RNA analysis of patients with benign and malignant pulmonary nodules

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Abstract. Pulmonary nodules are the main manifestations of early lung cancer. Non-small cell lung cancer is the most common histological type of lung cancer, and the main histological classification of non-small cell lung cancer is lung adenocarcinoma. The present study aimed to analyze the differentially expressed genes between patients with benign and malignant pulmonary nodules, and to identify potential therapeutic targets for lung adenocarcinoma. Sequencing data for benign and malignant pulmonary nodule samples and samples with no nodules were obtained from the National Center for Biotechnology Information Gene Expression Omnibus GSE135304 dataset. Differential gene analysis showed that S100 calcium binding protein P (S100P), ribonuclease A family member 2 (RNASE2), cytochrome *c* oxidase subunit 7C and mast cell expressed membrane protein 1 (C19orf59) were significantly upregulated among the blood samples collected from patients with malignant pulmonary nodules. Results from Kaplan-Meier plotter datasets showed that S100P, RNASE2 and C19orf59 were associated with the prognosis of lung cancer. RNASE2 expression was positively associated with nodule size and negatively associated with lung cancer prognosis. Moreover, RNASE2 was highly expressed in lung adenocarcinoma tissues compared with that in normal tissues. CCK-8 and Transwell assays indicated that overexpressed RNASE2 promoted the proliferation, migration and invasion of lung adenocarcinoma cells. In lung adenocarcinoma cells, RNASE2 was identified as a downstream target of microRNA (miR)-185-5p and was regulated by it. Inhibited cell proliferation, migration and invasion were observed following overexpression of miR-185-5p. Overexpression of RNASE2 reversed the inhibitory effect of miR-185-5p overexpression. In conclusion, in blood samples from patients with malignant pulmonary nodules and lung adenocarcinoma tissues, RNASE2 was found to be upregulated. High RNASE2 expression was associated with poor overall survival. miR-185-5p inhibited the proliferation, migration and invasion of lung adenocarcinoma cells by downregulating RNASE2 expression. These findings have implications for guiding therapeutic strategies.

Introduction

Non-small cell lung cancer is the most common histological type of lung cancer, and the main histological classification of non-small cell lung cancer is lung adenocarcinoma (1). According to Global Cancer Statistics data published in 2020, lung cancer continues to pose a significant threat to human health as one of the most perilous malignant tumors, with an estimated 2.2 million new cases, and one of the leading causes of cancer-related mortality, with an estimated 1.8 million associated deaths (2). Typically, lung cancer has a poor prognosis. With the diverse treatment strategies now available, the survival prospects of individuals with advanced lung cancer have improved. However, despite these advancements, the overall 5-year survival rate remains at <20% (3-5). The primary factors contributing to this low survival rate include the fact that early symptoms that often go unnoticed, leading to a postponed diagnosis (6,7), and that, following surgery, lymphatic and distant metastases are common, with 34-45% of patients experiencing recurrence (8). In addition,

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although targeted therapy and immunotherapy have shown significant effectiveness and made some progress in lung cancer therapeutics, the available options remain limited (9). Therefore, the identification of molecular targets for lung cancer is vital.

Various bioinformatics analyses have been conducted to identify novel and potential markers that can detect cancers in their early stages, predict prognosis or serve as therapeutic targets (10,11). For example, Ma *et al* (11) reported that RUNX family transcription factor 1 is pivotal to non-small cell lung cancer by using The Cancer Genome Atlas (https://portal.gdc.cancer.gov/), Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/), Gene Expression Profiling Interactive Analysis (http://gepia.cancer-pku.cn/) and Kaplan-Meier (KM) plotter databases (https://kmplot.com/analysis/). Jiang *et al* (12) utilized bioinformatic approaches and found that chromatin licensing and DNA replication factor 1 could predict the prognosis of human lung adenocarcinoma. Furthermore, Gong *et al* (13) identified the therapeutic targets of aspirin in small cell lung cancer using bioinformatics analysis.

Pulmonary nodules are the main manifestations of early lung cancer (14). In the present study, the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) GSE135304 dataset was downloaded, which contains sequencing data of whole blood from patients with malignant nodules and benign nodules, and nodule-free patients, and analyzed differentially expressed genes (DEGs) to identify key genes involved in lung cancer. The associations between key genes and lung cancer prognosis were verified using online KM Plotter. Functions and mechanisms of lung cancer-associated genes were also investigated using molecular experiments.

Materials and methods

Bioinformatics analysis. Setting 'Homo sapiens' and 'pulmonary nodules' as keywords, the GSE135304 dataset (15) was downloaded from the NCBI GEO database (https://www.ncbi.nlm.nih.gov/). The dataset consisted of three main types of samples: Whole-blood samples from patients with malignant nodules, patients with benign nodules and nodule-free patients. After removing duplicate and treated samples, 587 samples remained in the following groups: Malignant (n=303), benign (n=196) and no nodules (n=88). Association between RNASE2 expression and nodule size was also analyzed.

The identification of statistically different genes was completed using the 'Limma' package (R Core Team) with false discovery rate <0.05 and log(fold-change) >0.05.

Overlapping DEGs from malignant/benign and malignant/(benign + no nodules) nodules were identified using VENNY 2.0 (https://bioinfogp.cnb.csic.es/tools/venny/index2.0.2.html). The KM Plotter (http://kmplot.com/analysis/index.php?p=service) was used to assess the association between the expression of all genes and survival in lung cancer, and all analysis of results has been taken directly from Kaplan-Meier Plotter without reanalysis.

