expression with clinicopathological factors in non-small cell lung cancer patients.

Clinicopathological features	Number of cases	DUXAP8 expression		*p*-value
		Low (n=39)	High (n=)	
Age				
> 60 years	30	13	17	0.625
≤ 60 years	24	12	12	
Gender				
Male	35	16	19	0.627
Female	19	10	9	
Tumor size(cm)				
> 3	25	10	15	0.266
≤ 3	29	16	13	
Clinical stage				
I+II	30	21	9	0.001
III+IV	24	6	18	
Lymph node metastasis				
Positive	26	4	22	<0.001
Negative	28	23	5	

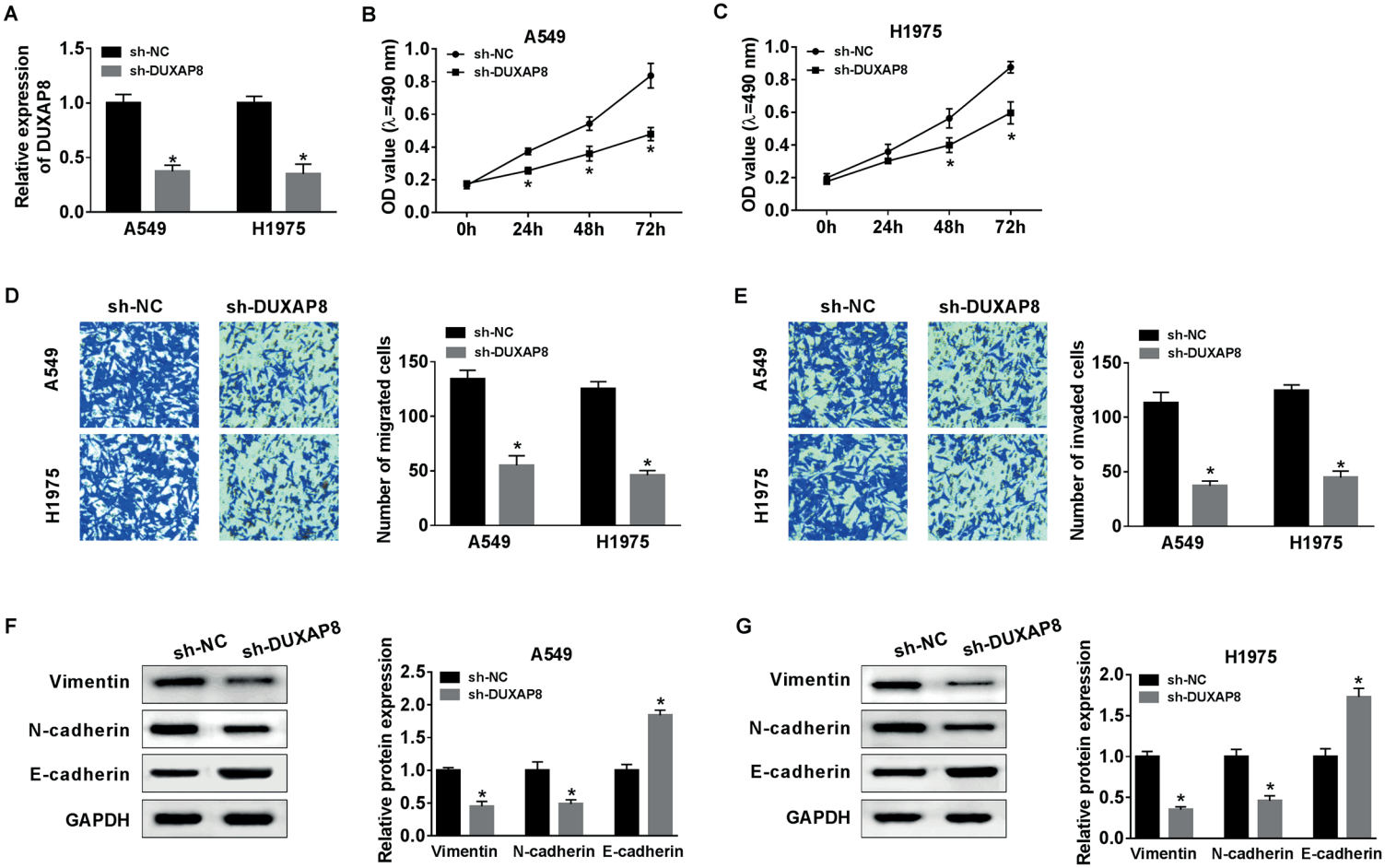

Figure 2. Knockdown of DUXAP8 inhibited cell proliferation, migration, invasion and EMT in NSCLC cells. **(A)** The DUXAP8 expression in A549 and H1975 cells transfected with sh-DUXAP8 or sh-NC was tested. **(B-C)** The cell proliferation of A549 and H1975 cells with sh-DUXAP8 or sh-NC was measured by MTT assay. **(D-E)** Transwell assays were performed to detect migration and invasion of A549 and H1975 cells transfected with sh-DUXAP8 or sh-NC, and the numbers of cells that migrated or invaded were calculated. **(F-G)** Western blot assay was conducted to detect the protein expression of Vimentin, N-cadherin and E-cadherin in A549 and H1975 cells transfected with sh-DUXAP8 or sh-NC. *p<0.05.

