European Review for Medical and Pharmacological Sciences 2020; 24: 8893-8901

MiRNA-488-3p inhibits malignant progression of NSCLC by modulating ADAM9

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Abstract. – OBJECTIVE: The purpose of this study was to investigate the role of microRNA-488-3p in the proliferation, invasion and migration of lung cancer cells and to further explore the potential regulatory mechanisms.

PATIENTS AND METHODS: MicroRNA-488-3p expression in 46 pairs of tumor tissue and paracancerous tissue specimens collected from non-small cell lung cancer (NSCLC) patients were measured through quantitative real-time polymerase chain reaction (qRT-PCR) method, and the interplay between microRNA-488-3p expression and some clinical indicators of these subjects was also analyzed. In addition, microRNA-488-3p overexpression models were constructed in NSCLC cells, and then Cell Counting Kit-8 (CCK-8) test, transwell assays were carried out to evaluate the effect of microRNA-488-3p on the NSCLC cell functions. Furthermore, bioinformatics analysis and luciferase reporter gene assay were carried out to uncover the potential interaction between microRNA-488-3p and its downstream gene ADAM9.

RESULTS: QPCR results revealed that microRNA-488-3p showed a significantly low expression in NSCLC tissue samples than adjacent normal ones. In comparison to patients with high expression of microRNA-488-3, patients with low expression of microRNA-488-3 exhibited higher incidence of lymph node or distant metastasis and lower survival rate. *In vitro* cell experiments showed that in comparison to control group, overexpression of microRNA-488-3p significantly weakened the proliferation ability as well as the invasion and migration of NSCLC cells. Subsequently, a significant increase in ADAM9 expression in NSCLC samples was found, which indicated its negative correlation with microRNA-488-3p. In addition, rescue experiment demonstrated that overexpression of ADAM9 could counteract the impact of microRNA-488-3p upregulation on proliferation and invasion ability of NSCLC cells, and the two may thus together affect the malignant progression of NSCLC.

CONCLUSIONS: It can be concluded that microRNA-488-3p, which is associated with the incidence of metastasis in NSCLC patients, can inhibit the malignant progression of NSCLC cells by modulating ADAM9 expression.

Key Words:
MicroRNA-488, ADAM9, Non-small cell lung cancer, Invasion, Migration.

Introduction

The mortality caused by lung cancer ranks first in the world among cancer-induced deaths, and the areas with high-incidence are mainly located in more developed countries and regions, such as North America, Europe, Australia, and New Zealand. Meanwhile, males are more subjected to this cancer than female[1,2]. There are many risk factors that may contribute to the occurrence of lung cancer, including smoking, ionizing radiation, air pollution, pollution from coal smoke and oil smoke, occupational hazards, carcinogens in food and genetic factors, among which smoking is the most dangerous[1,3,4]. There are few symptoms in the early stage of lung cancer, and most patients who come for symptoms have been in the middle and advanced stage. As a result, early detection, diagnosis and treatment are the keys to improve the cure rate of lung cancer and reduce patients' mortality[1,5,6]. However, so far, there is currently no mature lung cancer screening program in the world[7,8]. In recent years, with the progress in imaging diagnosis and clinical treatment of non-small cell lung cancer (NSCLC), the 5-year survival rate of patients admitted to the hospital with NSCLC that can be radically resected has reached about 25%-30%[9,10]. The 5-year recurrence rate of early NSCLC after radical treatment is 40%, and the overall 5-year survival rate reaches about 15% in developed countries and less than 10% in developing countries, for most patients in advanced stage and losing the opportunity of surgery treatment[11]. Therefore, it is of great significance to search for targets for accurate diagnosis, prognosis evaluation and drug treatment of NSCLC[12,13].