The GSE68465 dataset, which contained expression data for 462 samples [lung adenocarcinoma (n=443) and normal (n=19)] and survival information, was also downloaded from NCBI GEO in order to verify the expression of RNASE2.

The online databases miRDB (https://mirdb.org/) and TargetScanHuman (http://www.targetscan.org/) were used to predict the microRNA (miR/miRNA) targets.

Cell culture and transfection. Human lung adenocarcinoma cell lines (A-549 and ABC-1) were purchased from Nanjing Cobioer Gene Technology Co., Ltd. The A-549 cells were cultured in F12K + 10% FBS (Nanjing Cobioer Gene Technology Co., Ltd.) and the ABC-1 cells were cultured in MEM + 1% non-essential amino acids + 10% FBS + 1 mM sodium pyruvate (Nanjing Cobioer Gene Technology Co., Ltd.), both in a 37˚C incubator with 5% CO_2.

The ribonuclease A family member 2 (RNASE2) pVITRO2 overexpression vector (RNASE2 OE), small interfering RNA (siRNA) against RNASE2 (RNASE2 siRNA), miR-185-5p mimic and negative controls were designed, synthesized and obtained from GenePharma Co., Ltd. Empty vector and miR-NC were used as negative controls.

A-549 and ABC-1 cells were infected with RNASE2 siRNA, RNASE2 OE, RNASE2 OE, miR-185-5p mimic using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 24 h according to the manufacturer's instructions. The cells were collected after 48 h of culture for subsequent experiments.

The sequence of the miR-185-5p mimic was as follows: Sense, 5'-UGGAGAGAAAGGCAGUUCCUGA-3' and antisense, 5'-AGGAACUGCCUUUCUCUCCAUU-3'. The sequence of the mimic-NC was as follows: Sense, 5'-UUC UCCGAACGUGUCACGUTT-3' and antisense, 3'-TTAAGA GGCUUGCACAGUGCA-5'.

RNA immunoprecipitation (RIP) assay. An EZ Magna RNA immunoprecipitation Kit (cat. no. 17-701; MilliporeSigma) was used according to the manufacturer's guidelines. Briefly, A-549 and ABC-1 cells were lysed in 500 μl RIP lysis buffer. Centrifugation was performed at 21,367 x g at 4˚C for 15 min to extract the supernatant. Magnetic beads (100 μl) were preincubated with 5 μg anti-protein argonaute-2 (AGO2) or 5 μg immunoglobulin G (IgG) antibodies for 30 min at room temperature. Cell lysates (100 μl) were immunoprecipitated with beads for 6 h at 4˚C. After incubation and brief centrifugation, the EP tube was placed on a magnetic rack, waiting for the solution to clear, and the supernatant discarded. A total of 500 μl RIP Wash Buffer was added, and then the contents of the EP tube was vortexed and placed back on the magnetic rack. The supernatant was discarded again and this step was repeated five times for a total of six washes. qPCR was used to confirm the target protein.

Cell counting Kit-8 (CCK-8) assay. The proliferation of the two cell lines (A-549 and ABC-1) was measured using the CCK-8 assay (MilliporeSigma). The cells were routinely digested using trypsin and seeded onto 96-well plates. After 24 h, 10 μl CCK-8 solution was added per well and incubated for 2 h at 37˚C. Finally, absorbance was measured at 450 nm using a microplate reader (Molecular Devices, LLC).

Transwell cell migration and invasion assay. After coating the upper chamber with 50 mg/l Matrigel (BD Biosciences) and leaving to air dry at 4˚C, $1x10^5$ A-549 or ABC-1 cells

with 100 μl serum-free medium (Thermo Fisher Scientific, Inc.) were seeded into Transwell chambers (8.0-μm pore size; MilliporeSigma). The complete medium was added to the lower chamber. After 48 h of incubation at 37˚C, the upper chamber was cleaned using cotton swabs, whereas the cells in the lower chamber were fixed with 70% ethanol for 20 min and stained with 0.1% crystal violet for 15 min, both at 25˚C. The number of invasive cells was counted using a light microscope (Olympus Corporation). The steps for the cell migration assay were the same as those for the invasion assay, except that the upper chamber was not pre-coated.

Luciferase reporter gene analysis. pmirGLO vectors (Promega Corporation) were inserted with binding site-amplified RNASE2 3'UTR, through which RNASE2 3'UTR wild-type reporter (RNASE2-Wt) and a mutated isoform (RNASE2-Mut) with altered binding sites were constructed. Transfection of RNASE2-Wt and RNASE2-Mut with Lipofectamine was conducted along with the aforementioned miR-185-5p mimic. Next, the fluorescence intensity indicating luciferase activity was measured using a Reporter Assay Kit (cat. no. 16186; Thermo Fisher Scientific, Inc.) at 36 h post-transfection, while normalization was performed with reference to *Renilla* luciferase activity, according to the manufacturer's protocol.

