

shRNA-mediated silencing of TARBP2 inhibits NCI-H1299 non-small cell lung cancer cell invasion and migration via the JNK/STAT3/AKT pathway

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Abstract. Metastasis is a major cause of lung cancer-associated mortality. The current study aimed to investigate the effects and mechanisms of TAR (human immunodeficiency virus 1) RNA binding protein 2 (TARBP2) in the invasion and migration of non-small cell lung cancer *in vitro*. The highly metastatic cell clone H1299/M02 was obtained by TARBP2 overexpression. Expression of TARBP2 in H1299/M02 was also downregulated to different levels via small hairpin RNAs (shRNAs). Subsequent to TARBP2 silencing, the proliferation of H1299/M02 cells was predominantly unaffected, while invasion and migration were significantly inhibited. A positive correlation was observed between invasion and migration and the level of TARBP2 silencing *in vitro*. Western blotting and reverse transcription-quantitative polymerase chain reaction indicated that the protein expression levels of amyloid β (A4) precursor protein (APP) and zinc finger protein 395 (ZNF395) were upregulated, while expression levels of pro-metastatic proteins including interleukin (IL)-1 β , IL-8, cyclooxygenase (COX)-2, matrix metalloproteinase 2 (MMP2) and MMP9 were downregulated. Phosphorylation of c-Jun N-terminal kinase (JNK), signal transducer and activator of transcription 3 (STAT3) and protein kinase B (AKT) were also inhibited. Overexpression of TARBP2 was suggested to be involved in the metastasis of H1299/M02 cells. Silencing of TARBP2 was able to upregulate levels of APP and ZNF395, in addition to inhibiting metastasis-promoting cyto-

kines, the JNK/STAT3/AKT pathway and COX-2 to attenuate the invasion and migration of cancer cells.

Introduction

Lung cancer is a common malignancy and has the highest rate of cancer-associated mortality worldwide (1). Metastasis is the predominant cause of mortality in patients with cancer and accounts for approximately 90% of cases of cancer associated-mortality (2). In patients with lung cancer, the cancer cells frequently metastasize to the brain, bone, liver, adrenal glands and other organs (3). The clonal growth of cancer cells in these organs considerably impairs organ function and thus is life-threatening. The metastatic cancer cells exhibit biological characteristics different from cancer cells in the primary lesion and tend to have higher rates of malignancy (4). At present, the therapeutic methods used for metastatic cancer including chemotherapy, radiotherapy, targeted therapy, biological therapy and combination therapy remain based on the characteristics of cancer cells in the primary lesion. It is therefore difficult to use these methods to effectively treat metastatic cancers due to the lack of specificity. The prognosis is often much poorer in patients with metastatic cancer than in those with non-metastatic cancer. Therefore, it has important clinical significance to further investigate the key cell regulatory proteins in metastatic cancers.

TAR [human immunodeficiency virus (HIV)-1] RNA binding protein 2 (TARBP2) is a double-stranded RNA-binding protein and serves an important role in the physiological functions of microRNAs. The discovery of this protein is attributable to its ability to bind with TAR, a RNA regulatory element regulating gene expression in the HIV genome, in addition to TAT protein, a trans-activating factor of HIV (5). A previous study identified that TARBP2 had a tendency to bind with guanine-cytosine-rich double-stranded RNAs (6). Taking these binding characteristics into account, the intracellular TARBP2 has been suggested to bind with microRNA hairpin precursors and serve a role in the maturation process. In addition, it has been reported that the Dicer complex can be recruited into Ago2 complexes to implement miRNA-mediated gene

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silencing (7). By comparing the differences in RNA expression profiles between metastatic and non-metastatic breast cancer cells, Goodarzi *et al* (8) demonstrated that TARBP2, as a regulatory factor for mRNA stability, was overexpressed in metastatic breast cancer cells. The results of the current study indicated that TARBP2 was able to bind with the mRNA hairpin structure to mediate the expression of amyloid β (A β) precursor protein (APP), zinc finger protein 395 (ZNF395) and additional metastasis-suppressor proteins, in addition to down-regulating the expression of pro-metastatic interleukin (IL)-1 β , IL-8, cyclooxygenase (COX)-2 and associated proteins. Due to the fact that the roles of TARBP2 in metastatic cancers remain unclear, the present study was designed to investigate the roles of TARBP2 in metastatic non-small cell lung cancer, identifying a basis of the mechanisms of action at a molecular level.

