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MiR-486-5p specifically suppresses SAPCD2 expression, which attenuates the aggressive phenotypes of lung adenocarcinoma cells

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Summary. Background. MiR-486-5p expression is restrained in lung adenocarcinoma (LUAD). However, much less has been understood on its role in LUAD. We aimed to explore the biofunctions of miR-486-5p in LUAD.

Methods. A differential expression analysis based on The Cancer Genome Atlas-LUAD dataset was done to screen the differently expressed miRNAs and mRNAs. MiR-486-5p and SAPCD2 mRNA expression was analyzed by qRT-PCR, and protein level of SAPCD2 was assayed by western blot. Upregulation and downregulation of miR-486-5p or SAPCD2 were achieved by cell transfection. For cell function assays, the proliferation of cancer cells was examined by MTT assay. Cell apoptosis was assessed by flow cytometry and microscopy. Transwell assay was applied to evaluate cell migration and invasion. A dual-luciferase detection was employed to determine the miRNA-mRNA targeting relationship.

Results. MiR-486-5p expression was notably reduced in LUAD tissue and cell lines. Upregulating miR-486-5p restrained the anti-apoptotic and proliferative abilities, as well as cell migratory and invasive phenotypes in LUAD cells. SAPCD2 was determined as one target of miR-486-5p. Also, SAPCD2 forced expression was able to attenuate the inhibitory impacts of miR-486-5p on the malignant phenotypes of LUAD cells.

Conclusion. MiR-486-5p suppressed cell malignant progression in LUAD by targeting SAPCD2, suggesting that the two may be targets for LUAD treatment.

Key words: Lung adenocarcinoma, miR-486-5p, SAPCD2, Migration, Proliferation, Apoptosis, Invasion

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Introduction

Lung cancer is a prevalent cancer across the globe with high mortality and morbidity (Bray et al., 2018). Most lung cancer cases are non-small cell lung cancer (NSCLC), and the main type of NSCLC is lung adenocarcinoma (LUAD) (Relli et al., 2019). LUAD originates in small airway epithelium II type alveolar cells (Noguchi et al., 1995). Although the diagnostic methods and treatment of lung cancer have been developed recently, the 5-year relative survival rate of lung cancer patients is still not ideal (Siegel et al., 2015). Hence, a more comprehensive understanding of the mechanisms on LUAD malignant progression is of great importance for identifying new effective therapeutic targets.

MicroRNAs (miRNAs) can inhibit mRNA degradation or protein translation by negatively modulating gene expression at post-transcriptional level (Huntzinger and Izaurralde, 2011). Studies revealed the regulatory function of miRNAs in LUAD progression, providing potential therapeutic targets for LUAD (Yerukala Sathipati and Ho, 2017). For example, miR-222, which has a pro-cancer effect, promotes the development of LUAD cells by targeting ETS1 (Sun et al., 2017), whereas miR-576-3p and miR-374a, serving as tumor suppressors, target TGFA and SGK1, respectively, to hamper LUAD cell proliferation and invasion (Greenawalt et al., 2019). MiR-486-5p which we are interested in also plays a modulatory role in many cancers. For instance, it constrains cell proliferation in esophageal squamous cell carcinoma (Yi et al., 2016). Via targeting PIK3R1, it represses cell migration and invasion in colorectal cancer (Zhang et al., 2018). Additionally, miR-486-5p level is prominently reduced in lung cancer (Tessema et al., 2017). However, the research on its regulatory mechanisms in LUAD is not comprehensive enough and needs to be further explored.

SAPCD2 is a cell cycle-dependent gene (Xu et al., 2007; Jiang et al., 2018). A recent finding indicated that

SAPCD2 modulated cell division via controlling spindle orientation in cells (Chiu et al., 2016), which seems to be associated with cell proliferation. Also, some studies showed that SAPCD2 promoted the malignant progression of many cancers. For instance, overexpressed SAPCD2 facilitates malignant phenotypes of nasopharyngeal carcinoma cells (Jia et al., 2019). SAPCD2 overexpression in colorectal cancer notably promotes cell progression (Luo et al., 2020). However, the mechanisms of SAPCD2 in LUAD have not been explained in detail.

Herein, through a set of cell assays, the influence of miR-486-5p on LUAD cells was studied, and its downstream target was predicted and verified. We studied the mechanism of miR-486-5p in LUAD, which helps expand understanding of the mechanisms in LUAD.

