

Epididymal protein 3A is upregulated and promotes cell proliferation in non-small cell lung cancer

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Received September 10, 2019; Accepted February 11, 2020

DOI: 10.3892/ol.2020.11517

Abstract. Lung cancer is one of the most common cancer types and a major contributor to cancer-associated mortalities worldwide. The aim of the present study was to investigate the function of the epididymal protein 3A (EDDM3A) in non-small cell lung cancer (NSCLC). Data from patients with NSCLC were retrieved from The Cancer Genome Atlas and analyzed, and the differences in EDDM3A expression level between 30 NSCLC tissues and matched adjacent non-tumor tissues (>5 cm) were assessed via tissue microarray analysis. It was revealed that, compared with adjacent non-tumor tissues, EDDM3A expression was significantly increased in NSCLC tissues ($P=4.19 \times 10^{-2}$). To knock down EDDM3A expression in a human NSCLC cell line, lentivirus-mediated short hairpin RNAs (shRNAs) were used, and the knockdown efficiency was assessed via reverse transcription-quantitative PCR and western blotting. Moreover, cell proliferation was evaluated with an MTT assay and Celigo imaging cytometry. In addition, cell apoptosis was detected by Annexin V staining. It was demonstrated that knockdown of EDDM3A inhibited the proliferation of A549 cells. Furthermore, compared with the control group, the apoptotic rate of the EDDM3A-shRNA group was significantly higher. Collectively, the present results indicate the potential role of EDDM3A in NSCLC and suggest that EDDM3A may represent a potent therapeutic target for treating patients with NSCLC.

Introduction

As a leading cause of cancer-associated mortalities globally (1), lung cancer is an important and highly investigated area in cancer research. Moreover, non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases (2,3). Despite improvements in diagnosis and standard therapy, the 5-year survival rate of NSCLC patients is only 18% (4). A possible reason for this low survival rate is the uncontrolled proliferation and metastatic potential of NSCLC cells (5). While numerous molecular genetic studies have been conducted in NSCLC, the precise molecular mechanisms underlying NSCLC progression remain unknown (6). Thus, the aim of the present study was to identify valuable biomarkers for NSCLC.

Epididymal protein 3A (EDDM3A) is located on human chromosome (chr) 14q11.2 and there is only one transcript (NC_000014.9) for EDDM3A (7). Damyanova *et al* (8) reported that the loss of chr14q11.2 affects proteins that are synthesized and secreted by epididymal epithelial cells, which are upregulated in the epididymis of male patients with non-obstructive azoospermia. Despite these previous findings, there is an overall lack of evaluation of the clinicopathological significance of EDDM3A in NSCLC, and the molecular mechanisms underlying its role are not fully understood.

In the present study, it was demonstrated that EDDM3A expression is significantly upregulated in NSCLC by using human NSCLC tissues and data from The Cancer Genome Atlas (TCGA). Moreover, the aim of the present study was to identify whether EDDM3A was significantly associated with cell proliferation, cell cycle progression and apoptosis in the A549 lung cancer cell line. It was indicated that EDDM3A is an oncogene in NSCLC, which may represent a novel diagnostic and therapeutic target for the treatment of NSCLC. Therefore, the present study may have identified a potential new therapeutic and prognostic target for NSCLC.

Materials and methods

Gene expression datasets. TCGA datasets TCGA_LUNG_exp_HiSeq_V2-2015-02-24, TCGA_LUAD_exp_HiSeqV2-2015-02-24 and TCGA_LUSC_exp_HiSeqV2-2015-02-24 were downloaded from TCGA (cancergenome.nih.gov/) database and these contained 51 pairs of NSCLC tissues and matched non-tumor tissues (9).