Materials and methods

Reagents and instruments. The human lung cancer cell line NCI-H1299 was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The viral vectors and Lipofectamine 2000 (Thermo Fisher Scientific, Inc. (Waltham, MA, USA) for small hairpin RNA (shRNA)-mediated silencing were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR) kits were purchased from Promega Corporation (Madison, WI, USA). The *in vitro* invasion test kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The monoclonal antibodies were purchased from Abcam (Cambridge, MA, USA). The Enhanced Chemiluminescence (ECL) Immunoblotting Substrate kit was purchased from EMD Millipore (Billerica, MA, USA). The Multiskan FC microplate reader and Arktik Thermal Cycler for PCR were purchased from Thermo Fisher Scientific, Inc. .

Cell culture and induction of the highly metastatic cell line. The cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (Corning Incorporated, Manassas, VA, USA) and were cultured in a 37°C incubator under 5% CO₂ and saturated humidity. The cells were digested with 0.25% trypsin-EDTA (Beyotime Institute of Biotechnology, Shanghai, China) for passaging. Cells in the logarithmic growth phase were used in all experiments. In order to screen out the highly metastatic cell line, H1299 cells were cultured in the upper chamber of Transwell (Corning Incorporated, Corning, NY, USA) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) for 24 h. Subsequently, the cells migrating to the lower chamber were collected for clone culture. The above procedures were repeated 10 times to screen out the highly metastatic cell clones.

shRNAs silences TARBP2. Two different shRNAs, termed sh1 and sh2, were used to achieve different TARBP2 silencing effects. The Sigma-Aldrich clone numbers for sh1 and sh2 were TRCN0000330642 and TRCN000019339, respectively, and they were incorporated into the lentivirus vector (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) using Invitrogen

Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). Subsequent to culture to the logarithmic growth phase, these cells were transfected in accordance with the manufacturer's instructions (Sigma-Aldrich), then the successfully transfected cells were screened out.

MTS cell proliferation assay. Cells in the logarithmic growth phase (5×10^4 cells/ml) were seeded into 96-well microplates (100 μ l/well) and cultured overnight to allow cell adhesion. Subsequent to continued culture for 48 h at 4°C, the medium was removed and MTS was added in accordance with manufacturer's instructions, with continued culture for 4 h at 4°C. Finally, the optical density value was determined to measure the cell counts at a wavelength of 490 nm with the microplate reader.

In vitro invasion and migration test. The CultreCoat® 96-Well BME-Coated Cell Invasion Optimization Assay kit supplied by R&D Systems, Inc. was used to evaluate the *in vitro* invasive ability of cancer cells. Subsequent to starvation in serum-free RPMI-1640 for 16 h, the cells were collected and seeded into the upper chamber of the Transwell system at a density of 2.5×10^4 cells/well. Following continued culture for 48 h, the number of invasive cells was assessed in accordance with the manufacturer's instructions. The *in vitro* migrative ability of cancer cells was evaluated with the wound-healing assay by culturing the cells in 6-well plates until monolayer fusion occurred. Subsequent to starvation in serum-free medium overnight, 200- μ l pipette tips were used to score the cell layer. Subsequent to continued culture for 48 h, the distance between the scorings was observed and measured under an SZX16 stereo microscope (Olympus Corporation, Tokyo, Japan).