Materials and methods

Bioinformatics approaches

Mature miRNA expression data were provided by The Cancer Genome Atlas (TCGA)-LUAD dataset (https://portal.gdc.cancer.gov/), in which 513 cancer samples along with 45 para-cancerous samples were obtained. Also, mRNA sequencing data were accessed, where 519 cancer samples and 58 para-cancerous samples were acquired. Differentially expressed miRNAs (DEmiRNAs) and mRNAs (DEmRNAs) in LUAD were screened from the obtained samples and corresponding expression data. Differential analysis was conducted by "edgeR" ($|logFC|>2$, $padj<0.05$). The target genes of miRNA were predicted by using miRTarBase (http:// mirtarbase.cuhk. edu.cn/) and miRWalk (http:// mirwalk.umm.uni-heidelberg.de/) databases. Then, the correlation between miRNA and candidate mRNAs was calculated to determine the target gene. The relationship between the target gene expression, and T stage, N stage and clinical stage was analyzed.

Cell Culture

Human LUAD cell lines NCI-H1975 (BNCC280755), NCI-H441 (BNCC292357), and NCI-H1792 (BNCC322781) were bought from BeNa Culture Collection. Human normal lung epithelial cell line BEAS-2B (CL0044) and LUAD cell line SPC-A1 (CL0296) were purchased from Fenghui Biological Co. Ltd. Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS) and 100 mg/mL streptomycin/penicillin was applied for culture BEAS-2B at 37°C with 5% CO_2. LUAD cell lines were incubated in 90% Roswell Park Memorial Institute (RPMI)-1640 medium plus 10% FBS. The cells were collected when the bottom of the bottle was 90% covered, and the medium was replaced once every 2-3 days.

Cell transfection

NCI-H1975 cells were incubated in 6-well plates until 80% fusion was achieved. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was utilized to transfect miR-486-5p-mimic (miR-mimic) (Genepharma, Shanghai, China) and NC-mimic into NCI-H1975 cell line, respectively. The transfected cells were cultured at 37°C with 5% CO_2 for 2 days, and then collected after centrifugation for subsequent functional assays.

NCI-H1975 cells treated with miR-mimic or NC-mimic were transfected with oe-SAPCD2 plasmid or oe-NC plasmid (Shanghai Gene Chem Corporation, China). After being cultured with 5% CO_2 at 37°C for 2 days, cells were collected for subsequent functional assays.

qRT-PCR assay

TRIzol reagent (Life Technologies, Grand Island, NY, USA) was utilized to extract total RNA. Then, RNA was reverse-transcribed to obtain complementary DNA using the High-Capacity cDNA synthesis kit (Takara). qRT-PCR was performed on Applied Biosystems® 7500 Real-Time PCR Systems (Thermo Fisher, Waltham, MA) using the SYBR Green PCR Kit (Takara Bio, Otsu, Japan). GAPDH and U6 served as the internal parameters. Relative expression was expressed using $2^{-\Delta\Delta Ct}$ method. Table 1 displays the sequences of primers.

Western blot assay

Cells were centrifugally collected and rinsed with phosphate-buffered saline (PBS). Then, cells were treated with radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) to obtain protein samples. The samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. After the membrane was blocked with 5% skimmed milk, it was incubated overnight with primary antibody rabbit anti-SAPCD2 (1:1000, ab241047, Abcam, UK) or rabbit anti-GAPDH (1:10000, ab181602, Abcam, UK) at 4°C. Afterward, the membrane was reacted with HRP-labeled

Table 1. Primer sequences of qRT-PCR.

Gene	Primer sequence (5'→ 3')
miR-486-5p	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-ACGCTTCACGAATTTGCGT-3'
U6	F: 5'-ACGCTTCACGAATTTGCGT-3' R: 5'-CATCTTCAAAGCACTTCCCTT-3′
SAPCD2	F: 5'-GAGGTGACCGAGAAGAGTGAG-3' R: 5'-GATGAAGGTGGAATCCAGAGG-3'
GAPDH	F: 5'-TGACTTCAACAGCGACACCCA-3' R: 5'-CACCCTGTTGCTGTAGCCAAA-3'

secondary antibody goat anti-rabbit IgG H&L (ab6721, Abcam, UK) at 37°C for 1 h. Then the membrane was washed with PBST buffer 15 min×3 times. Finally, an enhanced chemiluminescence kit (GE Healthcare, Chicago, IL, USA) was used for color development. The imaging was performed using the Chemical Mp Imaging System (Bio-Rad, Hercules, CA, USA). GAPDH was employed as the internal reference.

MTT assay

The transfected NCI-H1975 cells (2×10^4 cells/well) were seeded into 96-well plates. The cells were cultured for 1, 2 and 3 days, respectively. Twenty μl MTT solution (5 mg/ml, Sigma, USA) was supplemented to each well every day. Each group was set with 5 replicates. The medium was removed after 4 h of culture. 150 μl dimethyl sulfoxide was added to each well to dissolve the crystal completely. A microplate reader was utilized to measure the absorbance (A_{490}).