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Abbreviations: EDDM3A, epididymal protein 3A; NSCLC, non-small cell lung cancer; TCGA, The Cancer Genome Atlas; shRNA short hairpin RNA; IHC, immunohistochemistry; RT-qPCR, reverse transcription-quantitative PCR

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Key words: EDDM3A, NSCLC, shRNA, proliferation, apoptosis

Patients and tissues. A total of 30 patients (46.6% females and 53.4% males; mean age 59.8 and age range 46-82) diagnosed with NSCLC at The First Affiliated Hospital of Soochow University (Suzhou, China) were enrolled between July 2018 and June 2019. The paraffin-embedded slides, including 30 pairs of NSCLC tissues and adjacent healthy lung tissues, were obtained from the Department of Pathology, The First Affiliated Hospital of Soochow University. The current study was approved by The Institutional Ethical Committee of The First Affiliated Hospital of Soochow University (approval no. 2018011), and a signed informed consent form was obtained from each participant prior to the study.

Immunohistochemistry (IHC). 5- μ m continuous slides were incubated with anti-EDDM3A antibody produced in rabbit (1:25; Sigma-Aldrich; Merck KGaA) at 4°C overnight, followed by incubation for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:500; cat. no. ab7090; Abcam) at room temperature, then 3,3'-diaminobenzidine staining solution (1:25, cat. no. 070004-D; Beijing CellChip Biotechnology Co., Ltd.) for 10 min at room temperature. Slides were blocked using 10% goat serum (Thermo Fisher Scientific, Inc.) for 10 min at room temperature, and then treated according to the manufacturer's instructions for the Rabbit/Rabbit Streptomyces vitellogenin-Biotin Detection system (OriGene Technologies, Inc.). Then, the slides were mounted and imaged with an optical microscope at x400 magnification.

Cell lines and culture conditions. The human NSCLC cell line A549 was obtained from the American Type Culture Collection and was assessed by short tandem repeat analysis. All cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) and 100 IU/ml penicillin/streptomycin and cultured at 37°C in a humidified incubator containing 5% CO₂.

Reverse transcription-quantitative PCR (RT-qPCR). Analysis of relative gene expression was conducted using RT-qPCR. RNeasy Mini Kit was utilized (Qiagen, USA) to isolate RNA according to the manufacturer's protocol and the concentration of RNA was identified by spectrophotometer Nanodrop 2000 (Thermo Fisher Scientific, USA). 1 μ g RNA was reverse transcribed to cDNA using PrimeScript™ RT Reagent Kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. EDDM3A and GAPDH primer sequences were as follows: EDDM3A forward, 5'-CATTGTGGCGTAGATGGATA-3' and reverse, 5'-ATAAATGTAAGCGGGGAGTG-3'; and GAPDH forward, 5'-TGACTTCAACAGCGACACCCA-3' and reverse, 5'-CACCTGTTGCTGTAGCCAAA-3'. The C_q values were normalized to that of β -actin, which was used as a reference to estimate the different gene expression levels. The expression of EDDM3A was calculated by the 2^{- $\Delta\Delta$ C_q} method (10). To ensure quantitative accuracy, each sample was assayed three times.

Lentiviral short hairpin (sh)RNA vector construction. C-terminal HA-tagged full-length cDNA encoding human EDDM3A (ID: 16978) and the deletion plasmids (UBA

and CULLIN) were amplified by PCR using Phusion DNA Polymerases (Thermo Fisher Scientific, Inc.). The thermocycling were as follows: Initiation at 94°C for 30 sec denaturation at 95°C for 6 min, annealing at 60°C for 30 sec and elongation at 73°C for 1.5 min for 40 cycles. The sequences of forward and reference primers: Forward, 5'-TGTGGCGTAGATGG-3' and reverse, 5'-ATGTAAGCGGGGAG-3'. Then the amplified cDNA was cloned into the lentiviral expression vector pGVX115-GFP (Shanghai GeneChem Co., Ltd.). Primers for PCR were designed to include BamHI and XhoI restriction sites. Overall, 10 μ g pGV115-shControl (Ctrl)/pGV115-shEDDM3A were transfected into 293T cells (Thermo Fisher Scientific, Inc.) with the pHelper system (Shanghai GeneChem Co., Ltd.) to produce lentiviral particles. Lentiviruses were harvested after 48 h and filtered using a 0.45 mm filter.