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With the discovery and confirmation of more and more tumor-related molecular markers, researchers have paid more and more attention to the screening of NSCLC markers, some of which have been applied in the clinical diagnosis and NSCLC treatment[13,14]. Currently, the mechanism of the occurrence and development of NSCLC has not been fully uncovered, but more and more evidences indicate that miRNA is engaged in the progression of malignant tumors including NSCLC[15,16]. MiRNAs are a kind of non-coding single-stranded small RNA widely existing in animals and plants, with a total length of 20-24 nt and containing 18-24 nucleotides. They are mainly derived from protein-coding gene sequences or protein-coding intergene sequences, with phosphate groups at the 5' end and hydroxyl groups at the 3' end[17,18]. MicroRNA-488-3p, a member of the miRNA family, is located in the Xp38.13 region of the human chromosome and distributed in clusters[17,18]. Some studies[19-21] have revealed that microRNA-488-3p is abnormally expressed in a variety of solid malignancies, but its mechanism of action in NSCLC remains elusive. [illegible] through bioinformatics analysis, it was hypothesized that microRNA-488-3p may modulate the malignant progression of NSCLC through directly binding to ADAM9, which has been reported to be highly expressed in a variety of malignant tumors and associated with tumor invasion, metastasis and poor prognosis[22,23].

Based on the above results, in this study, we explored the role of microRNA-488-3p and ADAM9 in NSCLC and the potential molecular mechanism, which may contribute to bringing new ideas for the diagnosis and treatment of NSCLC.

Patients and Methods

Patients and NSCLC Samples

Tumor tissue samples and paracancerous tissues of 46 NSCLC patients aged 45-88 years old were collected. The patients did not receive any radiotherapy or chemotherapy before surgery. Pathological classification and staging criteria for NSCLC were performed in accordance with the International Association of Cancer (UICC) lung cancer staging criteria. Patients and their families had been fully informed and signed informed consents. This investigation was approved by the Ethics Committee of Huadong Hospital.

Cell Lines and Reagents

Five NSCLC cell lines (A549, H1299, PC-9, H358, SPC-A1) and one normal human bronchial epithelial cell (BEAS-2B) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), while Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were from Life Technologies (Gaithersburg, MD, USA). Cells were cultured with DMEM medium containing 10% FBS in incubator with 5% CO2 at 37°C.

Transfection

Control (NC mimics) and microRNA-488-3p overexpression sequences (microRNA-488-3p mimics) were designed by Shanghai GenePharma Company (Shanghai, China). Cells were plated in a 6-well plate and grown to a cell density of 70%. Transfection was carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, cells were collected for subsequent experiments.

Cell Counting Kit-8 (CCK-8) Assay

The cells after 48 h of transfection were harvested and plated into 96-well plates at 2000 cells per well. After cultured for 24 h, 48 h, 72 h, and 96 h, the cells were added with CCK-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan), and the optical density (OD) value of each well was measured in the microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 490 nm absorption wavelength after 2 hours of incubation.

Transwell Cell Migration and Invasion Assay

After transfection for 48 h, the cells were trypsinized and resuspended in serum-free medium. Following cell counting, the diluted cell density was adjusted to 3.0×10^5/mL, and the transwell chamber containing Matrigel (Corning, Corning, NY, USA) and no Matrigel was placed in a 24-well plate. Then, 200 µL cell suspension was added to the upper chamber, while 500 µL complete medium was added to the lower chamber. After incubated in a 37°C incubator for 48 h, the chamber was removed, fixed with 4% paraformaldehyde, and stained with crystal violet. Subsequently, the perforated cells stained in the outer layer of the basement membrane of the chamber were observed under the microscope (Nikon, Tokyo, Japan), with 5 fields of view randomly selected.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. QRT-PCR was performed according to the SYBR® Premix Ex Taq™ kit instructions (TaKaRa, Otsu, Shiga, Japan). The primers are as follows: microRNA-488-3p: F: 5'-CGGGGCAGCUCAGUACAG-3', R: 5'-CAGTGCGTGTCGTGGAGT-3', U6: F: 5'-CTGCGAATGGCGTCATTAAATCAG-3', R: 5'-CCGTCGGCATGTATTAGCTCTAG-3', ADAM9: F: 5'-GCTGTCTTGCCACAGACCCGGTATGTGGAG-3', R: 5'-TGGAATATTAAGAAGGCAGTTTCCCTTT-3', glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: 5'-GCACCGTCAAGGCTGAGAAC-3', R: 5'-TGGTGAAGACGCCAGTGGA-3'. Three replicate wells were set for each sample and the assay was repeated twice. Bio-Rad PCR instrument was used to analyze (Bio-Rad, Hercules, CA, USA).