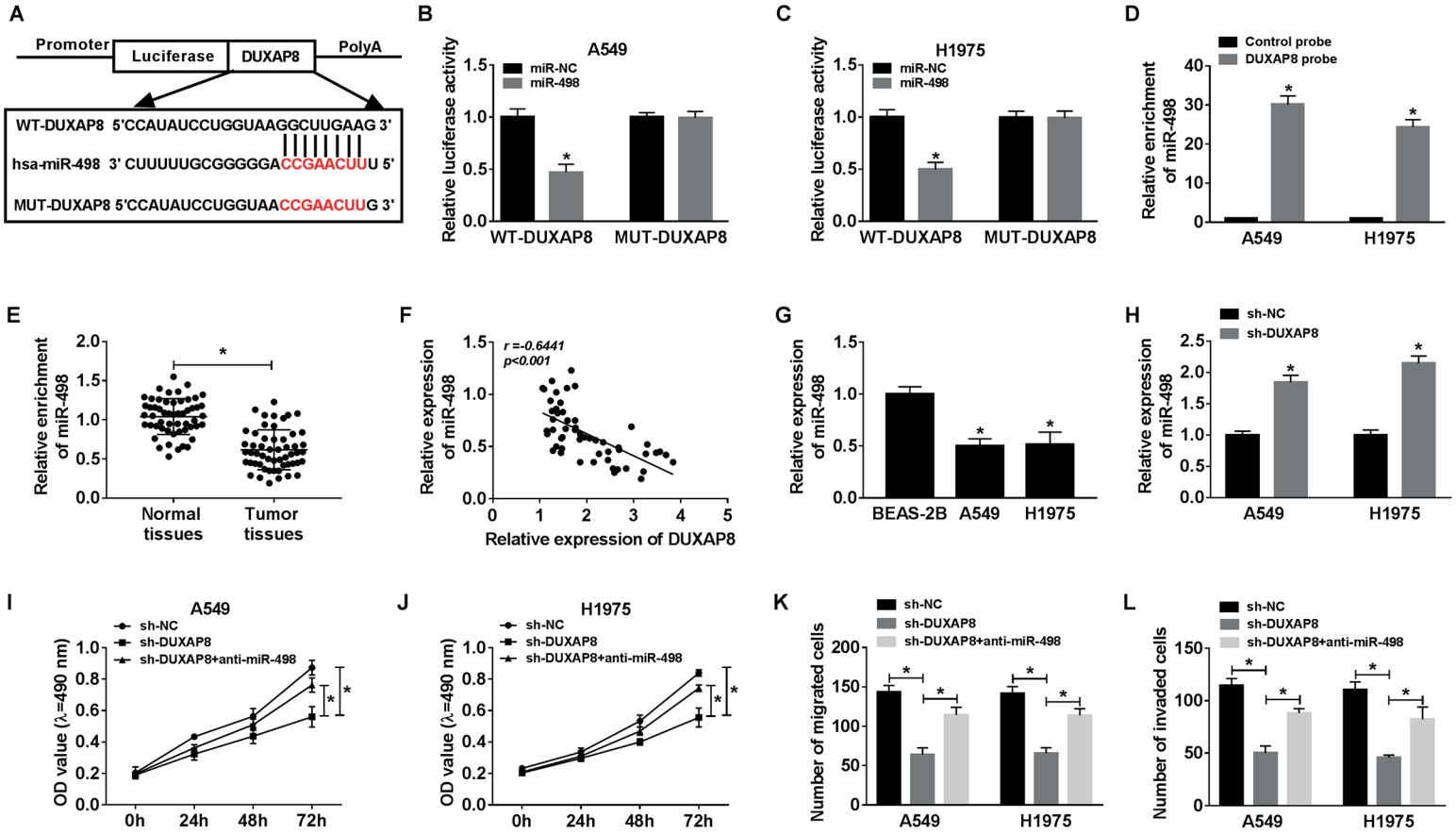

Figure 3. Identification of DUXAP8 targeting miR-498. **(A)** The putative binding site between DUXAP8 and miR-498 was predicted by starBase v2.0. **(B-C)** Relative luciferase activity of A549 and H1975 cells co-transfected WT-DUXAP8 or MUT-DUXAP8 with miR-498 or miR-NC was checked. **(D)** MiR-498 expression in A549 and H1975 cells transfected with DUXAP8 probe or control probe was detected by qRT-PCR. **(E)** Relative expression of miR-498 in NSCLC tissues was measured by qRT-PCR in comparison with corresponding non-tumor normal tissues (n=54). **(F)** The correlation between DUXAP8 and miR-498 was analyzed by Pearson's coefficient correlation (R =-0.6441, p<0.001). **(G)** MiR-498 expression in NSCLC cell lines of A549 and H1975 was down-regulated compared with that in normal cell line BEAS-2B. **(H)** MiR-498 expression in A549 and H1975 cells transfected with sh-DUXAP8 or sh-NC was measured. **(I-J)** The cell proliferation of A549 and H1975 cells co-transfected with sh-DUXAP8 and anti-miR-498 or not was detected by MTT. **(K-L)** The cell migration and invasion of A549 and H1975 cells co-transfected with sh-DUXAP8 and anti-miR-498 or not were measured by transwell assay. *p<0.05.