Transwell assay

Cell migration and invasion were analyzed in Transwell chambers (8 μm, BD Biosciences, USA). Matrigel gel (BD Biosciences, San Jose, CA, USA) was added to the upper chamber for invasion assay, but not for migration assay. Firstly, 2×10^5 cells were resuspended in 200 μl serum-free RPMI-1640 medium and added into the upper chamber, while 600 μl medium containing 10% FBS was placed to the lower chamber. After being cultured at 37°C for 24 h, the cells remaining in the upper chamber without migration or invasion were removed. Then, cells were fixed with 4% paraformaldehyde for 10 min and dyed with 0.5% crystal violet. Finally, the membrane was washed with clean water. The stained cells were counted under an inverted microscope.

Apoptosis assessment

Annexin V-FITC and prodium iodide (PI) were used to stain cells, and apoptosis was assayed by flow cytometry. Transfected NCI-H1975 cells were collected and incubated in medium without serum for 24 h to induce apoptosis. After the floating cells were removed, adherent cells were collected. After being washed with PBS, the cells were resuspended with a binding buffer to obtain cell suspension (1×10^6 cells/ml). After cell staining with Annexin V-FITC and PI for 15 min in the dark, the cell fluorescence was detected by FACS Calibur flow cytometry (BD Biosciences, San Jose, CA), and the proportion of apoptosis was analyzed. The apoptotic features of the cells were measured by a microscope (Olympus, Japan).

Dual-luciferase assay

Wild type (WT) SAPCD2 3'-untranslated region (UTR) and mutant (MUT) SAPCD2 3'-UTR plasmids were constructed by dual-luciferase reporter plasmid psiCHECK (Sangon Co., Ltd, Shanghai, China). Lipofectamine 2000 (Invitrogen, USA) was used to co-transfect miR-mimic with psiCHECK-SAPCD2 WT or psiCHECK-SAPCD2 MUT into NCI-H1975 cells. In addition, NC-mimic was co-transfected with psiCHECK-SAPCD2 WT or psiCHECK-SAPCD2 MUT, respectively. The above cell groups transfected with psiCHECK-SAPCD2 MUT or psiCHECK-SAPCD2 WT were named as SAPCD2-MUT or SAPCD2-WT, respectively. After 48h, the activities of Renilla luciferase and firefly luciferase in each group were measured using the Dual-Luciferase Reporter Assay System (Promega, USA).

Statistics

Data were processed using GraphPad Prism 6.0 (La Jolla, CA) and each assay was repeated 3 times. All data are displayed as mean ± standard deviation. Data between two groups were compared by *t*-test. $P<0.05$ represents a statistically prominent difference.

Results

MiR-486-5p is lowly expressed in LUAD

Studies put forward that miR-486-5p was downexpressed in many cancers. Bioinformatics analysis result exhibited that miR-486-5p level was notably low in the cancer tissue compared with that in normal tissue ($p<0.05$) (Fig. 1A). Also, the correlations between miR-486-5p level and T, N, stage were analyzed, in which no significant correlation was observed (Supplementary data). Then, qRT-PCR result showed that miR-486-5p was prominently less expressed in LUAD cell lines ($p<0.05$) (Fig. 1B). The above stated that miR-486-5p was downexpressed in LUAD tissue and cell lines. Subsequently, NCI-H1975 and NCI-H1792 were selected to construct miR-486-5p up-regulated and down-regulated cell lines respectively for further study.

Overexpressed miR-486-5p restrains proliferation, migration, invasion, and anti-apoptosis of LUAD cells

MiR-mimic and miR-inhibitor were respectively transfected into NCI-H1975 and NCI-H1792 cell lines to examine the changes of malignant phenotypes of LUAD cells. Firstly, the transfection efficiencies were verified by qRT-PCR, in which miR-486-5p expression was evidently upregulated and downregulated by miR-mimic and miR-inhibitor, respectively ($p<0.05$) (Fig. 2A). The result of MTT assay revealed that miR-486-5p overexpression restrained LUAD cells from proliferation, while its downregulation simulated proliferation ($p<0.05$) (Fig. 2B). Transwell assay results suggested that up-regulated miR-486-5p weakened the migration and invasion of LUAD cells, yet the results

were the opposite in the miR-inhibitor group (p<0.05) (Fig. 2C,D). Flow cytometry analysis illustrated that miR-486-5p was able to facilitate LUAD cell apoptosis (p<0.05) (Fig. 2E), and the apoptosis-related cell morphology features were observed by transfecting miR-mimic, in which the cell shrinkage was found (Fig. 2F). Taken together, overexpressed miR-486-5p hampered LUAD cell malignant phenotypes.