Immunoblot Analysis

The transfected cells were lysed using PROPREPTM protein lysate, shaken on ice for 30 [illegible] and centrifuged at 14,000 × g for 15 min[illegible]. Total protein concentration was calculated b[illegible]S-CLCA Protein Assay Kit (Pierce, Rockford[illegible], USA). Immunoblotting was carried out using s[illegible]pecific antibody against ADAM9 ([illegible] Cruz Bi[illegible] technology, Santa Cruz, CA, [illegible]) [illegible] GAPDH as an internal reference con[illegible]. Finall[illegible]e intensity of protein expressio[illegible]s deter[illegible]ing alpha SP image analysi[illegible] so[illegible].

Dual-Luciferase [illegible]porting A[illegible]

HEK293T c[illegible] seeded in 2[illegible]l plates and co-transf[illegible]d wi[illegible]roRNA-488-3p mimics/NC an[illegible]MIR lucife[illegible] reporter plasmids. Prior to [illegible], the plasmid [illegible]aired with the ADAM[illegible] mutati[illegible] binding si[illegible]e 3′-untranslated regi[illegible]3′-UTR[illegible]y insertion of other wild-type AD[illegible]ar[illegible]e muta[illegible] binding site was constructe[illegible]MIR. [illegible] plasmid was then introl[illegible] into [illegible]sel[illegible]sing Lipofectamine 2000. A[illegible]48 h, [illegible] of firefly luciferase activity wer[illegible]alculated [illegible]or results (Promega, Madison, W[illegible].

[illegible]tistical Analysis

[illegible]tistical analysis was conducted using Statist[illegible] Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA). Univariate analysis was carried out using the χ^2-test and the exact probability Fisher test, and multivariate analysis was performed using COX regression analysis. Besides, patient survival was analyzed through the Kaplan-Meier m[illegible] the intergroup curves were compared[illegible]he Log-rank test. Data were expressed a[illegible]an ± standard deviation, and $p<0.05$ was [illegible]ered to be statistically significant.

[illegible]lts

Downregula[illegible] Micro[illegible]488 in NSCLC T[illegible]s, and Re[illegible] Expressio[illegible]roRNA-48[illegible]p Connec[illegible] wit[illegible]anced Clinical Procedure and Po[illegible]tient Prognosis

[illegible]parison to a[illegible]nt normal tissues, q[illegible] detected a significant reduction in miRNA-488-3p[illegible]pression in NSCLC tissues (Figure 1A). Si[illegible]arly, in NSCLC cell lines, e[illegible]ially in A[illegible] and SPC-A1 cells, microRNA[illegible] showed a lower expression than that in [illegible]2B cells (Figure 1B). According to microRNA-488-3p expression, the NSCLC [illegible]pecimens were divided into high- and [illegible]ression groups to explore the relationship between microRNA-488-3p and the prognosis of patients with NSCLC. Kaplan-Meier survival curves showed that low expression of microRNA-488-3p was remarkably positively correlated with poor prognosis of NSCLC patients ($p<0.05$, Figure 1C). Subsequently, the interplay between microRNA-488-3p level and some clinical indexes, such as age, gender, pathological stage, incidence of lymph node or distant metastasis of NSCLC patients was further analyzed. As shown in Table I, the low expression of microRNA-488-3p was positively correlated with metastasis incidence of patients with NSCLC, but not with other indicators. Therefore, the above observations suggest that microRNA-488-3p may serve as a new biological indicator for predicting a malignant progression of NSCLC.

Upregulation of MicroRNA-488-3p Inhibited Cell Proliferation, Migration and Invasion

To clarify the influence of microRNA-488-3p on the function of NSCLC cells, microRNA-488-3p was overexpressed in NSCLC cell lines (Figure 1D) and cell proliferation and invasion abili-