Statistical analysis. Using GraphPad Prism (version 8.0.1; Dotmatics), data from three independent experiments (with the exception of clinical data) were analyzed and exhibited as the mean ± SD. The statistically significant differences between tumor tissues and adjacent normal tissues were determined using unpaired Student's t-test. The statistically significant differences between other two groups comparisons were determined using Mann-Whitney U-test or unpaired Student's t-test, where appropriate. ANOVA with Tukey's post hoc test was used in datasets containing three or more groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Gene profiling in whole blood samples from patients with malignant nodules and benign nodules, and from nodule-free patients. Pulmonary nodules are the main manifestations of early lung cancer (14). The GSE135304 dataset, which contains the sequencing data of whole blood from patients with malignant nodules and benign nodules, and from nodule-free patients, was downloaded from the GEO database. Duplicates and treated samples were removed, leaving 587 samples (303 samples with malignant nodules, 196 with benign nodules and 88 with no nodules). Fig. 1A shows that 14 DEGs were upregulated in the malignant nodule blood samples compared with the benign nodule blood samples. Fig. 1B shows that 5 DEGs were upregulated and one was downregulated in the malignant nodule blood samples compared with the benign nodule and no nodules blood samples. Four overlapping DEGs [S100 calcium binding protein P (S100P), RNASE2, cytochrome *c* oxidase subunit 7C (COX7C) and mast cell expressed membrane protein 1 (C19orf59)] from malignant/benign and malignant/(benign + no nodules) nodules were obtained using VENNY (Fig. 1C).

Expression characteristics of the four overlapped DEGs. The four overlapping DEGs (S100P, RNASE2, COX7C and C19orf59) were upregulated in the malignant nodule blood samples compared with those in the benign nodule samples and no nodules samples, and their RNA expression levels did not differ between the benign and no nodules blood samples (Fig. 2A). Using the KM Plotter, the connection between these DEGs and the overall survival of patients with lung cancer was analyzed. Patients with high S100P and RNASE2 expression exhibited a significantly poorer prognosis compared with those patients with low expression, whereas patients with high C19orf59 expression exhibited a better prognosis compared with the patients with low expression (Fig. 2B). The survival of patients with lung cancer was not affected by COX7C expression.

RNASE2 promotes cell proliferation, migration and invasion. A literature search revealed that, to the best of our knowledge, no study has yet reported the association between RNASE2 and lung cancer. Therefore, RNASE2 was selected as a novel target to explore its role in lung cancer. The analysis of the GSE135304 dataset revealed an association between RNASE2 expression and nodule size; in general, the larger the nodule, the higher the gene expression level (Fig. 3A). Using the GSE68465 dataset, it was found that the expression of RNASE2 was significantly higher in lung adenocarcinoma tissues than that in normal tissues (Fig. 3B). RNASE2 was knocked down and RNASE2 was overexpressed in A-549 and ABC-1 cells (Fig. 3C), and the proliferation of transfected A-549 and ABC-1 cells was measured using a CCK-8 assay. Proliferation was inhibited by RNASE2 siRNA and promoted by RNASE2 overexpression (Fig. 3D). The ability of the cells to migrate and invade was also tested, which similarly showed inhibition after RNASE2 siRNA treatment and promotion by RNASE2 overexpression (Fig. 3E and F).

RNASE2 is a target gene of miR-185-5p. The upstream regulatory mechanism of RNASE2 was also explored. As a number of mRNAs are regulated by miRNAs that play a role in the occurrence and development of tumors (16-18), 28 miRNAs were predicted to target RNASE2 in miRDB (https://mirdb.org/) (Table SI). The 18 miRNAs with target scores of >60 were analyzed. Additionally, the miRNA targets were predicted using TargetScanHuman (http://www.targetscan.org/) (Table SII). In TargetScanHuman, the context++ score is an indicator used to assess the reliability and strength of the binding between a miRNA and its target genes. Specifically, the context++ score is calculated based on various features within the target mRNA sequence, including the position of binding sites, sequence specificity, and whether there are evolutionary conservation features such as immunohistochemistry staining. A lower context++ score indicates a stronger predicted binding and a more likely miRNA-target relationship. In total, 17 miRNAs with a context ++ score percentile of 99 were selected. Using VENNY, 10 overlapping miRNAs were identified between the miRDB and TargetScan miRNAs (Fig. 4A). Among these, miR-185-5p is closely associated with lung cancer (19-21). The miR-185-5p mimic was therefore used to transfect A-549 and ABC-1 cells to upregulate miR-185-5p level (Fig. 4B), and RNASE2 was found to be inhibited by