MiR-486-5p targets and restrains SAPCD2 level in LUAD cells

To identify the downstream target of miR-486-5p in LUAD cells, a differential analysis was done based on the accessed TCGA dataset. Then 2,473 DEmRNAs were obtained, where 533 were upregulated and 1,940 were downregulated (p<0.05) (Fig. 3A). The targets of miR-486-5p were predicted by using the miRWalk and miRTarBase databases, and 2 candidates were screened out from the predicted upregulated DEmRNAs (Fig. 3B). Among them, SAPCD2 showed the most significant correlation with miR-486-5p, with a correlation of -0.2 (Fig. 3C,D). The bioinformatics method was applied to predict a binding site of miR-486-5p in SAPCD2 3'-UTR, and the mutated one (MUT) was synthesized as a control for the subsequent dual-luciferase reporter gene detection (Fig. 3E). Moreover, TCGA data presented that SAPCD2 level in the cancer tissue was evidently higher than that in the para-cancerous tissue (p<0.05) (Fig. 3F). Also, a consistent result was observed in LUAD cells (p<0.05) (Fig. 3G). Besides, according to the clinical data from TCGA, the significant correlations between SAPCD2 expression and T, N stage, and clinical stage were studied (p<0.05) (Fig. 3H). According to the dual-luciferase detection, it was observed that the luciferase activity of the WT SAPCD2 3'-UTR was evidently restrained by transfection of miR-mimic, while that of the MUT SAPCD2 3'UTR was not influenced (p<0.05) (Fig. 3I), indicating miR-486-5p targeted SAPCD2 mRNA. Additionally, SAPCD2 level in different transfected groups was detected by qRT-PCR and western blot, finding that miR-486-5p notably constrained both the protein and mRNA expression levels of SAPCD2 in LUAD cells (p<0.05 for qRT-PCR assay) (Fig. 3J,K). The above results demonstrated that miR-486-5p was able to target SAPCD2 and suppress SAPCD2 level in a post-transcriptional manner.

MiR-486-5p affects LUAD cell progression through downregulating SAPCD2

To verify the modulatory mechanism of miR-486-5p and SAPCD2 in LUAD cells, a cell line with overexpressed miR-486-5p alone, and a cell line with overexpressed SAPCD2 and miR-486-5p were constructed. qRT-PCR and western blot results presented that SAPCD2 was prominently suppressed by miR-486-5p overexpression alone, while facilitated by the simultaneous overexpression of SAPCD2 and miR-486-5p (p<0.05) (Fig. 4A). MTT assay revealed that overexpressed miR-486-5p constrained LUAD cells from proliferation, which was alleviated by overexpression of SAPCD2 and miR-486-5p (p<0.05) (Fig. 4B). Experimental results of cell migration and invasion uncovered that the migratory and invasive abilities of LUAD cells were notably constrained after transfection with miR-486-5p, which was weakened after co-transfection of SAPCD2 and miR-486-5p (p<0.05) (Fig. 4C,D). Finally, the proportion of cell