Western blotting. Western blotting was performed to measure the protein expression in transfected A549 cells. Cells were lysed with RIPA buffer (Boster Biological Technology Co., Ltd.) and the total protein concentration was measured with a bicinchoninic acid kit (Abbkine Scientific Co., Ltd.). GAPDH was used as the internal reference. Overall, 5 μ g proteins per lane were loaded on a 10% SDS-PAGE and then transferred to PVDF membranes. Subsequently, the PVDF membranes were blocked with 5% BSA (Abbkine Scientific Co., Ltd.) for 2 h at room temperature. The primary antibodies, including anti-EDDM3A (1:500; cat. no. ab151083; Abcam) and anti-GAPDH (1:500; cat. no. ab8245; Abcam), were incubated for 1 h at room temperature. The blots were then incubated with the secondary goat anti-rabbit antibody (HRP goat anti-mouse IgG H&L, 1:1,000, cat. no. ab6708, Abcam; or goat anti-rabbit IgG; 1:1,500, cat. no. bs-0295G, BIOSS) for 1 h at room temperature and developed with an ECL reagent (Abcam). Densitometry was performed using a bio-imaging system (DNR Bio-Imaging Systems, Ltd.). Experiments were performed in triplicate.

Cell proliferation. Transfected A549 cells reached 80% confluency, the fluorescence of these cells was detected and the number of cells was automatically calculated using the Celigo Imaging Cytometry system version 2.0 (Nexcelom Bioscience LLC). The cell viability was monitored using a Cell Proliferation kit I (MTT) (Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions and 0.25% trypsin was used to dissolve the purple formazan. Cells were seeded at a density of 3x10³ cells/well and cultured for 5 days. Subsequently, the optical density (OD) was measured by microplate reader under 450 nm. For each experimental condition, 5 parallel wells were assigned to each group. The experiments were performed in triplicate.

Colony formation assays. A549 cells transfected with shEDDM3A or shCtrl were plated in 6-well plates at a density of 1,200 cells/well in culture medium 24 h after transfection, and the medium was changed every 3 days. After 2 weeks, cells were fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet for 15 min at (both at room temperature). Colonies with >50 cells per colony were counted under the light microscope at x40 magnification manually and each experiment was repeated three times.