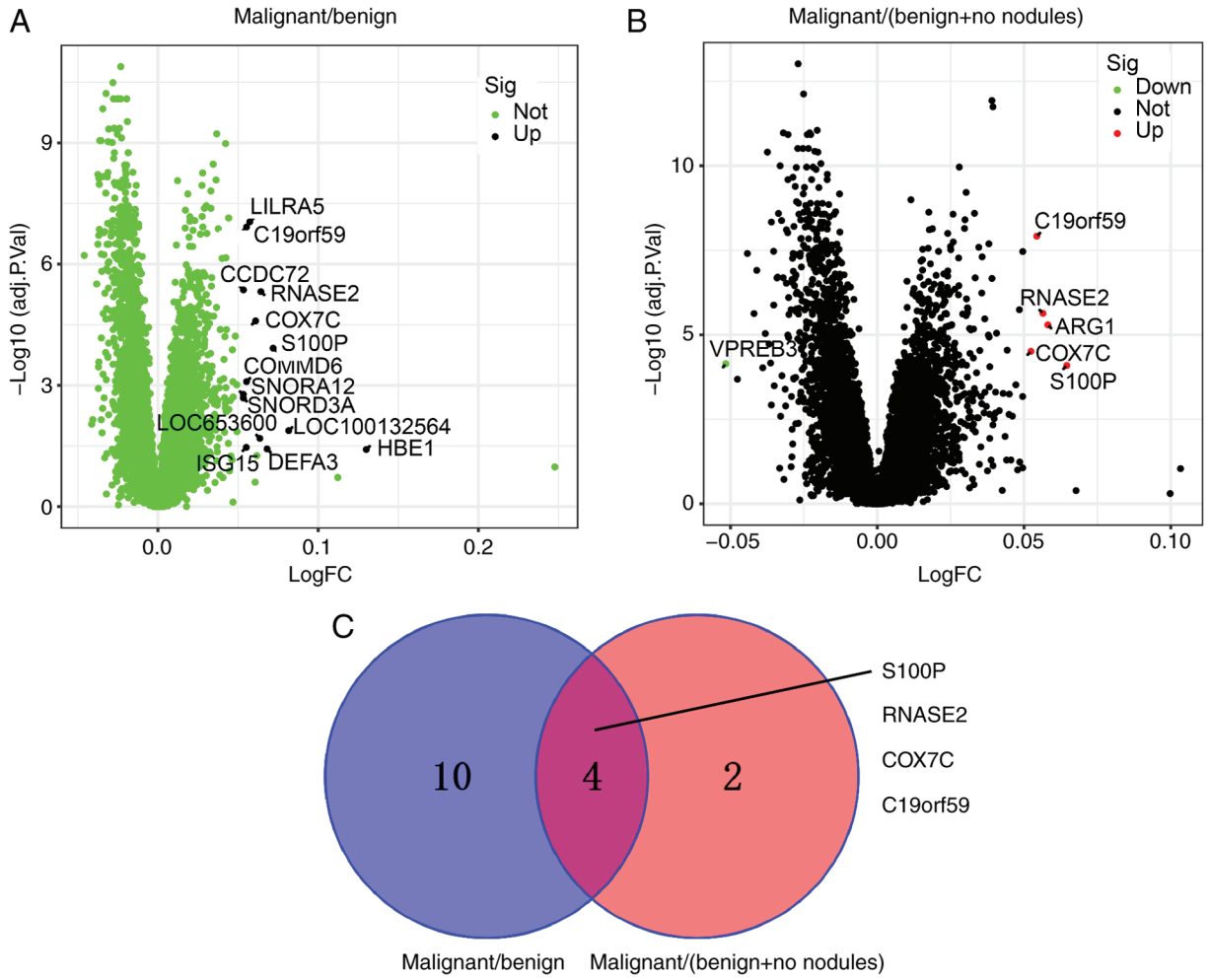

Figure 1. Gene profiling in whole blood samples from patients with malignant nodules and benign nodules, and from nodule-free patients. (A) DEGs between malignant nodule blood samples and benign nodule blood samples [fdr <0.05 and log(fold-change) >0.05]. (B) DEGs between malignant nodule blood samples and benign nodule + no nodule blood samples [fdr <0.05 and log(fold-change) >0.05]. (C) The overlapped DEGs from malignant/benign nodules and malignant/(benign + no nodules) were obtained using VENNY. DEG, differentially expressed gene; fdr, false discovery rate; S100, S100 calcium binding protein P; RNASE2, ribonuclease A family member 2; COX7C, cytochrome *c* oxidase subunit 7C; C19orf59, mast cell expressed membrane protein 1

the miR-185-5p mimic (Fig. 4C). Next, RNASE2-Wt and RNASE2-Mut were constructed (Fig. 4D). The results of a luciferase reporter assay showed that the luciferase activity of RNASE2-Wt was inhibited by the miR-185-5p mimic, whereas no such effect was shown for RNASE2-Mut (Fig. 4E). The RNA-induced silencing complex (RISC) is a complex protein complex in cells that regulates gene expression. This complex can target a specific mRNA through miRNA guidance, inhibiting its translation or promoting its degradation, thus affecting gene expression levels. RISC contains miRNA, mRNA and Ago2 protein. To confirm whether miR-185-5p and RNASE2 form a RISC, RIP experiments were conducted, which found that both miR-185-5p and RNASE2 were enriched in the Ago2 group (Fig. 4F). This indicates that RNASE2 can bind to miR-185-5p.

miR-185-5p inhibits the proliferation, migration and invasion of lung adenocarcinoma cells by targeting RNASE2. Using the miR-185-5p mimic alone or in combination with RNASE2 OE, transfection was conducted in the ABC-1 cells. RNASE2 expression was significantly inhibited by the miR-185-5p mimic in comparison with the control (Fig. 5A). Moreover, significantly suppressed cell proliferation was observed in the ABC-1 cells transfected with miR-185-5p mimic compared with the control, which partially reversed the enhanced cell proliferation induced by RNASE2 OE (Fig. 5B). The migration and invasion of ABC-1 cells were also inhibited by the miR-185-5p mimic, which also partially reversed the enhanced cell migration and invasion induced by RNASE2 OE (Fig. 5C and D).