Western blotting. Using β -actin (1:5,000; ab8227; Abcam) as an internal control, the expression levels of the following proteins were detected by western blotting with the following antibodies from Abcam: Mouse monoclonal TARBP2 (1:2,000; ab129325), IL-1 β (1:2,000; ab8320) and IL-8 (1:2,000; ab18672); rabbit polyclonal APP (1:2,000; ab59592), ZNF395 (1:2,000; ab75727), phosphorylated c-Jun N-terminal kinase (p-JNK; 1:1,000; ab4821), matrix metalloproteinase 2 (MMP2; 1:2,000; ab37150) and MMP9 (1:2,000; ab38898); goat polyclonal COX-2 (1:2,000; ab35995); mouse monoclonal signal transducer and activator of transcription 3 (STAT3; 1:2,000; ab119352); and rabbit monoclonal JNK (1:1,000; ab76125), p-STAT3 (1:1,000; ab76315), protein kinase B (AKT; 1:2,000; ab32505), and p-AKT (1:1,000; ab81283). The cell lysates were collected by centrifugation at $13,000 \times g$ for 30 min at 4°C prior to extraction of the proteins with a protein extraction kit (Beyotime Institute of Biotechnology) and separation using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnology). The proteins were then transferred onto a polyvinylidene difluoride membrane to detect the target proteins incubating with the antibodies at 4°C overnight. Subsequent to washing with PBS with 5% Tween 80 (Beyotime Institute of Biotechnology), the goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2,500; W4011; Promega Corporation) was added with incubation for 1 h at 37°C. Following several washes, the ECL kit was used to develop the immunoreactive bands.

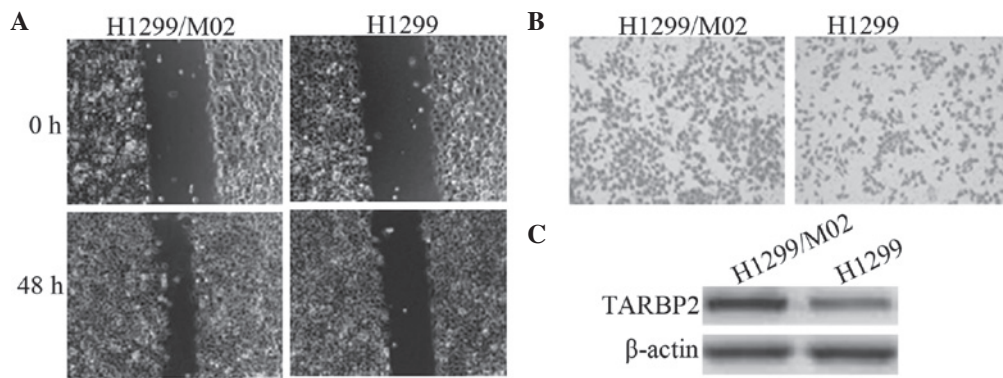


Figure 1. TARBP2 promoted metastasis of lung cancer cell lines. The migration and invasion of H1299/M02 and H1299 cells were analyzed using the (A) wound healing assay and (B) Transwell assay. (C) In addition, the expression levels of TARBP2 were also detected by western blotting with β -actin as the internal control. TARBP2, TAR (human immunodeficiency virus 1) RNA binding protein 2.