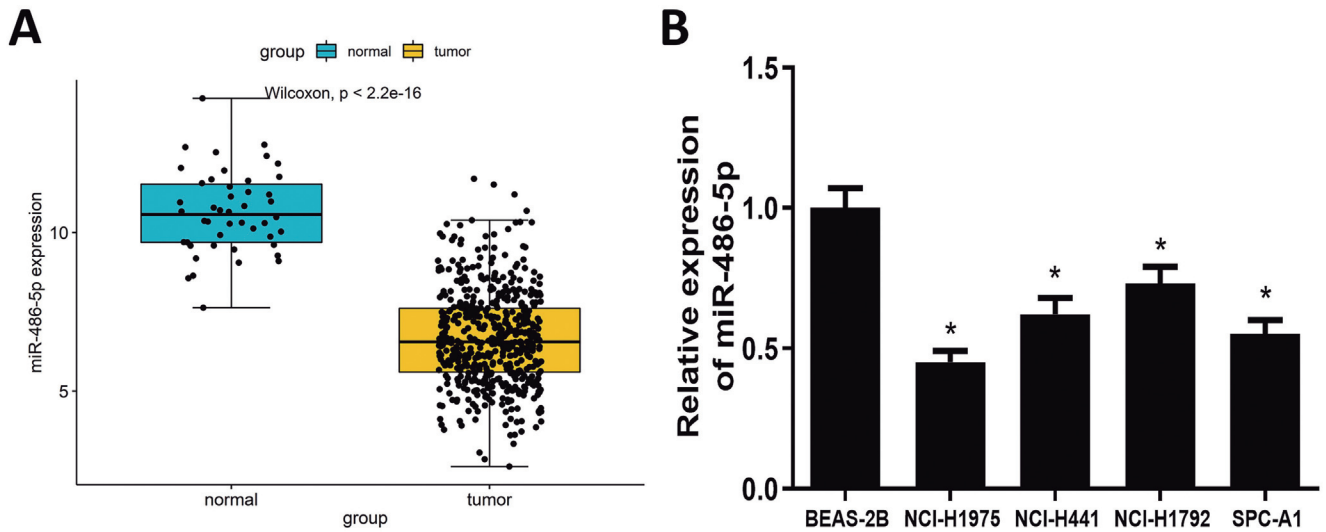

Fig. 1. MiR-486-5p is less expressed in LUAD cells. **A.** Box plot of miR-486-5p expression. Blue: normal group, yellow: tumor group. **B.** MiR-486-5p expression in normal lung epithelial cell line BEAS-2B and LUAD cell lines NCI-H1975, NCI-H441, NCI-H1792 and SPC-A1; * p<0.05.

apoptosis in each group was detected, finding that overexpressed miR-486-5p stimulated the apoptotic ability of LUAD cells, while the simultaneous overexpression of SAPCD2 and miR-486-5p hampered this promoting effect (p<0.01) (Fig. 4E). These results proved that miR-486-5p was able to target and inhibit SAPCD2 expression, thereby repressing LUAD cell malignant phenotypes.

Discussion

MiRNAs can function as novel diagnostic and prognostic biomarkers for various cancers (Huang, 2017). Several recent studies detected the expression of multiple miRNAs in LUAD. For example, miR-133 (Wei et al., 2018) and miR-485 (Mou and Liu, 2016) are suppressed, while miR-210 (Xie et al., 2019) and miR-590 (Liu et al., 2017) are boosted in LUAD. MiR-486-5p is prominently constrained in both renal cell carcinoma (He et al., 2019a) and hepatocellular carcinoma (He et al., 2019b). Herein, bioinformatics methods and qRT-PCR uncovered notably restrained miR-486-5p expression in LUAD tissue and cells, which is similar to the result of Tian et al. (2016). We also observed that lowest miR-486-5p expression in NCI-H1975 cell line, a LUAD cell line with T790M EGFR mutation, among the