Discussion

In the present study, DEGs in blood samples with or without malignant nodules were analyzed to identify potential biomarkers for the treatment of lung cancer. Four key genes (S100P, C19orf59, COX7C and RNASE2) were identified that may play important roles in lung cancer. Using KM Plotter datasets, it was found that S100P, C19orf59 and RNASE2 were associated with the prognosis of patients with lung cancer. High S100P and RNASE2 expression was associated with a poor prognosis, whereas high C19orf59 levels indicated a better prognosis. As S100P, C19orf59 and RNASE2 are upregulated in malignant

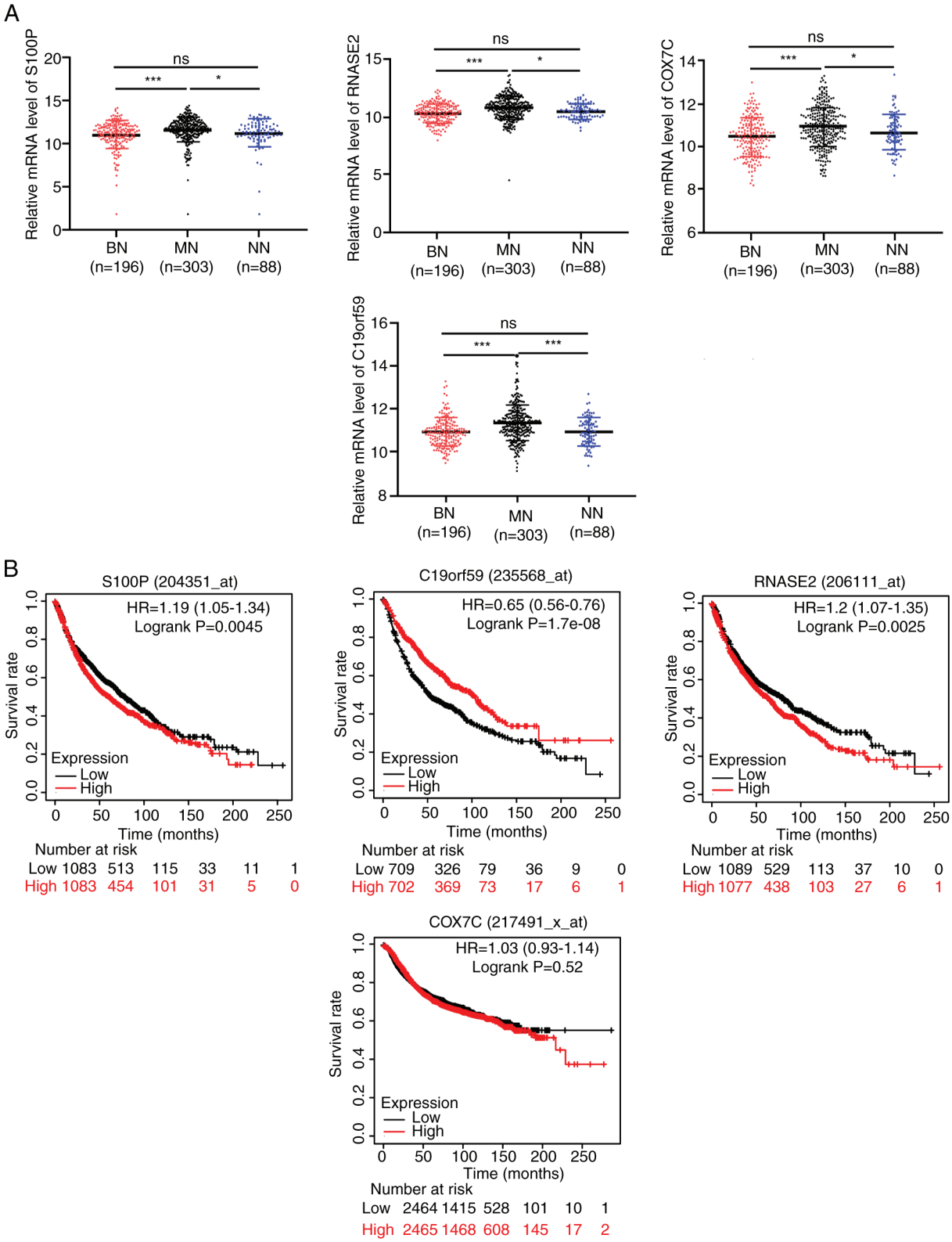

Figure 2. Expression characteristics of the four overlapped DEGs. (A) The expression of S100P, RNASE2, COX7C and C19orf59 in malignant and benign blood samples, and those with no nodules. (B) The connection between these DEGs and the overall survival of lung cancer patients was analyzed though Kaplan-Meier Plotter. *P<0.05 and ***P<0.001. ns, not significant; BN, benign nodules; MN, malignant nodules; NN, no nodules; S100, S100 calcium binding protein P; RNASE2, ribonuclease A family member 2; COX7C, cytochrome *c* oxidase subunit 7C; C19orf59, mast cell expressed membrane protein 1; DEG, differentially expressed gene.

nodule samples, a focus was placed on S100P and RNASE2. C19orf59 was not a focus as its expression was inconsistent with the prognosis trend.