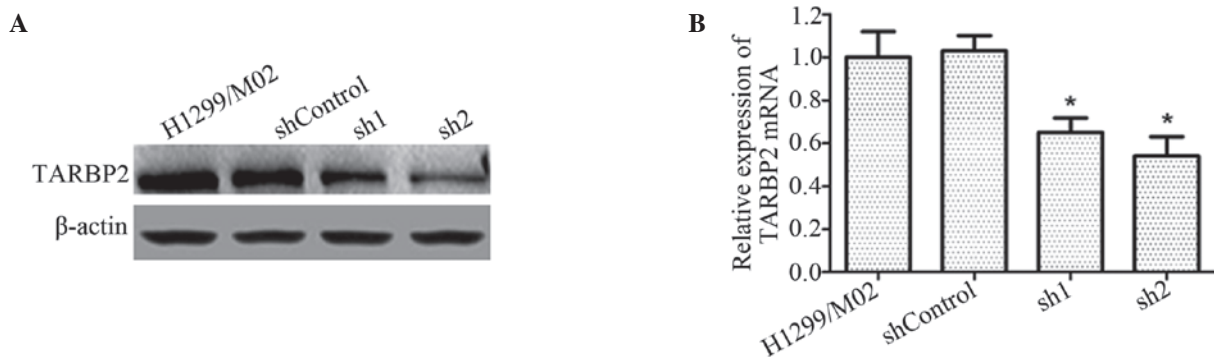


Figure 2. TARBP2 shRNA inhibited TARBP2 protein and mRNA expression in lung cancer cell lines. H1299/M02 cell lines were transfected with TARBP2 shRNA1 (sh1) and shRNA2 (sh2) or the negative control, then TARBP2 protein and mRNA expression levels were measured by (A) western blotting and (B) RT-qPCR, respectively. β -actin was the internal control for western blotting, and glyceraldehyde 3-phosphate dehydrogenase was the internal control for RT-qPCR assay. * $P < 0.05$ vs. H1299/M02. TARBP2, TAR (human immunodeficiency virus 1) RNA binding protein 2; sh, small hairpin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

RT-qPCR. Subsequent to extraction of the total RNA in each group using Invitrogen TRIzol (Thermo Fisher Scientific, Inc.), reverse transcription was conducted using the RT-qPCR kit (Takara Biotechnology, Co., Ltd., Dalian, China) to obtain the cDNA (1 μ l). mRNA expression levels of TARBP2, APP, ZNF395, IL-1 β , IL-8 and COX-2 were then detected. The primer sequences used were as follows: TARBP2, forward 5'-CAGGAGTATGGGACCAGAATAGG-3' and reverse 5'-ACCCGGAAGGTGAAATTAGGC-3'; APP, 5'-AAC CACCGTGGAGCTCCTT-3' and reverse 5'-ATGCCACGG CTGGAGATC-3'; ZNF395, forward 5'-TCATGGCTTTGA GACCGATCC-3' and reverse 5'-CCACAATGGAGCGCA GAACT-3'; IL-1 β , forward 5'-GCACGATGCACCTGTACG AT-3' and reverse 5'-CACCAAGCTTTTTTGCTGTGAGT-3'; IL-8, forward 5'-CAAGAGCCAGGAAGAAACCA-3' and reverse 5'-GTCCACTCTCAATCACTCTCAG-3'; COX-2, forward 5'-CAGCCATACAGCAAATCCT-3' and reverse 5'-TCT CCATAGAATCCTGTCCG-3'; MMP2, forward 5'-TGATCT TGACAGAATACCATCGA-3' and reverse 5'-GGCTTG CGAGGGAAGAAGTT-3'; MMP9, forward 5'-GTGCTGGGC TGCTGCTTTGCTG-3' and reverse 5'-GTCGCCCTCAA GGTGTTGGAAT-3'; GAPDH, forward 5'-CTTAGATTGGT CGTATTGG-3' and reverse 5'-GAAGATGGTGATGGGATT -3'. PCR was conducted using an ABI7500 Real-Time PCR

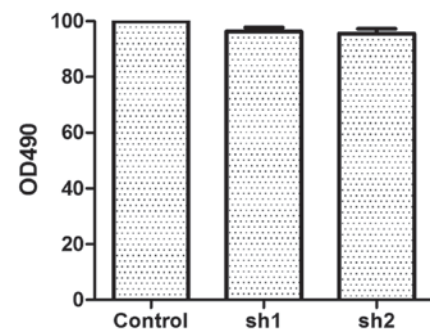


Figure 3. TARBP2 silencing does not significantly affect cell growth in the lung cancer cell lines. H1299/M02 cell lines were transfected with TARBP2 shRNA1 (sh1) and shRNA2 (sh2) or negative control, then cell proliferation was analyzed using the MTT assay. TARBP2, TAR (human immunodeficiency virus 1) RNA binding protein 2; sh, small hairpin; OD, optical density.