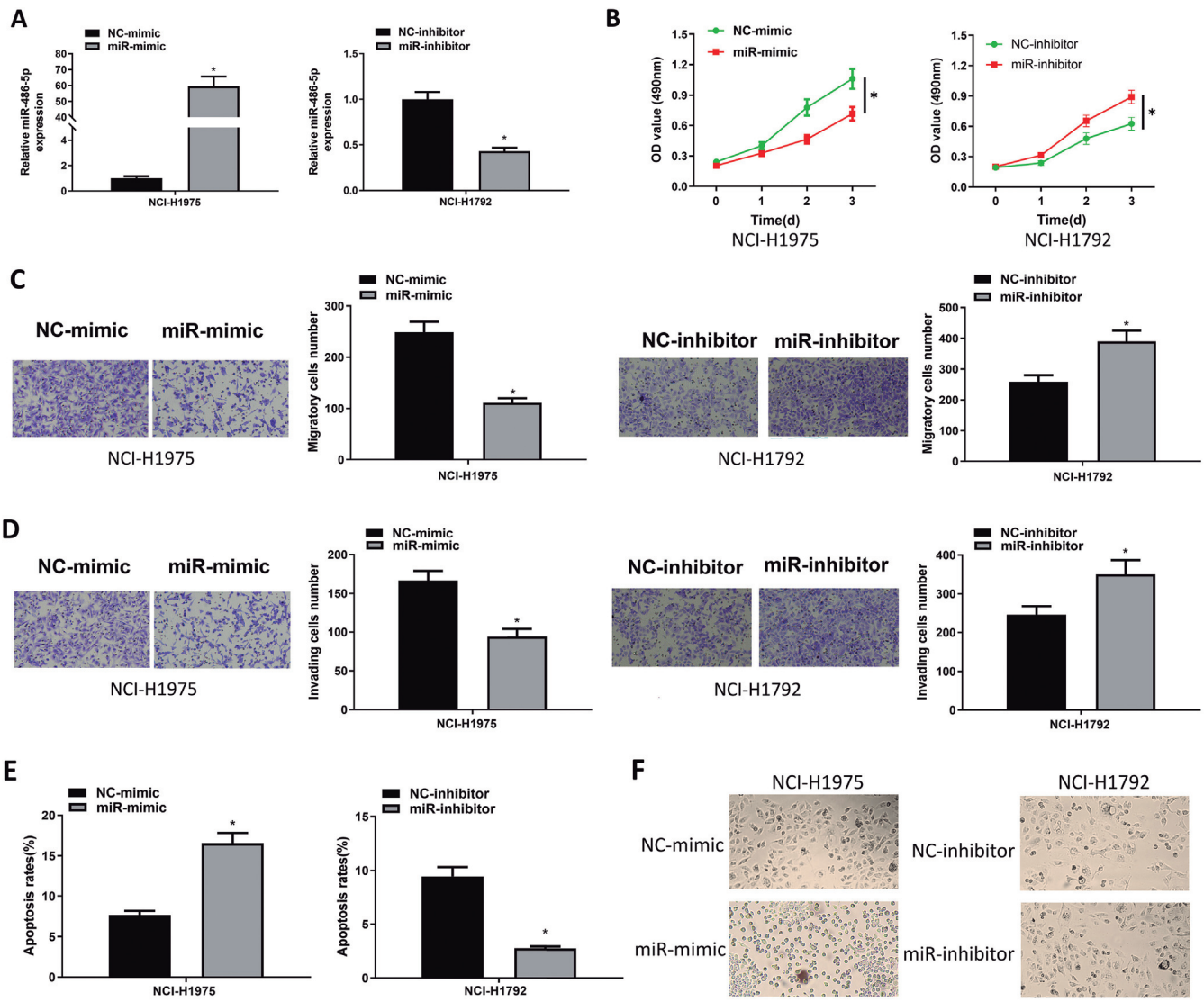

Fig. 2. Overexpressed miR-486-5p inhibits proliferation, migration, invasion and anti-apoptosis of LUAD cells. LUAD cells were treated with miR-mimic or miR-inhibitor. **A.** Transfection efficiency detected by qRT-PCR. **B.** Cell proliferation in each transfection group. **C, D.** The migration and invasion of cells (100×). **E.** The apoptotic rate of cells . Apoptotic rate = (number of early apoptotic cells + number of late apoptotic cells) / the total number of tested cells. **F.** Microscopy was applied to examine cell morphology * p<0.05.

different LUAD cell lines. As EGFR is well understood as one crucial tumorigenesis for LUAD, we assume that its mutation may suppress miR-486-5p level to some extent.

After determination of miR-486-5p level in LUAD, its role in this cancer should be verified. A growing number of studies have pointed out that miR-486-5p constrained growth of multiple cancer cells. Ma et al. (2016) uncovered that downregulating miR-486-5p facilitated proliferation, while it inhibited apoptosis of thyroid cancer cells. Moreover, cisplatin efficacy was reported to be strengthened by upregulating miR-486-5p in bladder cancer (Salimian et al., 2020). Herein, miR-486-5p overexpression was achieved in LUAD cell line NCI-H1975. Then, *in vitro* cell assays described that the upregulated miR-486-5p level constrained LUAD cell malignant phenotypes. Gao et al. (2018) revealed that in NSCLC, forced miR-486-5p expression suppresses cell