The role of S100P in the development and occurrence of lung cancer was established through a literature search. Rehbein *et al* (22) reported that S100P induction is vital to

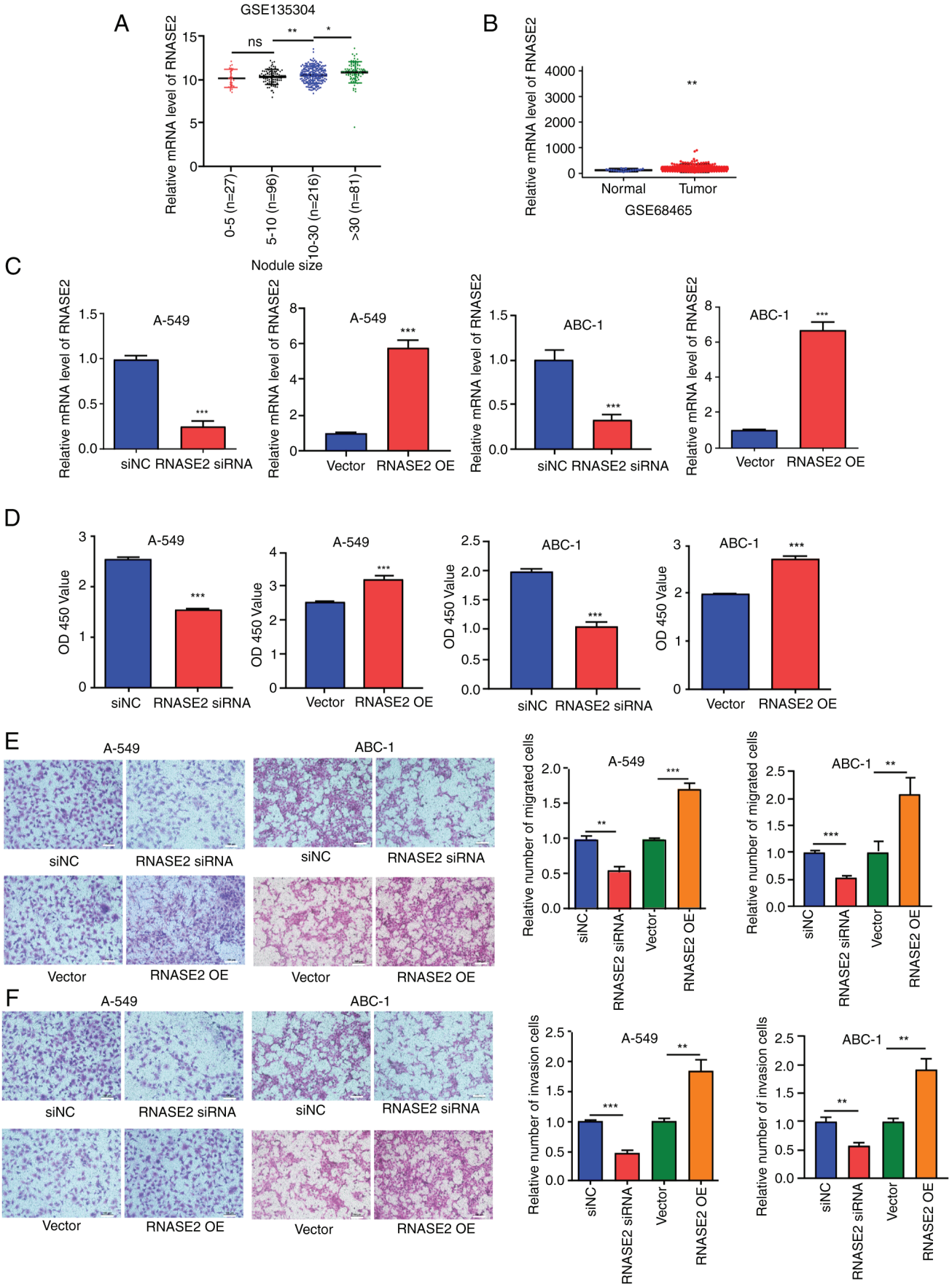

Figure 3. RNASE2 promotes cell proliferation, migration and invasion. (A) RNASE2 expression relative to nodule size in the GSE135304 dataset. (B) The expression level of RNASE2 in lung adenocarcinoma tissues and normal tissues from the GSE68465 dataset. (C) The expression level of RNASE2 was knocked down or overexpressed in A-549 and ABC-1 cells. (D) The proliferation of transfected A-549 and ABC-1 cells was measured using Cell Counting Kit-8. (E) Migration and (F) invasion were tested using Transwell assays. Crystal violet staining; scale bar, 100 μm. *P<0.05, **P<0.01 and ***P<0.001. ns, not significant; RNASE2, ribonuclease A family member 2; siRNA, small interfering RNA; OE, overexpression; NC, negative control.