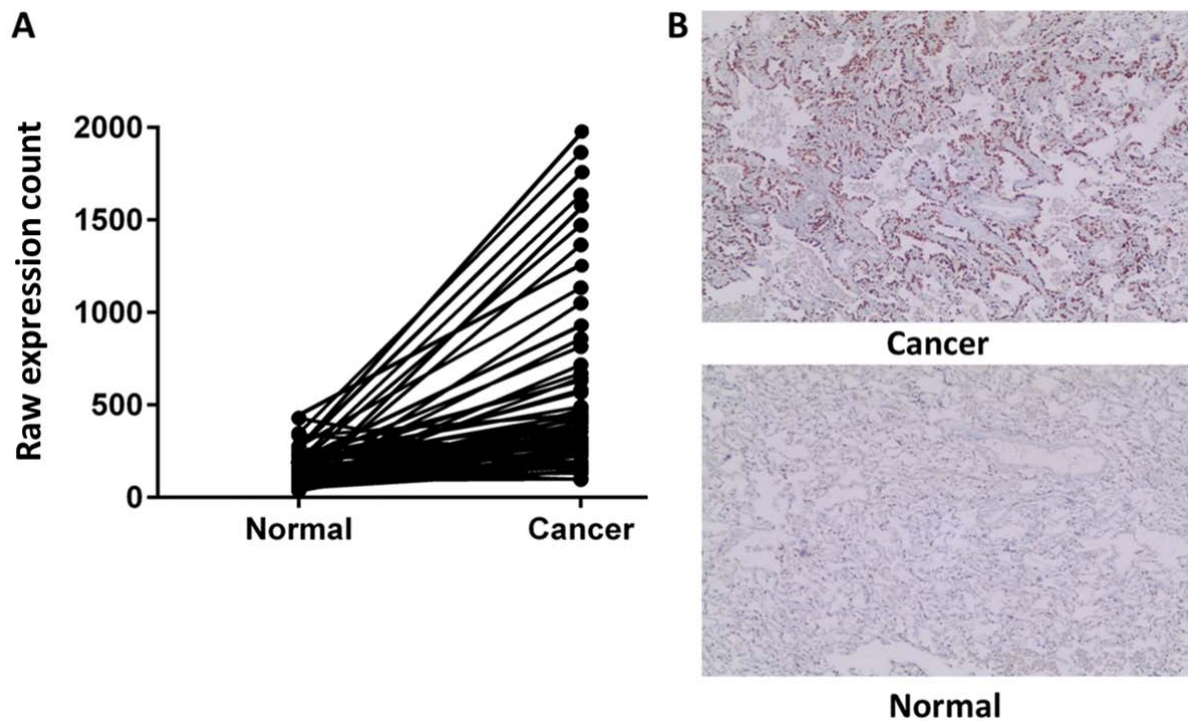


Figure 1. EDDM3A is upregulated in tumor tissues compared with non-tumor tissues in NSCLC. (A) Expression of EDDM3A in NSCLC compared with adjacent healthy tissues based on TCGA dataset. (B) Expression of EDDM3A in NSCLC tissues compared with adjacent healthy tissues based on immunohistochemistry (Olympus IX71) at magnification, x40. Brown represents EDDM3A expression and blue represents cell nucleus. NSCLC, non-small cell lung cancer; EDDM3A, Epididymal Protein 3A.

Apoptosis assay. A total of 1×10^5 cells/well were incubated in 6-well plates for 24 h and treated with shEDDM3A and shCtrl for 48 h. Then cells were collected using centrifuge at $1,000 \times g$. After washing the cells with PBS, an Annexin V-APC Apoptosis Detection kit (eBioscience; Thermo Fisher Scientific, Inc.) was used to assess apoptosis using a flow cytometer (FACScalibur; BD Biosciences) following the manufacturer's instructions and the percentage of apoptotic cells was determined using ModFit LT version 5.0 software (Verity Software House, Inc.).

Statistical analysis. SPSS software (version 19.0; IBM Corp.) was used to analyze data for statistical significance. Data are presented as the mean \pm SEM from one representative experiment out of three independent experiments, and every representative experiment had three replicates. Unpaired two-tailed Student's t-test or one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test were performed to assess differences among the variables. $P < 0.05$ was considered to indicate a statistically significant difference. GraphPad Prism 5 (GraphPad Software) was used for image editing.

Results

EDDM3A expression is significantly increased in NSCLC tissues. To investigate whether EDDM3A expression was altered in NSCLC tissues, the mRNA expression profiles of NSCLC tissues and healthy tissues were retrieved from TCGA database. It was demonstrated that EDDM3A expression

was significantly increased ($P = 4.19 \times 10^{-2}$) in NSCLC tissues compared with healthy tissues (Fig. 1A). Furthermore, the expression of EDDM3A was detected in 30 pairs of NSCLC tissues and adjacent healthy lung tissues via IHC. The results indicated that expression of EDDM3A was increased in NSCLC tissues compared with healthy lung tissues (Fig. 1B), which was consistent with the results from TCGA database.

Knockdown efficiency of EDDM3A via shRNA infection in A549 cells. To investigate the role of EDDM3A in A549 cells, lentiviruses expressing shEDDM3A or shCtrl were used to infect A549 cells. The transfection efficiency was evaluated using RT-qPCR, and it was revealed that EDDM3A mRNA expression was significantly reduced by 80% in the shEDDM3A group (Fig. 2A). Moreover, compared with the shCtrl group, EDDM3A protein expression was knocked down in the shEDDM3A lentivirus group of A549 cells (Fig. 2B).

Knockdown of EDDM3A inhibits the proliferation of A549 cells. The present study examined the role of EDDM3A in the proliferation and colony formation of A549 cells after infection with shEDDM3A or shCtrl in a 5-day study. It was identified that the proliferation of shEDDM3A-infected cells was inhibited compared with the shCtrl-infected cells, as demonstrated by the Celigo cell imaging system (Fig. 3).