system (Thermo Fisher Scientific, Inc.) with 2 μ g cDNA and the following cycling conditions: Annealing at 54°C for 60 sec, and 40 cycles of 95°C for 2 min, 94°C for 10 sec, 68°C for 1 min. The $2^{-\Delta\Delta C_q}$ method was used for quantification (9).

Statistical analysis. The experimental data were presented as the mean \pm standard deviation and were analyzed using SPSS

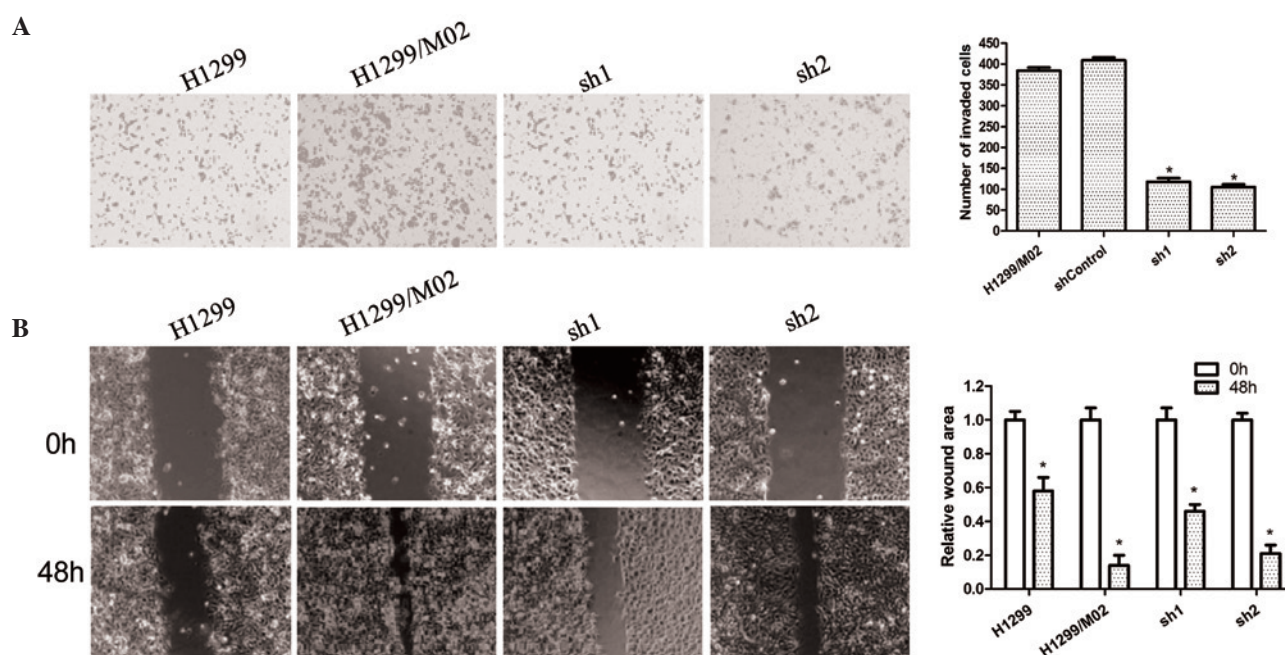


Figure 4. TARBP2 silencing inhibits the *in vitro* invasion and migration of lung cancer cell lines. H1299/M02 cell lines were transfected with TARBP2 shRNA1 (sh1) and shRNA2 (sh2) or negative control, then the migration and invasion of H1299/M02 and H1299 cells was analyzed using (A) a Transwell assay (* $P < 0.05$ vs. H1299/M02 group) and (B) a wound healing assay (* $P < 0.05$ vs. 0 h group). TARBP2, TAR (human immunodeficiency virus 1) RNA binding protein 2; sh, small hairpin.