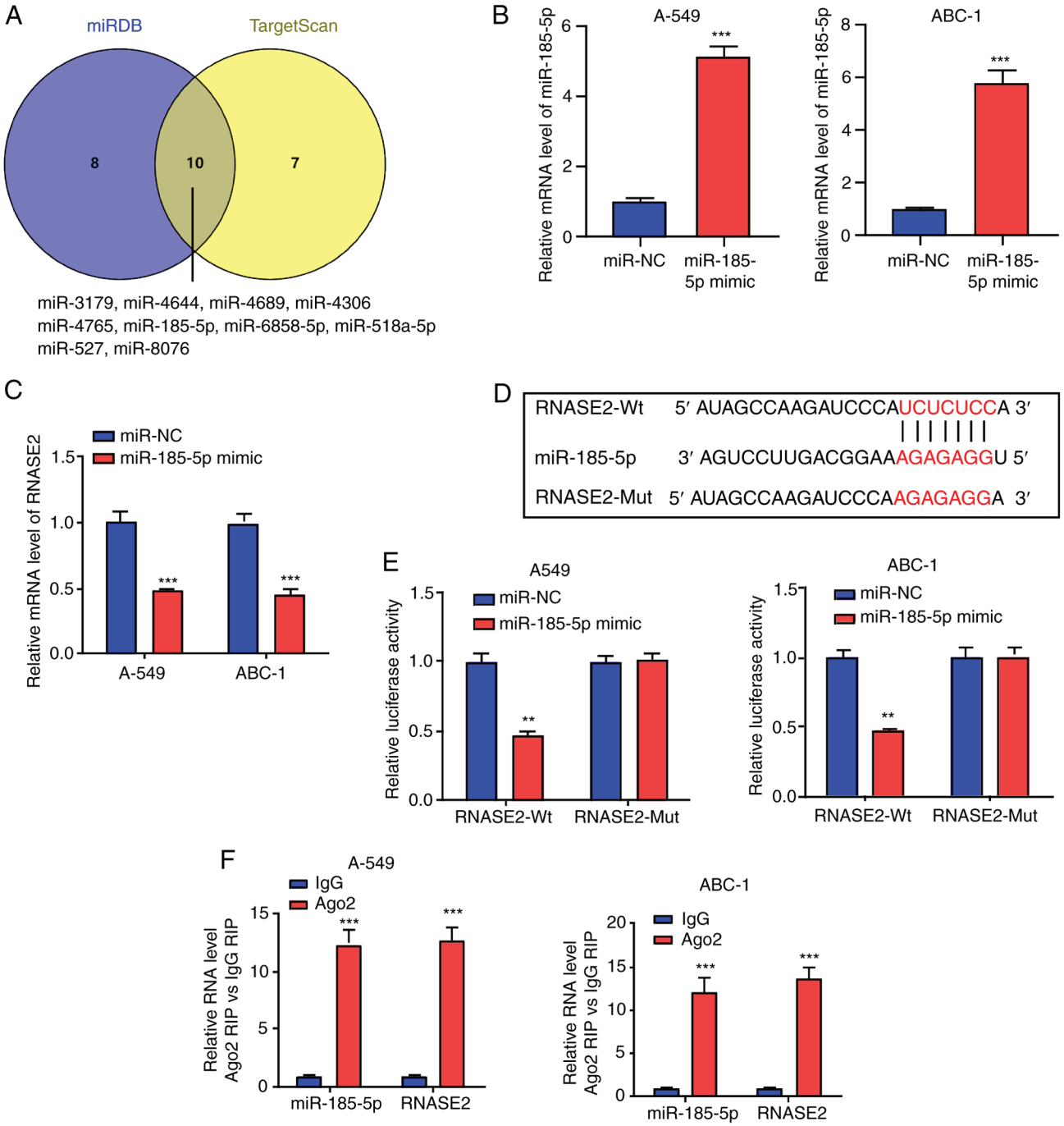

Figure 4. RNASE2 is a target gene of miR-185-5p. (A) A total of 10 overlapped miRNAs were found using VENNY. (B) Results of quantitative PCR showed that the transfections were successful. (C) Inhibition of RNASE2 by miR-185-5p mimic. (D) Construction of RNASE2-Wt and RNASE2-Mut. (E) Inhibition of RNASE2-Wt luciferase activity by miR-185-5p mimic. (F) Substantiation of the binding of RNASE2 to miR-185-5p by RIP. **P<0.01 and ***P<0.001. miR, microRNA; NC, negative control; RNASE2, ribonuclease A family member 2; wt, wild-type; mut, mutant; RIP, RNA immunoprecipitation; Ago2, protein argonaute-2; IgG, immunoglobulin G.

early lung adenocarcinomas, whereas in advanced stages, tumor progression partially relies on its downregulation. Tan *et al* (23) reported that the lncRNA NORAD, through the sequestering of S100P, inhibits the metastasis of lung and breast cancer (23). Hsu *et al* (24) revealed that in lung cancer, S100P interacts with integrin α7, leading to heightened migration and invasion activities (24). Chien *et al* (25) reported that, in lung adenocarcinomas, through the control of S100P, the Keap1-Nrf2 interaction can suppress cell motility. However, to the best of our knowledge, no studies have reported the association between RNASE2 and lung diseases.

Although no study has found an association between RNASE2 and lung cancer, RNASE2 was previously found to promote the progression of gliomas (26). In the present study, GSE135304 analysis revealed a significant association between RNASE2 expression and nodule size. Using the GSE68465 dataset, it was found that the expression levels of RNASE2 were higher in lung adenocarcinoma tissues than those in normal tissues. In A-549 and ABC-1 cells, the ability