MTT assay results indicated that EDDM3A knockdown inhibited the proliferative rate of A549 cells (Fig. 4). Moreover, a colony formation assay was performed to evaluate the role of EDDM3A in long-term survival, and

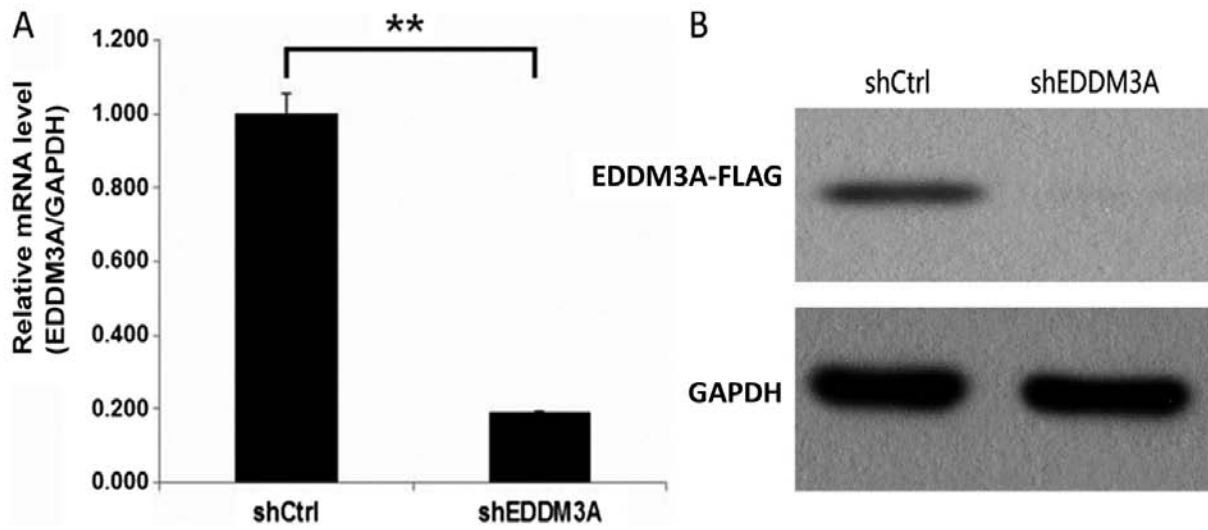


Figure 2. EDDM3A expression levels after shCtrl or shEDDM3A transfection in the A549 cell line. (A) EDDM3A mRNA expression in A549 cells was detected by reverse transcription-quantitative PCR analysis. (B) EDDM3A protein expression in A549 cells was detected by western blot analysis. ** $P < 0.01$. EDDM3A, Epididymal Protein 3A; shRNA, short hairpin RNA; Ctrl, control.