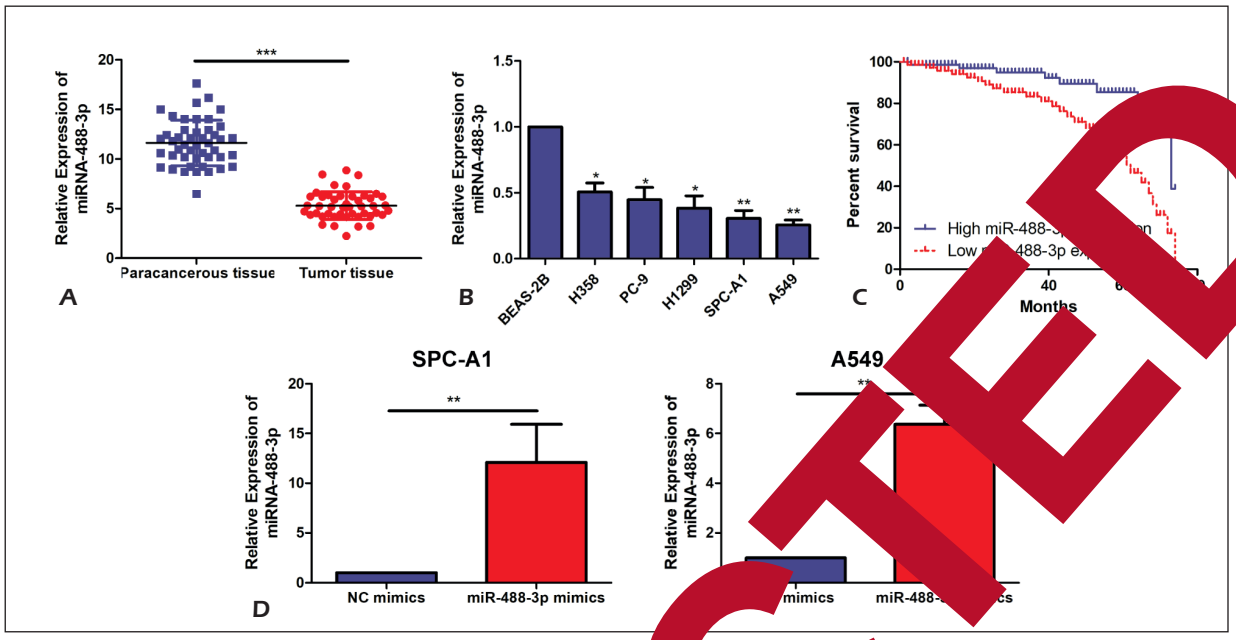

Figure 1. MiR-488-3p is under-expressed in lung cancer tissues cell lines. **A**, q CR is used to detect the differential expression of miR-488-3p in tumor tissues and adjacent tissues o cancer. **B**, qR CR is used to detect the expression level of miR-488-3p in lung cancer cell lines. **C**, Kaplan-Meier su urve of lu ncer patients based on miR-488-3p expression. **D**, qRT-PCR verifies the transfection efficiency after tra d miR-488-3p mimics in A549 and SPC-A1 cell lines. Data are mean ± SD, *p<0.05, ** 0.01, ***p<0.001.

ties were measured. Consequently, CCK-8 a showed that overexpression of microRNA-488 markedly attenuated the prolife apacity NSCLC cells in comparison t l group (Figure 2A), and same tend was rved in cell invasion as well as m ion ab was indicated by trans ll a (Figure 2B).

D was Highly Expressed in NSCLC Tissues and Cell Lines

Bioinformatics analysis revealed that microRNA-488-3p may specifically bind to ADAM9 (Figure 3A). Western Blotting and qPCR detection suggested a significant decrease in both mRNA and protein expression

Table I. Associati A-488-3p exp with clinicopathologic characteristics of non-small cell lung cancer.

Parameters	No. of cases	miRNA-488-3p expression		*p*-value*
		High (%)	Low (%)	
Ag rs)				0.979
	18	11	7	
≥60	28	17	11	
Gender				0.331
	22	15	7	
le	24	13	11	
T				0.067
	28	20	8	
-14	18	8	10	
ymph node metastasis				0.001
	30	24	6	
	16	4	12	
Distance metastasis				0.009
No	35	25	10	
Yes	11	3	8	