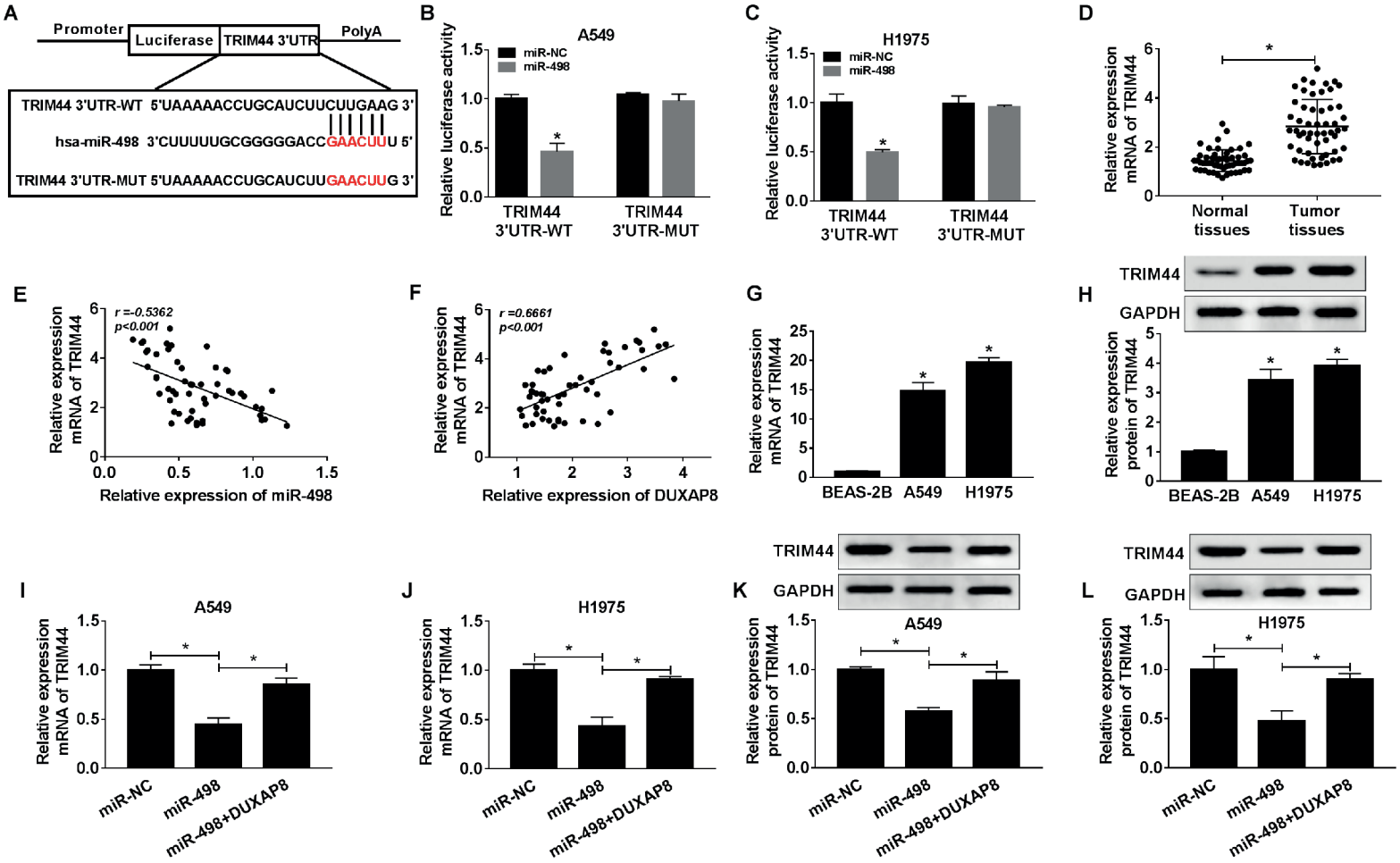

Figure 4. TRIM44 directly interacted with miR-498. **(A)** The putative binding site of TRIM44 and miR-498 was predicted by starBase v2.0. **(B-C)** Relative luciferase activity of A549 and H1975 cells co-transfected TRIM44 3'UTR-WT or TRIM44- 3'UTR-MUT with miR-498 or miR-NC was checked. **(D)** TRIM44 expression in NSCLC tissues and matched adjacent normal tissues was detected by qRT-PCR. **(E-F)** The correlation between TRIM44 and miR-498 expression (R =-0.5362, p<0.001), as well as between TRIM44 and DUXAP8 expression (R=0.6661, p<0.001) were analyzed by Pearson's coefficient correlation. **(G-H)** The mRNA and protein expression levels of TRIM44 in BEAS-2B, A549 and H1975 cells were detected. **(I-L)** The mRNA and protein expression of TRIM44 were assessed in A549 and H1975 cells transfected with miR-NC, miR-498, miR-498+DUXAP8. *p<0.05.

TRIM44 3' UTR (Figure 4A). Then, luciferase reporter assay was performed in A549 and H1975 cells. The result showed that the luciferase activity with TRIM44-3' UTR-WT and miR-498 was exceptionally constrained relatively to that in cells transfected with TRIM44-3' UTR-WT and miR-NC, while there was no distinct change in the cells transfected with TRIM44-3' UTR-MUT (Figure 4B-C). Additionally, the expression of TRIM44 in clinical samples was measured. The results indicated that TRIM44 was up-regulated in NSCLC tissues compared with adjacent normal tissues (Figure 4D). What's more, the TRIM44 expression was negatively correlated with the miR-498 expression (R =-0.5362, $p<0.001$) (Figure 4E), while it was positively correlated