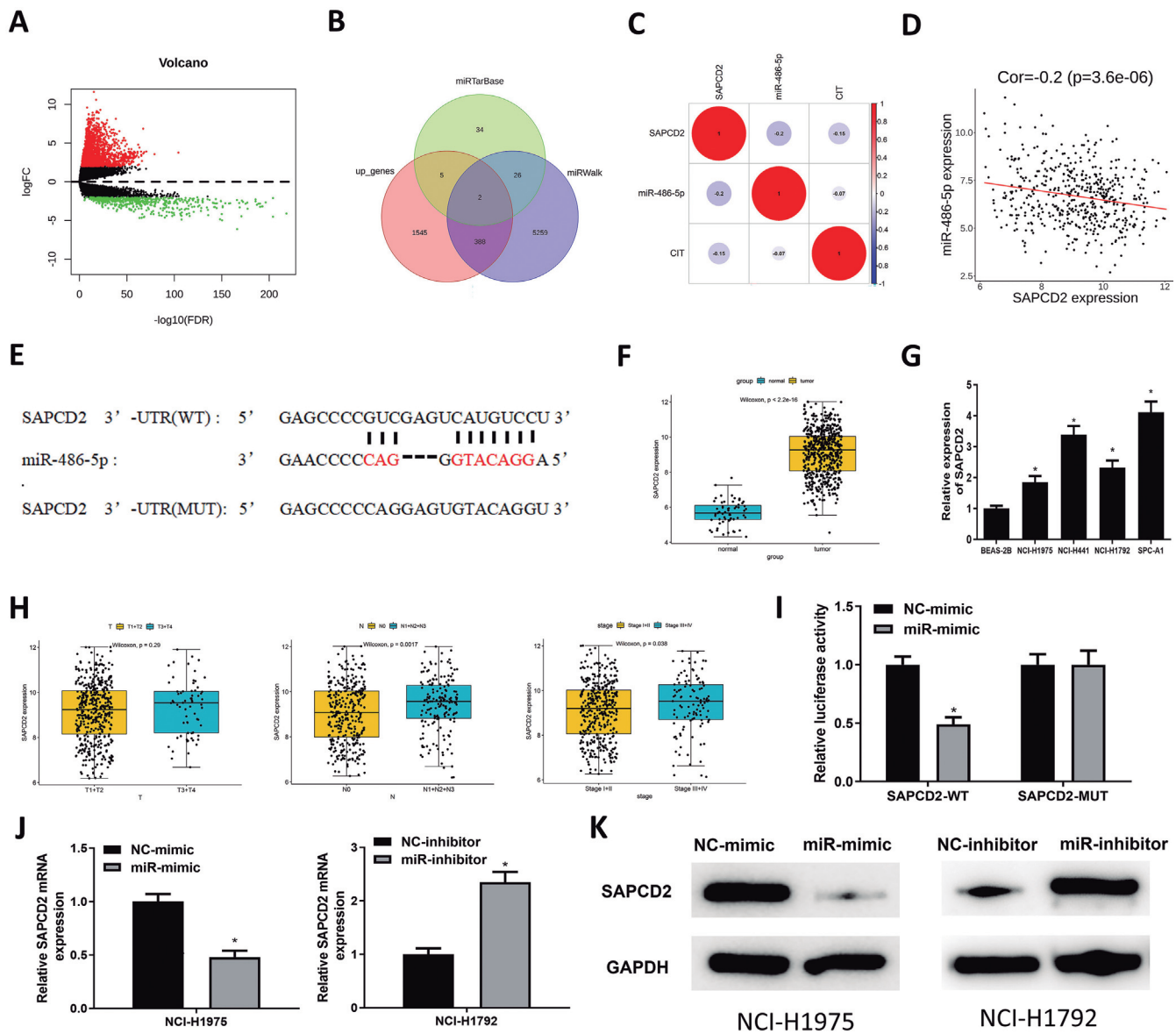

Fig. 3. SAPCD2 is targeted and regulated by miR-486-5p. **A.** Volcano map of LUAD DEmRNAs in normal and tumor groups. Red dots: upregulated mRNAs, green dots: downregulated mRNAs. **B.** Intersection of predicted target genes of miR-486-5p and DEmRNAs. **C.** Heat map of correlation between miR-486-5p and SAPCD2/CIT. **D.** Correlation diagram of SAPCD2 and miR-486-5p. **E.** Binding sites between SAPCD2 3'UTR and miR-486-5p. **F.** Box plot of SAPCD2 level in normal (blue) and the tumor groups (yellow). **G.** SAPCD2 expression in normal cells and LUAD cells. **H.** Boxplot of SAPCD2 expression in different clinical status (T, N, stage). **I.** Luciferase activity of NCI-H1975 cells in different treatment groups. **J, K.** The mRNA and protein expression of SAPCD2 after the overexpression or knock-down of miR-486-5p in LUAD cell lines; * $p<0.05$.