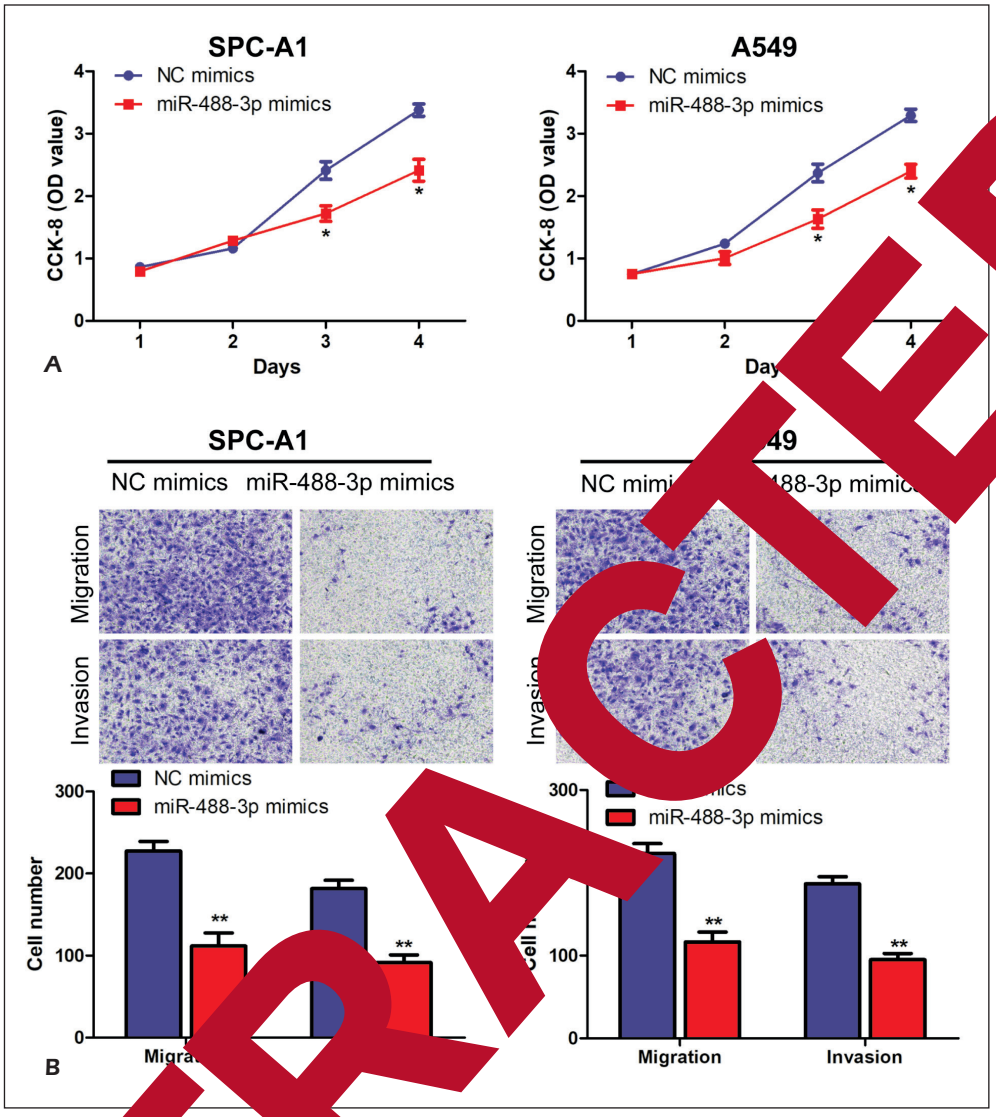

Figure 2. MiR-488 ibits prolifera vasion and migration of lung cancer cells. **A**, The CCK-8 assay detects the effect of overexpr R-488-3p on p ation of lung cancer cells in A549 and SPC-A1 cell lines. **B**, The transwell migration and i n ass ct the invasive ability of lung cancer cells after overexpression of miR-488-3p in A549 and SPC-A1 cell l es (magnifica ×). Data are mean ± SD, *$p<0.05$.

of A49 er ov pression of microR-NA-48 igure 3 Meanwhile, Luciferase er a e d that microRNA-488-3p ca eed co with ADAM9 through spec-ifi quences (Figure 3C and 3D). Moreover, e collected NSCLC tissues or in 49 and SPC-A1 cell lines, ADAM9 showed a her expression than the corresponding nor-nes (Figure 3E and 3F), which means, mi-cro A-488-3p and ADAM9 expression levels were negatively correlated both *in vivo* and *in vitro* (Figure 3G).