with DUXAP8 (R = 0.6661, $p<0.001$) (Figure 4F). Next, we found that both mRNA and protein expression of TRIM44 were drastically elevated in A549 and H1975 cells respect to that in normal cell line BEAS-2B (Figure 4G-H). To explore whether the expression of TRIM44 was modulated by DUXAP8 and miR-498 in NSCLC cells, the rescue experiments were conducted. The results showed that the mRNA and protein levels of TRIM44 were decreased in cells transfected with miR-498; however, in the miR-498 and DUXAP8 co-transfection group, the inhibition of miR-498 was recovered (Figure 4I-L). In conclusion, the above results indicated that DUXAP8 could up-regulate the expression of TRIM44 by inhibiting miR-498.

Overexpression of TRIM44 Almost Recovered the Inhibitory Effect of Down-Regulation of DUXAP8 in NSCLC cells

To further test the relationship between DUXAP8 and TRIM44, the recovery experiments were carried out by MTT and transwell assays. The results showed that interference with DUXAP8 by sh-DUXAP8 could inhibit the proliferation, migration and invasion of A549 and H1975 cells, whereas the inhibitory effect of sh-DUXAP8 could be restored by overexpression of TRIM44 (Figure 5A-D). More than that, the expression of EMT-related proteins Vimentin and N-cadherin was steeply repressed in sh-DUXAP8 groups compared with sh-NC groups of A549 and H1975 cells, while the protein expression of E-cadherin was enormously enhanced; however, this inhibition or promotion could be reversed by TRIM44 (Figure 5E-F). These data suggested that the DUXAP8 could regulate cell proliferation, migration, invasion and EMT by TRIM44 in NSCLC cells.