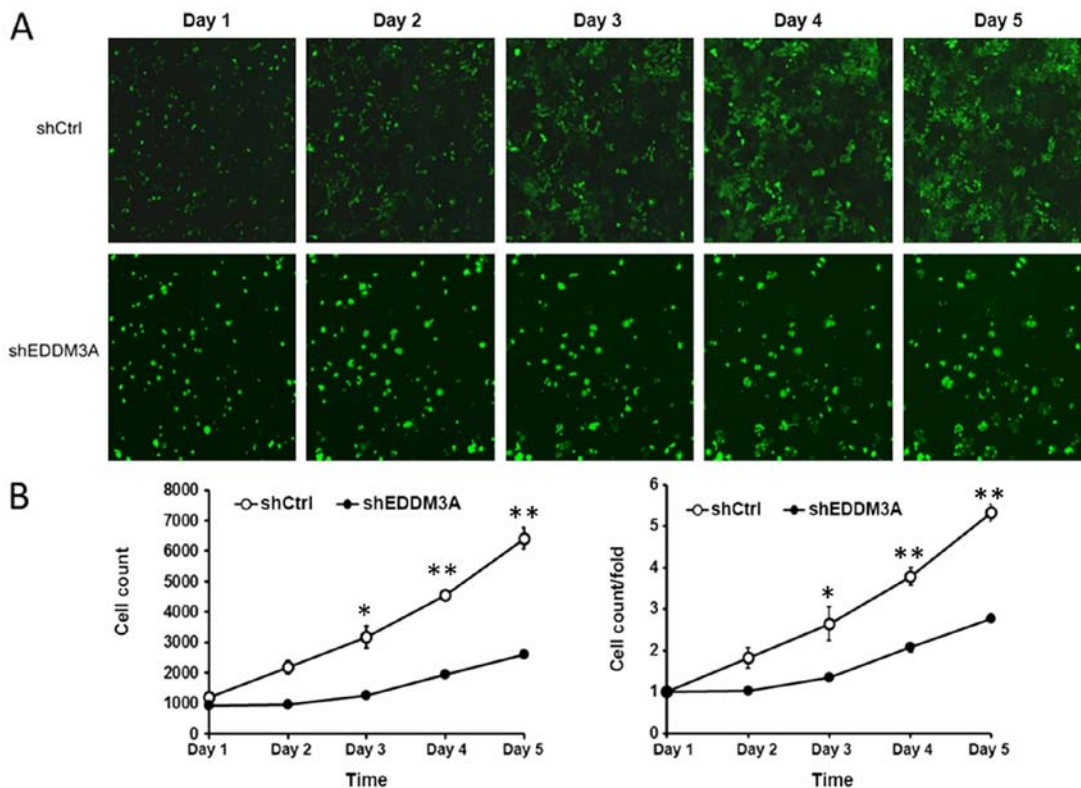


Figure 3. A549 cell proliferation after shCtrl or shEDDM3A transfection using the Celigo Imaging Cytometry system. (A) A549 cell proliferation after shCtrl or shEDDM3A infection from days 1-5 at magnification, x400 using a fluorescence microscope. (B) A549 cell proliferation curves were depicted according to the Celigo calculation. * $P < 0.05$, ** $P < 0.01$. EDDM3A, Epididymal Protein 3A; shRNA, short hairpin RNA; Ctrl, control.

it was found that knockdown of EDDM3A significantly attenuated the colony-forming ability of the cellular population (Fig. 5).

Knockdown of EDDM3A accelerates A549 cell apoptosis.

The effect of EDDM3A knockdown on A549 cell apoptosis was investigated by FACS, which identified that the apoptotic rate of the shEDDM3A group was elevated compared with the

shCtrl group ($P < 0.01$; Fig. 6). Therefore, it was suggested that shEDDM3A promoted A549 cell apoptosis.

Discussion

Lung cancer is the leading cause of cancer-associated mortality and has led advances in cancer therapy worldwide (11,12). The development of NSCLC results from both environmental and