MicroRNA-488-3p Exactly Inhibited ADAM9 Expression in NSCLC

Subsequently, co-transfection of microR-NA-488-3p and ADAM9 overexpression vectors were performed to further understand the role of their interaction in NSCLC progression, and mi-croRNA-488-3p transfection efficiency was veri-fied by qPCR (Figure 4A). As a result, upregulation of ADAM9 counteracted the influence of overex-pression of microRNA-488-3p alone on prolifera-tion and metastasis of NSCLC cell, measured by CCK-8 and transwell assays (Figure 4B and 4C).

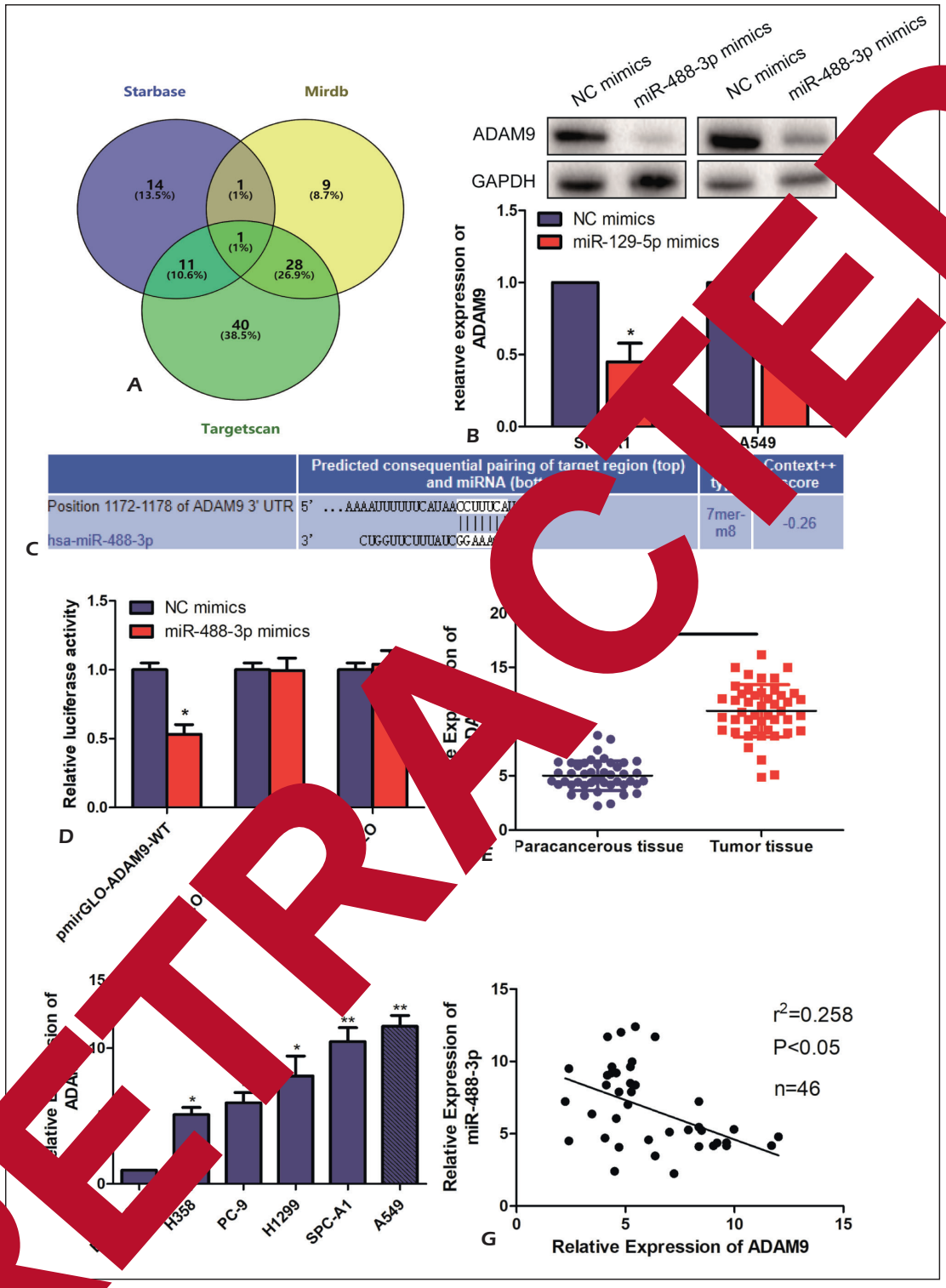

Figure 3. ADAM9 is highly expressed in lung cancer tissues and cell lines. **A**, Bioinformatics suggests potential genes that specifically bind to miR-488-3p. **B**, qRT-PCR and Western Blotting verifies the expression level of ADAM9 after transfection miR-488-3p overexpression vector in A549 and SPC-A1 cell lines. **C**, A sequence in which miR-488-3p specifically binds to ADAM9. **D**, Luciferase reporter gene experiments suggest that miR-488-3p specifically binds to ADAM9. **E**, qRT-PCR is used to detect the difference in expression of ADAM9 in lung cancer tumor tissues and adjacent non-tumor tissues. **F**, qRT-PCR is used to detect the expression level of ADAM9 in lung cancer cell lines. **G**, There is a significant negative correlation between miR-488-3p and ADAM9 expression in lung cancer tissues. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