Knockdown of DUXAP8 Inhibited AKT/mTOR Pathway by Affecting miR-498/TRIM44 Axis in NSCLC Cells

Previous study has shown that TRIM44 can regulate the progression of AKT/mTOR pathway (29). To investigate whether DUXAP8 involved in regulating AKT/mTOR phosphorylation in NSCLC cells, we detected changes in the expression of AKT/mTOR pathway-associated proteins by Western blot after transfected with sh-DUXAP8 or sh-NC. The data showed that the protein expression of p-AKT and p-mTOR was significantly induced in sh-DUXAP8 groups compared to sh-NC groups (Figure 6A-B). In other words, the interference with DUXAP8 could inhibit phosphorylation of AKT/mTOR. However, this inhibition can be restored by anti-miR-498 or overexpression of TRIM44 (Figure 6A-B). These results indicated that the interference with DUXAP8 inhibited AKT/mTOR pathway by regulating miR-498/TRIM44.

Silencing of DUXAP8 Retarded Tumor Growth and Metastasis In Vivo

To further examine the oncogenic activity of DUXAP8, H1975 cells stably transfected with sh-DUXAP8 or sh-NC (5×10^6 cells per injection site) were injected subcutaneously into nude mice. Tumor volume was measured every week, mice were euthanized 5 weeks after cell injection and the tumor weight was measured. The results showed that the knockdown of DUXAP8 could inhibit the tumor growth, reflected by reduced tumor volume and weight (Figure 7A-B). Additionally, the expression of DUXAP8, miR-498 and TRIM44 in resected tumor tissues was measured. In the sh-DUXAP8 group, DUXAP8 and TRIM44 expression were decreased and miR-498 was upregulated (Figure 7C). Additionally, the number of lung metastatic nodules in the sh-DUXAP8 group was significantly lower than that in the sh-NC group (Figure 7D). Above results indicated that DUXAP8 could inhibit NSCLC tumor growth and metastasis through the regulation of miR-498/TRIM44 *in vivo*.

Discussion

Recently, the role of lncRNAs in tumorigenesis, metastasis and potential molecular mechanisms has attracted great attention. Our study identified that DUXAP8 was drastically enhanced in NSCLC tissues and cell lines. We also