software, version 13.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used for comparison and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

H1299/M02 is highly metastatic and overexpresses TARBP2. Subsequent to *in vitro* screening, highly metastatic cell clones were successfully obtained. One of the clones was named as H1299/M02, and was identified by the *in vitro* invasion assay and the wound-healing assay to exhibit greater invasive ability than that of the parent cell line H1299. In addition, western blot analysis indicated that this clone overexpressed TARBP2 (Fig. 1).

sh1 and sh2 significantly inhibit TARBP2 expression. The shRNA-transfected cell clones were successfully obtained. Western blotting and RT-qPCR analysis indicated that sh1 and sh2 silencing resulted in a significant reduction in the expression levels of TARBP2 in H1299/M02 cells, to ~45% and 23% of the shControl group level in protein levels, respectively. No significant differences were observed between the shControl and H1299/M02 groups (Fig. 2).

TARBP2 silencing does not significantly affect cell growth. As indicated by the results of the MTS cell proliferation assay, although sh1 and sh2 downregulated TARBP2 expression in H1299/M02 cells, the cell growth was not affected significantly (Fig. 3).

TARBP2 silencing inhibits in vitro invasion and migration. As demonstrated by culture in Transwell for 24 h, invasive cells were clearly observed in the H1299/M02 group when

compared with the H1299 group; whereas the number of invasive cells was significantly reduced in the sh1 and sh2 groups when compared with the H1299/M02 group. The wound-healing assay also indicated that 48 h subsequent to scoring, no significant alterations in the wound widths were observed in the H1299 group. Wound width was the smallest in the H1299/M02 group while the widths in the sh1 and sh2 groups were significantly reduced compared with that of 0 h (Fig. 4). These assay results indicated that silencing of TARBP2 was able to inhibit the *in vitro* invasion and migration of cancer cells, which was associated with the extent of TARBP2 silencing.

TARBP2 silencing upregulates the expression of APP and ZNF395, and downregulates the expression levels of IL-1 β , IL-8, COX-2, MMP2 and MMP9 in H1299/M02 cells. Western blot analysis results indicated that APP and ZNF395 protein expression was significantly reduced in the H1299/M02 cells when compared with that of the H1299 cells. The APP and ZNF395 expression levels were then significantly increased when TARBP2 was silenced by sh1 and sh2. In contrast, IL-1 β , IL-8, COX-2, MMP2 and MMP9 expression levels were significantly increased in H1299/M02 cells, whereas expression of these proteins was significantly reduced in cells in the sh1 and sh2 groups. RT-qPCR results indicated that the regulation of expression of these proteins occurred at the transcriptional level (Fig. 5).

TARBP2 inhibits the phosphorylation of JNK, STAT3 and AKT. Western blot analysis indicated that JNK and STAT3 expression levels were not significantly affected by TARBP2 silencing. As presented in Fig. 6, the phosphorylation of JNK, STAT3 and AKT was reduced when TARBP2 was silenced in