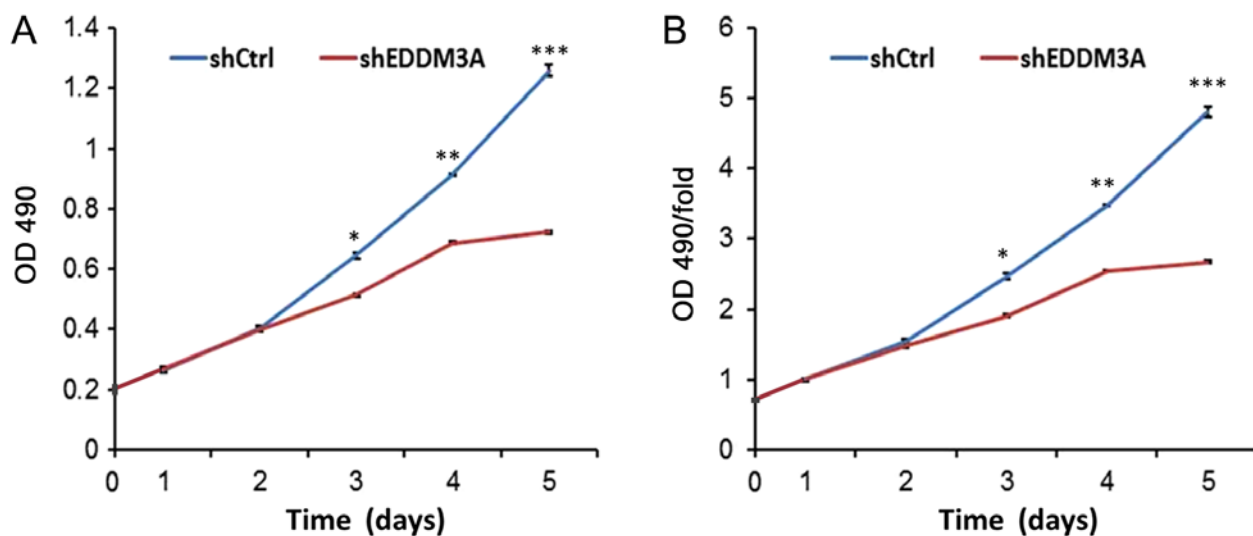


Figure 4. A549 cell proliferation after shCtrl or shEDDM3A transfection by MTT assay. (A) A549 cell proliferation curves were depicted according to OD490 value as measured via the MTT assay. (B) A549 cell proliferation curves were depicted according to OD490 fold. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. OD, optical density; EDDM3A, Epididymal Protein 3A; shRNA, short hairpin RNA; Ctrl, control.

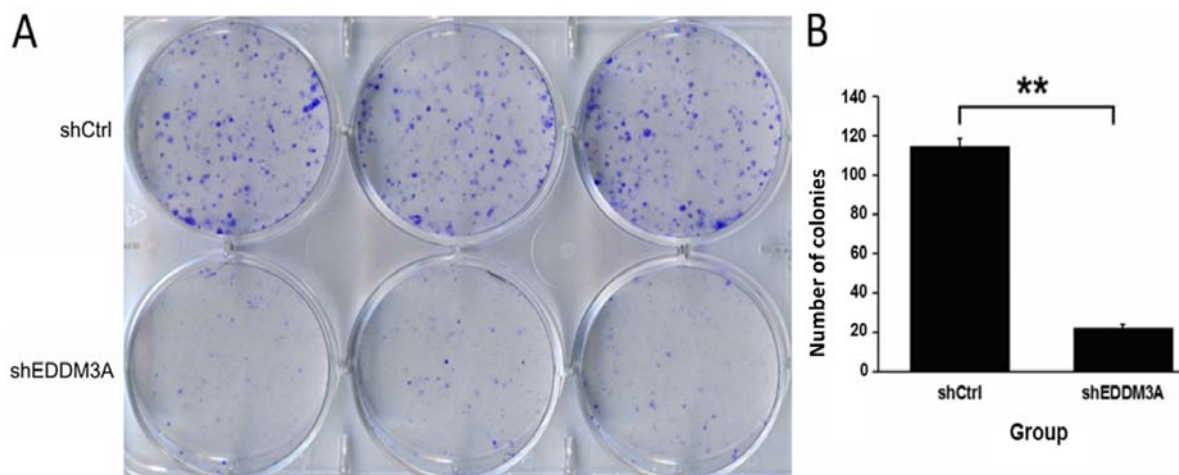


Figure 5. Efficiency of A549 colony formation following shCtrl or shEDDM3A transfection after 11 days. (A) Cell colony numbers of A549 cells in the shCtrl and shEDDM3A groups. (B) Analysis of cell colony number of A549 cells in the shCtrl and shEDDM3A groups. ** $P < 0.01$. EDDM3A, Epididymal Protein 3A; shRNA, short hairpin RNA; Ctrl, control.