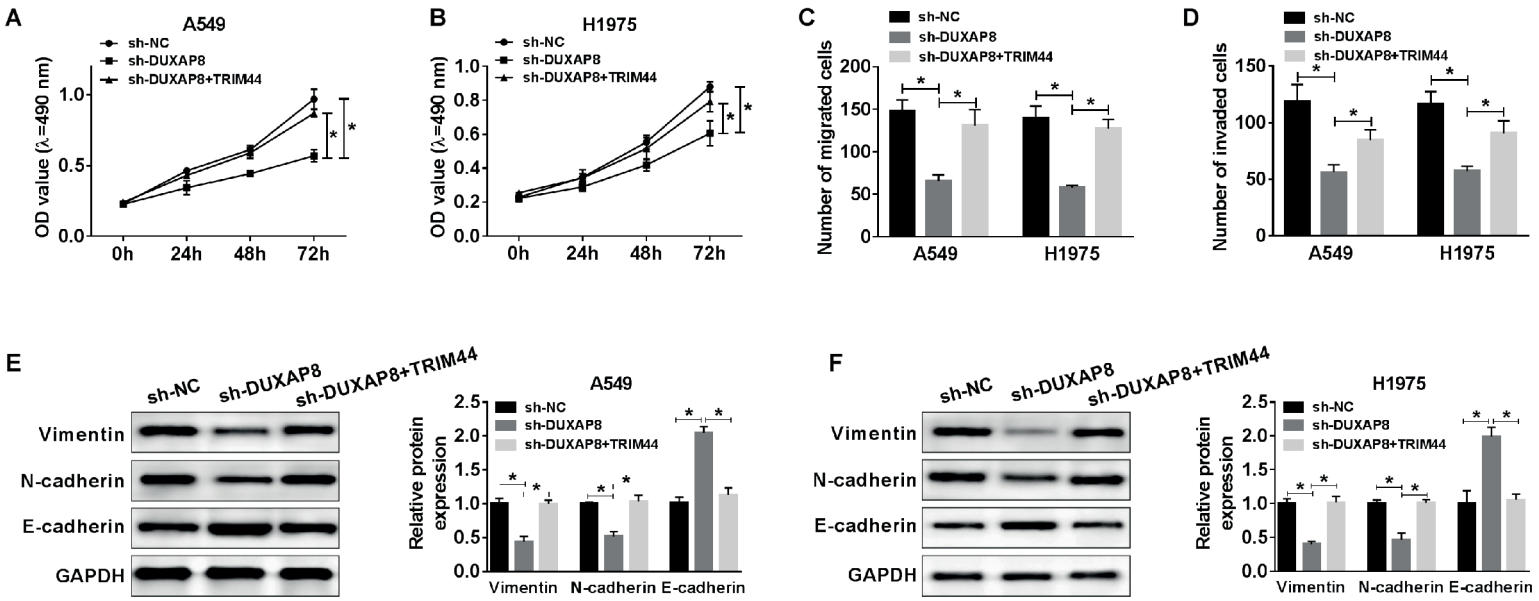

Figure 5. Overexpression of TRIM44 almost reversed the effect of sh-DUXAP8 on proliferation, migration, invasion and EMT in NSCLC cells. **(A-B)** The cell proliferation of A549 and H1975 cells co-transfected with sh-DUXAP8 and TRIM44 or not was detected. **(C-D)** Transwell assay was used to measure the cell migration and invasion in A549 and H1975 cells. (**E-F**) Western blot assays were applied to evaluate the protein expression of Vimentin, N-cadherin and E-cadherin in A549 and H1975 cells transfected with sh-NC, sh-DUXAP8 or sh-DUXAP8 +TRIM44. *p<0.05.