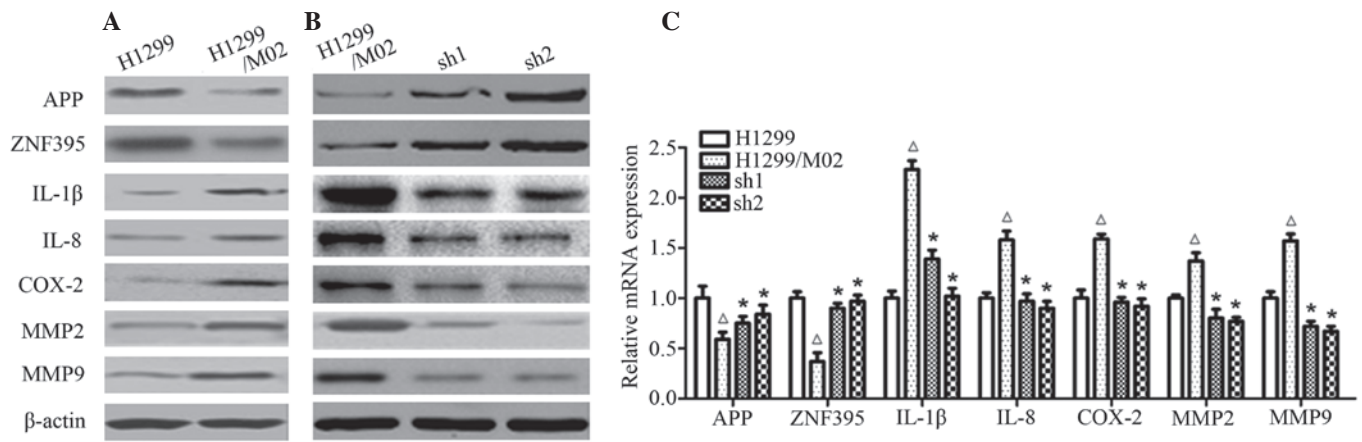


Figure 5. The effect of TARBP2 silencing on the expression of tumor-associated genes in lung cancer cell lines. (A) Immunoblotting for the proteins associated with migration and invasion in H1299/M02 and H1299 cell lines. Cell lysates were prepared and subjected to enhanced chemiluminescence-western blotting using β -actin as the internal control. Results are representative of three independent experiments. (B) H1299/M02 cell lines were transfected with TARBP2 shRNA1 (sh1) and shRNA2 (sh2) or negative control, then the proteins associated with migration and invasion were analyzed by immunoblotting, with β -actin as the internal control. (C) mRNA expression levels of the assessed genes were analyzed by RT-qPCR assay. Glyceraldehyde 3-phosphate dehydrogenase was used as the internal control for RT-qPCR. $^{\Delta}P < 0.05$ vs. H1299 group; $^*P < 0.05$ vs. H1299/M02 group. TARBP2, TAR (human immunodeficiency virus 1) RNA binding protein 2; sh, small hairpin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; APP, amyloid β (A4) precursor protein; ZNF395, zinc finger protein 395; IL, interleukin; COX-2, cyclooxygenase 2; MMP, matrix metalloproteinase.

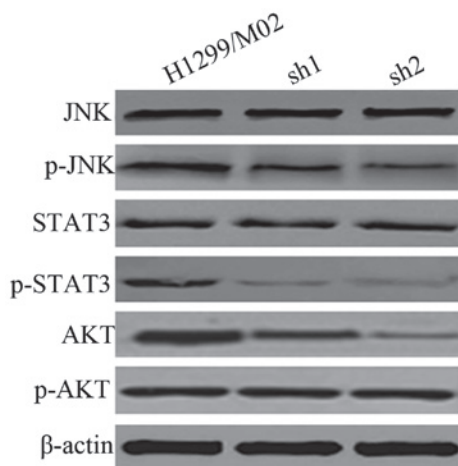


Figure 6. TARBP2 inhibits the phosphorylation of JNK, STAT3 and AKT in lung cancer cell lines. H1299/M02 cell lines were transfected with TARBP2 shRNA1 (sh1) and shRNA2 (sh2) or negative control, then the levels of total and phosphorylated proteins of JNK, STAT3 and AKT were analyzed by immunoblotting. β -actin was used as the internal control. TARBP2, TAR (human immunodeficiency virus 1) RNA binding protein 2; JNK, c-Jun N-terminal kinase; STAT3, signal transducer and activator of transcription 3; AKT, protein kinase B; sh, small hairpin; p-, phosphorylated.

highly metastatic cancer cells, indicating that activity of the JNK/STAT3/AKT signaling pathway was inhibited.