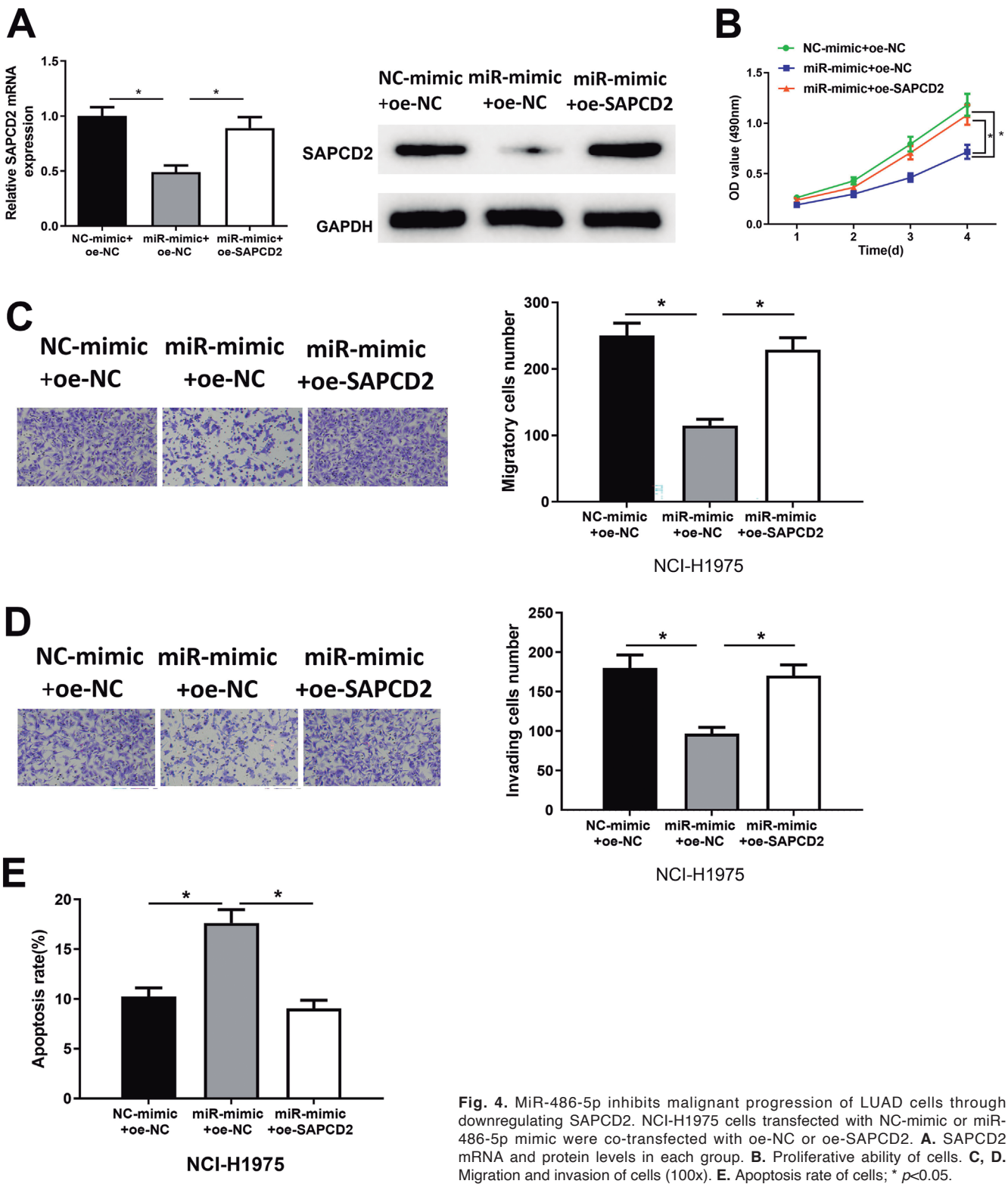

Fig. 4. MiR-486-5p inhibits malignant progression of LUAD cells through downregulating SAPCD2. NCI-H1975 cells transfected with NC-mimic or miR-486-5p mimic were co-transfected with oe-NC or oe-SAPCD2. **A.** SAPCD2 mRNA and protein levels in each group. **B.** Proliferative ability of cells. **C, D.** Migration and invasion of cells (100x). **E.** Apoptosis rate of cells; * $p<0.05$.

invasion and proliferation, which provides a reference for us, indicating that miR-486-5p can hamper LUAD malignant progression.

Looking for the downstream targets of miR-486-5p will provide a more comprehensive understanding of its regulatory mechanism in LUAD. For example, miR-486-5p binds to PIK3R1 and suppresses its expression in liver cancer cells (Gao et al., 2018). MiR-486-5p negatively modulates NEK2 in liver cancer (Fu et al., 2017). To further understand the regulatory mechanism of miR-486-5p in cancer, we continued to excavate its downstream targets. After the candidate targets of miR-486-5p were predicted, SAPCD2, the gene significantly negatively correlated with miR-486-5p, was obtained through correlation analysis. The interaction between the two was verified by dual luciferase analysis. Zhang et al. (2020) uncovered that inhibiting SAPCD2 expression hampered the progression of breast cancer cells. Overexpressed SAPCD2 induces invasion and proliferation of renal cell carcinoma cells, and accelerates the process of epithelial-mesenchymal transition (Li et al., 2014). Inhibition of SAPCD2 prominently weakens the invasion and proliferation of colorectal cancer cells *in vitro* (Shi et al., 2020). In this work, overexpressed SAPCD2 weakened the inhibitory effect of upregulated miR-486-5p on the malignant progression of LUAD cells, suggesting that miR-486-5p inhibited LUAD cell malignant processes by targeting SAPCD2.

In short, miR-486-5p expression was low in LUAD cells while SAPCD2 expression was high, and SAPCD2 was the downstream target of miR-486-5p. Overexpressed miR-486-5p inhibited the malignant phenotypes of LUAD cells, while overexpressed SAPCD2 reversed this effect. The modulatory mechanism of miR-486-5p/SAPCD2 in LUAD was revealed in this paper, which may contribute to a more comprehensive understanding of the mechanism of malignant progression of LUAD.

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Conflict of Interest Statement. The author declare that they have no potential conflicts of interest.

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Authors' contributions. The author contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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