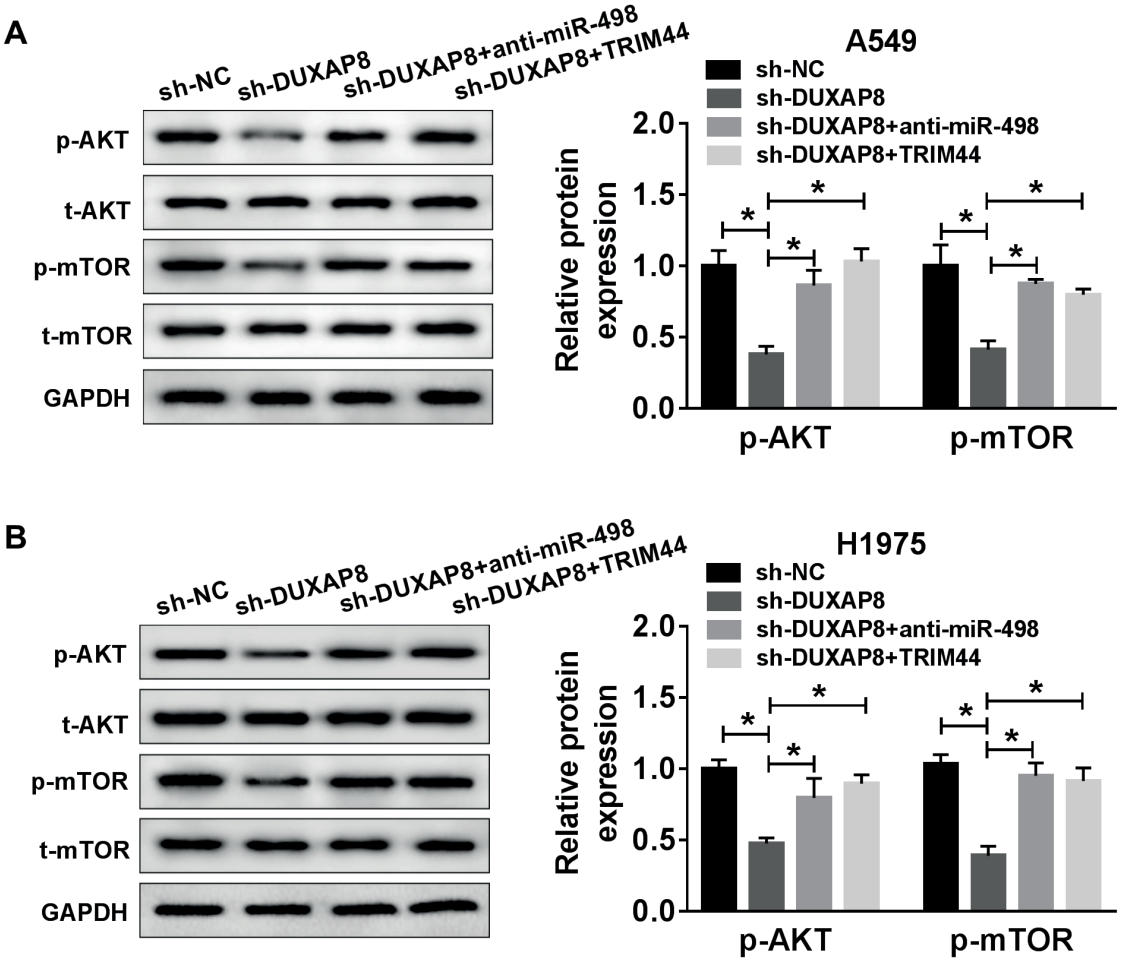

Figure 6. DUXAP8 regulated the AKT/mTOR pathway through affecting miR-498/TRIM44 in NSCLC cells. **(A-B)** Western blot assay was conducted to detect the protein expression of p-AKT, t-AKT, p-mTOR and t-mTOR in A549 and H1975 cells transfected with sh-NC, sh-DUXAP8, sh-DUXAP8 + anti-miR-498 or sh-DUXAP8+TRIM44. *$p<0.05$.

explored the function of DUXAP8 by loss-of-function approaches in NSCLC cells. The results demonstrated that knockdown of DUXAP8 repressed the proliferation, metastasis and EMT in A549 and H1975 cells, and induced tumor growth arrest *in vitro* and *in vivo*, which was consistent with the data of reported studies in gastric cancer[30]. Previously, it has been shown that EMT plays an important role in cancer metastasis. Through EMT, epithelial cells lose cell polarity and connection with the basement membranes, thus gaining higher migration and invasion ability, which has important pathological significance for tumor development[31]. These results showed that DUXAP8 might act an oncogene part in the progression of NSCLC.

lncRNAs function as ceRNAs to sponge miRNAs to affect functional roles of miRNAs[32]. DUXAP8 has been reported to directly bind with miR-126 to enhance renal cell carcinoma progression[33]. We hypothesized that DUXAP8 could also act as a ceRNA with miRNA. Our results indicated that DUXAP8 had binding sites for miR-498 and demonstrated their interaction. Interestingly, the decline of DUXAP8 resulted in the overexpression of miR-498; however, a significant inverse correlation between DUXAP8 and miR-498 was also observed. Moreover, interfering with DUXAP8-mediated inhibition of cell proliferation, metastasis and EMT could be restored by anti-miR-498. In previous studies, it has been reported that