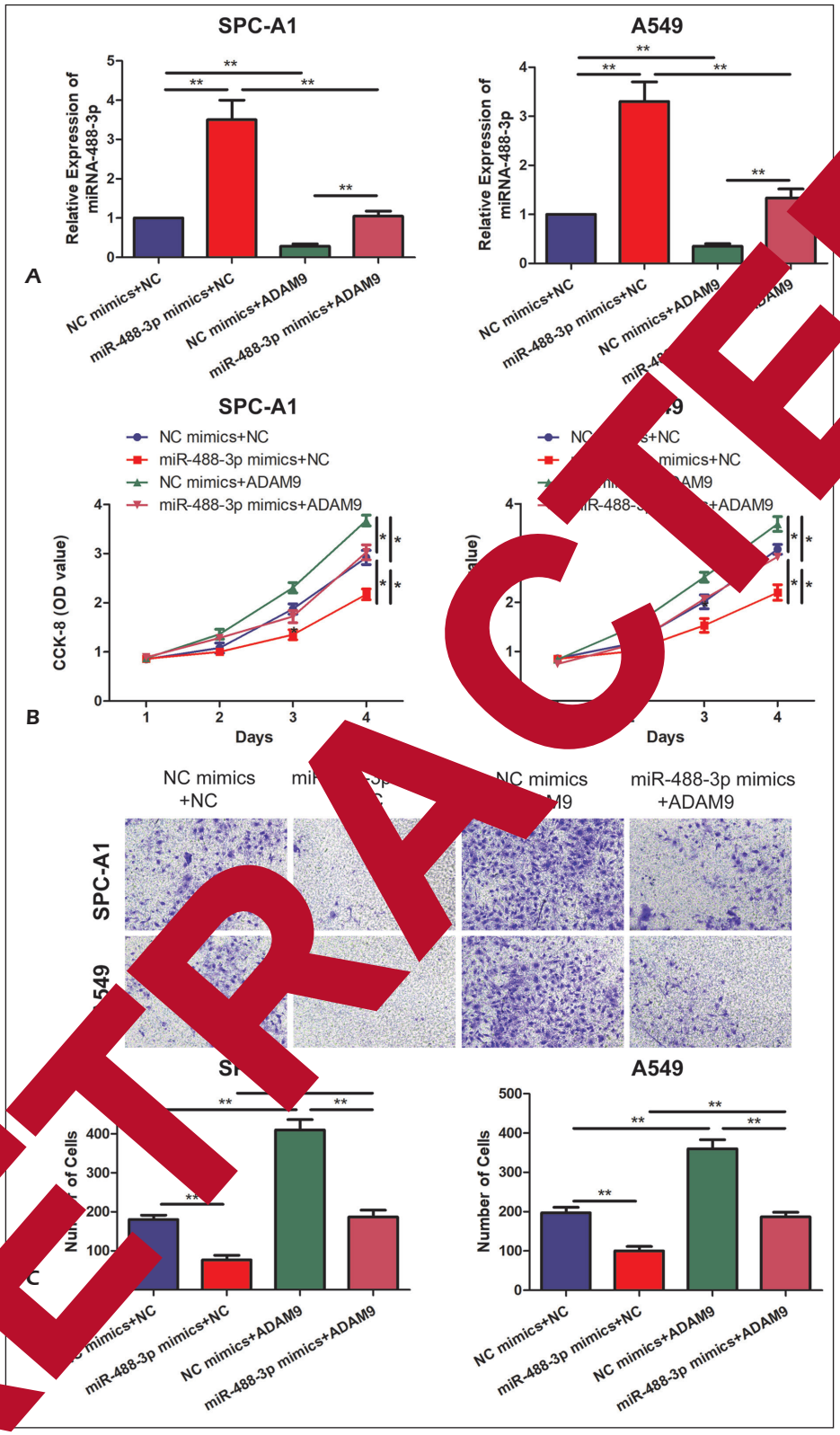

Figure 4. MiR-488-3p regulates the expression of ADAM9 in lung cancer cell lines. **A**, The expression level of miR-488-3p detected by qRT-PCR after co-transfection of miR-488-3p and ADAM9 overexpression vectors in A549 and SPC-A1 cell lines. CCK-8 assay detects the proliferation of lung cancer cells after co-transfection of miR-488-3p and ADAM9 overexpression vectors in A549 and SPC-A1 cell lines. **C**, Transwell migration assay is used to detect the invasion and migration of lung cancer cells after co-transfection of miR-488-3p and ADAM9 overexpression vectors in A549 and SPC-A1 cell lines (magnification: 40×). Data are mean ± SD, *p<0.05, **p<0.01.

Discussion

At present, the main causes of poor prognosis of NSCLC patients are recurrence and metastasis. About 80% of NSCLC patients have been in locally advanced stage or had distant metastasis when they were treated, and postoperative recurrence and metastasis are the primary causes of treatment failure or death[5-8]. With the in-depth study on NSCLC and the molecular mechanism of NSCLC cell invasion and metastasis, a number of tumor molecular markers have been found and confirmed to be applied in the early detection, tumor progression and treatment effect monitoring and prognosis judgment of clinical lung cancer patients[11-13]. However, the current sensitivity and specificity of various NSCLC tumor markers are still not ideal; as a result, more accurate and practical molecular markers need to be found[13,14].

In recent years, microRNAs, as a class of therapeutic and prognostic molecules with important potential application value, have attracted increasing attention[15,16]. MiRNAs are highly conserved non-coding short RNAs composed of 19-24 nucleotides, which mainly bind to the 3'UTRs region of target gene mRNA to degrade it or