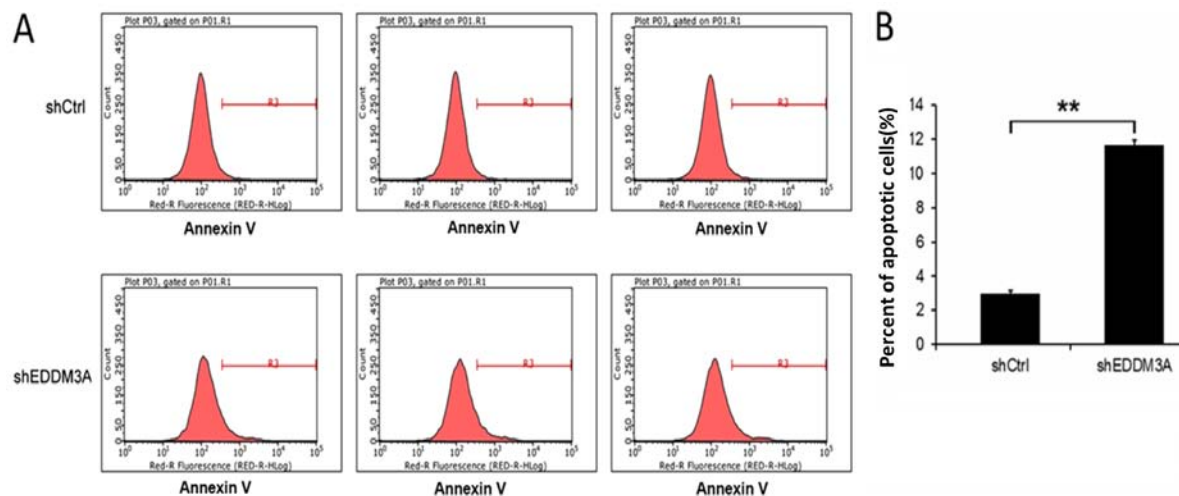


Figure 6. A549 cell apoptosis following shEDDM3A or shCtrl infection. (A) A549 cell apoptosis analysis by flow cytometry after shCtrl or shEDDM3A infection. (B) Percentage of cell apoptosis in A549 cells. ** $P < 0.01$. EDDM3A, Epididymal Protein 3A; shRNA, short hairpin RNA; Ctrl, control.

genetic changes (13), and the activation of oncogenes influences the proliferation, adhesion, motility and invasiveness of cancer cells and reconstructs the contact of tumor cells with the surrounding extracellular matrix, thus promoting epithelial-mesenchymal transition (14,15).

EDDM3A is a protein-coding gene located at chr14q11.2 and it has been shown that loci on chr14q11.2 are associated with a risk of childhood acute lymphoblastic leukemia (16). Moreover, double homeobox A pseudogene 10, located in chr14q11.2, promotes an aggressive phenotype by binding lysine-specific histone demethylase 1 and repressing large tumor suppressor kinase 2 and Ras-related glycolysis inhibitor and calcium channel regulator expression levels in NSCLC (17). Furthermore, previous studies have revealed that chr14q11.2 may participate in cancer development (18-20).

The present study examined the function of EDDM3A in NSCLC. EDDM3A expression levels in NSCLC tissues compared with healthy tumor tissues were assessed using TCGA database, and it was identified that EDDM3A was upregulated in NSCLC tissues. Next, lentiviruses harboring EDDM3A shRNA were constructed and transfected into A549 cells. The present results demonstrated that cell proliferation was inhibited in EDDM3A-silenced A549 cells, as demonstrated by the Celigo imaging cytometry system and MTT assay. Moreover, the apoptotic rate of the EDDM3A-shRNA group was significantly higher compared with the control group. Therefore, it was speculated that EDDM3A may regulate NSCLC cell proliferation and apoptosis *in vitro*.

However, certain limitations of the present study must be considered. For example, only the A549 cell line was used in the present study; therefore, in the future the present results should be assessed in other lung cancer cell lines, such as NCI-H1299, NCI-H460 and 95D, and healthy cell lines. Future studies will also perform additional downstream experiments to reveal the underlying molecular mechanism behind the observed effects.

In conclusion, the present study identified the functional role of EDDM3A in lung cancer progression. It was revealed that EDDM3A knockdown may promote cell apoptosis and inhibit cell proliferation and colony-forming abilities in the human NSCLC A549 cell line. Thus, it was speculated that EDDM3A expression in lung cancer may be a valuable biomarker for assessing aggressive features and poor prognosis. Moreover, the present results suggested that EDDM3A may represent a potential therapeutic target in NSCLC.

Acknowledgements

Not applicable.

Funding

The current study was supported by The National Natural Science Foundation of China (grant no. 81800279), the Natural Science Foundation of Jiangsu Province (grant no. BK20180197) and the Science and Education Youth Project of Suzhou (grant no. kxw2018002).

Authors' contributions

HM and YF designed the experiments. GL and XT performed the experiments and wrote the manuscript. LP and HH were responsible for data analysis. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Approval was obtained to review the data from The Institutional Ethical Committee of The First Affiliated Hospital of Soochow University (approval no. 2018011). Written informed consent was provided by each participant prior to the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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