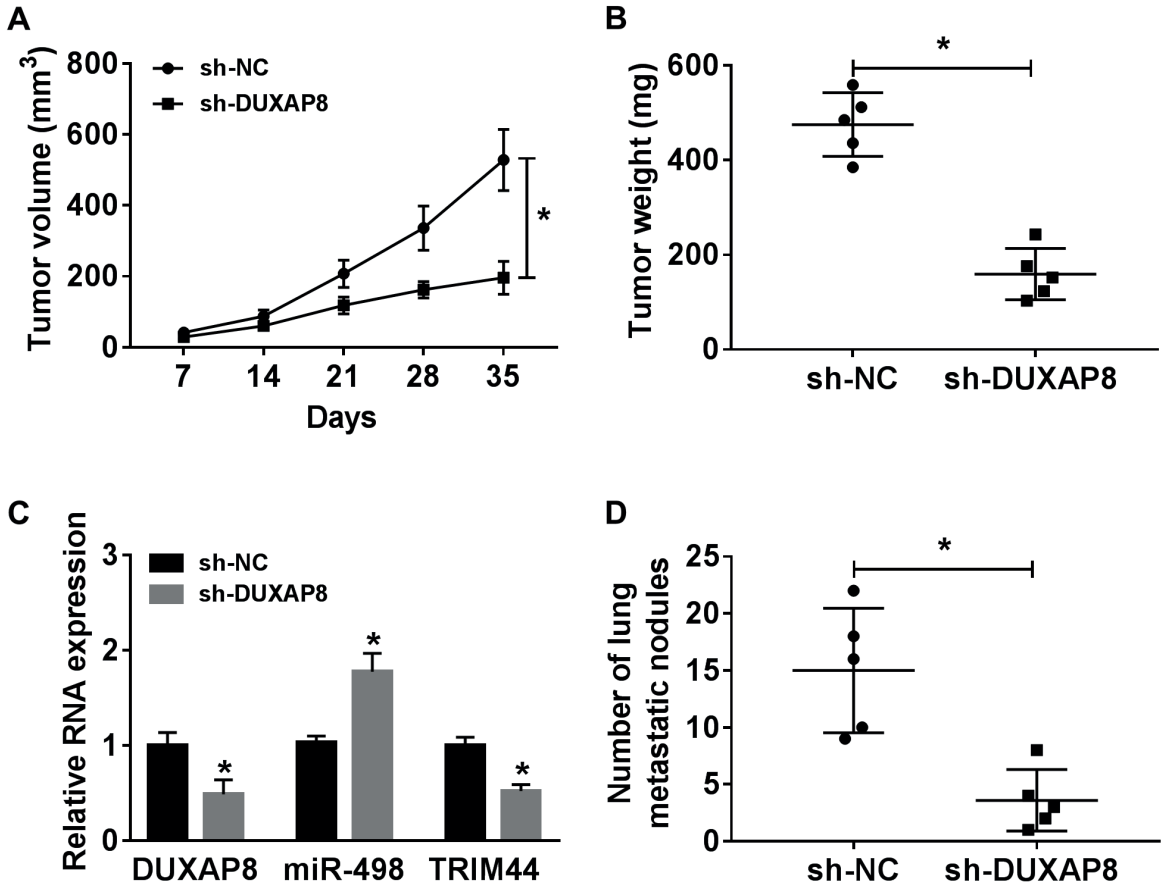

Figure 7. Silencing of DUXAP8 retards NSCLC cell growth and metastasis *in vivo*. **(A)** Tumor growth curves were measured after injection of H1975 cells transfected with sh-DUXAP8 or sh-NC. Tumor volume was calculated every 7 d (n=5). **(B)** Tumor weight was measured after mice were euthanized. Values are means of tumor weight±s.d. **(C)** The expression of DUXAP8, miR-498 and TRIM44 was measured in tumor tissues transfected with sh-DUXAP8 or sh-NC. **(D)** The number of metastatic nodules was counted after 5 weeks transfected with sh-DUXAP8 or sh-NC. *p<0.05.

miR-498 inhibitors were also able to reverse the restrained impact induced by si-circFADS2 on development and invasion of NSCLC cells[34]. Based on this evidence, we inferred that DUXAP8 might regulate the progression of NSCLC through the interaction with miR-498.

We indicated that TRIM44 was a target of miR-498 and was inversely related to the miR-498 expression in NSCLC tissues. Considering the interaction between DUXAP8 and miR-498, we suspected that DUXAP8 might also regulate TRIM44 expression. The results showed that TRIM44 was exceptionally facilitated in NSCLC and positively correlated with DUXAP8 expression. More than that, overexpression of TRIM44 could also restore the inhibitory effect of DUXAP8 on cell progression. Notably, the interference with DUXAP8 could inhibit AKT/mTOR signaling pathway, and this inhibition could be recovered by anti-miR-498 or overexpression of TRIM44. Also, Peng et al[35] indicated that TRIM44 could promote intrahepatic cholangiocarcinoma progression by inducing cell EMT (). TRIM44 has also been reported to promote cell proliferation and metastasis through EMT via AKT/mTOR pathway in human esophageal cancer. The results indicated that DUXAP8 could sequester miR-498 to protect their target RNA TRIM44 from inhibition. It is worth mentioning that a miRNA has many target genes Therefore, DUXAP8 might simultaneously target other targets to participate in the molecular mechanisms of NSCLC development, which is worthy of further study.

Conclusions

The present demonstrated that DUXAP8 could modulate TRIM44 expression by acting as a sponge of miR-498. Importantly, DUXAP8 was found to be a potential NSCLC tumor promoter by regulating cell proliferation, and crucially, it could facilitate the metastasis of NSCLC tumors through EMT, suggesting that DUXAP8 is a potential therapeutic target for cancer treatment.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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