Discussion

In the current study, the highly metastatic and TARBP2-overexpressing non-small cell lung cancer cell line H1299/M02 was obtained, and TARBP2-silencing cell clones were obtained using the shRNA transfection technique. Results of the Transwell assay and wound-healing assay indicated significant reductions of cell invasive and migratory abilities subsequent to TARBP2 silencing. By contrast, the MTS

assay indicated that silencing TARBP2 had no significant effect on cell growth, suggesting that TARBP2-mediated cell invasion and migration was not correlated with cell growth. Accordingly, the mechanisms by which TARBP2 inhibited H1299/M02 cell metastasis were further investigated.

APP is first a membrane protein and later forms a soluble product subsequent to digestion by proteases, upon which it serves a key role in Alzheimer's disease (10). Previous studies have identified that APP additionally served an important role in cancer. It was reported that TARBP2 overexpression in metastatic breast cancer resulted in downregulation of APP expression, resulting in a reduction of its ability to suppress cancer cell metastasis (11). Fan *et al* (12) identified in ovarian cancer that miR-20a was able to target and downregulate APP expression to promote cancer cell proliferation and invasion. However, additional studies on prostate cancer (13,14) suggested that APP exerted the opposite effect, promoting prostate cancer cell growth and invasion, suggesting that the role of APP in cancer may be associated with specific disease and cell types. In the current study, it was observed that APP expression was upregulated with the silencing of TARBP2 and the suppression of H1299/M02 metastasis, indicating that APP may serve a tumor suppressor role in these cancer cells. ZNF395 is associated with Huntington's disease and is involved in Huntington gene expression (15). Similar to that of APP, previous studies have indicated that ZNF395 is involved in the pathological progression of cancer. The report showed that TARBP2 over-expression in metastatic breast cancer was able to downregulate ZNF385 expression, suggesting a tumor suppressor role of TARBP2 (9). Pang *et al* (16) identified that miR-525-3p promoted the metastasis and invasion of liver cancer cells by downregulating ZNF395 expression. In the current study, downregulation of ZNF395 expression in highly metastatic H1299/M03 cells and upregulation of ZNF395 expression subsequent to TARBP2 silencing-mediated suppression of H1299/M03 metastasis was observed. This suggested that ZNF395 additionally exerted inhibitory effects on metastasis in these cells. It was demon-

strated that ZNF395 was able to inhibit the expression of IL-1 β , IL-8 and COX-2, which was observed in the H1299/M02 cells in the present study. Previous studies have indicated that IL-1 β and IL-8 serve important roles in lung cancer metastasis (17-21). MMPs are important in the promotion of angiogenesis, tumor invasion and tumor metastasis. MMP2 and MMP9, which can degrade collagen IV, the major extracellular membrane component of the basement membrane, have been suggested to be critical for the invasive and metastatic potential in lung carcinoma, activating JNK/STAT3/AKT and additional signaling pathways via autocrine or paracrine mechanisms (22-24). It has been previously observed that MMP2 and MMP9 were able to promote the growth and metastasis of tumor cells, in addition to microenvironment remodeling and angiogenesis (25,26). AKT, as an important downstream effector of STAT3, serves a key role in tumor-associated signaling pathways, in order to regulate the expression and activity of tumor-associated genes and transcription factors, respectively. In the current study, ZNF395 was observed to inhibit the activity of the JNK/STAT3/AKT signaling pathway.

In conclusion, the present study demonstrated that TARBP2 serves a role in the highly metastatic H1299/M02 cell line; silencing TARBP2 can inhibit the *in vitro* invasion and migration of H1299/M02 cells; and its mechanisms of action may be associated with the regulation of APP, ZNF395 and COX-2 expression and the JNK/STAT3/AKT signaling pathway.

Acknowledgements

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