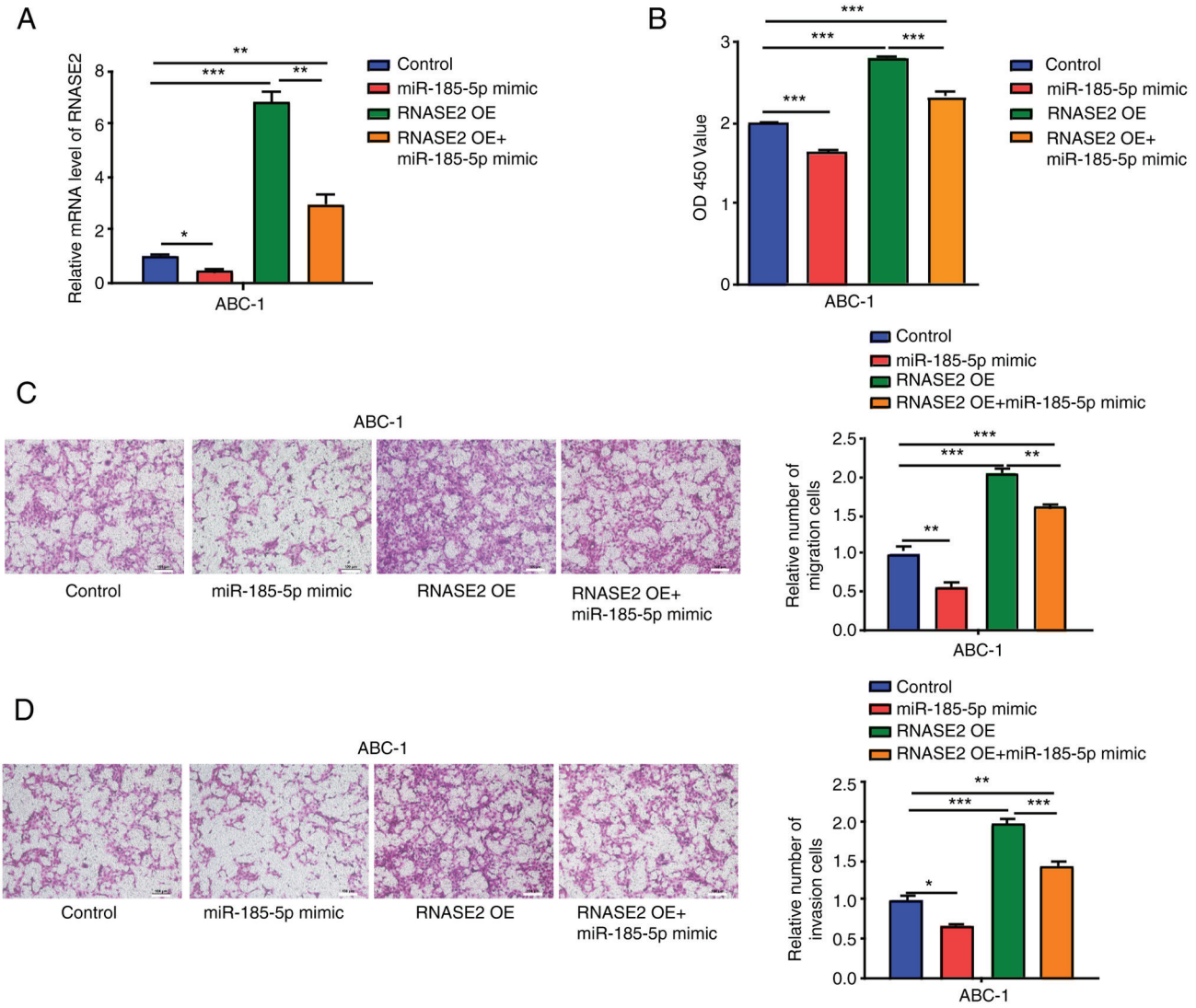

Figure 5. miR-185-5p inhibits the proliferation, migration and invasion of lung adenocarcinoma cells by targeting RNASE2. (A) The expression of RNASE2 in transfected ABC-1 cells. (B) The cell proliferation of transfected ABC-1 cells. The cell (C) migration and (D) invasion of transfected ABC-1 cells. Crystal violet staining; scale bar, 100 μm. *P<0.05, **P<0.01 and ***P<0.001. RNASE2, ribonuclease A family member 2; OE, overexpression; miR, microRNA; OD, optical density.

to proliferate, migrate and invade was promoted by the overexpression of RNASE2 and inhibited by RNASE2 siRNA. In addition, RNASE2 was regulated by miR-185-5p in lung adenocarcinoma cells. miR-185-5p was previously reported to target tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ to regulate non-small cell lung cancer progression (27). miR-185-5p has anticancer effects via the regulation of transgelin 2 in lung adenocarcinoma (21). Ras-associated binding protein 35 acts as a target of miR-185-5p, which subsequently regulates the proliferation, migration and invasion of non-small cell lung cancer cells, particularly through tumor cell-derived exosomes (28). In the present study, RNASE2 was highly expressed in lung adenocarcinoma tissues compared with normal tissues. Through the inhibition of RNASE2, miR-185-5p mimic could suppress the proliferation, migration and invasion of lung adenocarcinoma cells.

The present study has certain limitations. First, *in vivo* experiments were not performed, and these are required to elucidate the mechanism of action of RNASE2. Second, the analysis was based on public data, and clinical specimens should be collected and sequenced to verify the expression of RNASE2. Third, other candidate targets should be studied carefully to elucidate their roles in lung cancer. Fourth, in Fig. 2B, there is late-stage crossover of curves in the graph for RNASE2, and it would be better to re-analyse this dataset, either by restricting the analyzed period of time to exclude this late crossover event, or by using a weighted test, such as Renyi or Cramer-von Mises (29). Finally, genes other than miR-185-5p that may regulate RNASE2 and other miRNAs involved in lung cancer pathogenesis should be further explored.

In summary, the present study showed that miR-185-5p regulates RNASE2 to promote the proliferation, migration and invasion of lung adenocarcinoma cells. RNASE2 is a potential therapeutic target for the treatment of lung adenocarcinoma.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

FL conceived the study idea and designed the experiments. GL, YH, LW, DL and SX performed the experiments. QL, NZ and NG performed the bioinformatics analysis. GL, LZ and YH collected and analyzed the data. GL was a major contributor in writing the manuscript. All authors have read and approved the final manuscript. FL and GL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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