inhibit its translation process, thereby regulating the expression of target genes[17,18]. Current studies have demonstrated that more than 1/3 of human genes are regulated by miRNA, one miRNA can regulate as many as 200 genes, and one gene can also be regulated by multiple miRNA[16]. Increasing evidence suggests that miRNAs have different functions in different tumors, which are able to promote or inhibit tumor occurrence and development[16-18]. MicroRNA-488-3p is one of the most popular molecules recently and has been proved to play a pivotal role in a number of tumors, such as esophageal cancer and retinoblastoma[19-21]. To explore the role of microRNA-488-3p in NSCLC, here, the expression of microRNA-488-3p in tumor tissues and matching adjacent tissues of 46 patients with NSCLC were detected. It was found that the tumor tissues contained higher microRNA-488-3p levels which were also detected to be associated with the incidence of distant metastasis. Therefore, it was speculated that microRNA-488-3p may serve as a tumor suppressor in NSCLC. Moreover, to further explore the influence of microRNA-488-3p on the functions of NSCLC cells, a microRNA-488-3p overexpression vector was constructed and proved through CCK-8 and transwell experiments that microRNA-488-3p could inhibit the cell proliferation and migration ability of NSCLC cells.

MiRNAs have a variety of biological functions, and the realization of these biological functions mainly depends on the mutual recognition between the 5 '-end "seed sequence" and the 3' -non-coding region of the target gene, and then the degradation of target gene mRNA is through complete complementary pairing[15,16]. In addition, the "seed sequence" of microRNA can also bind to the 3 'non-coding region of target gene in an incomplete complementary form, ultimately achieving the purpose of inhibiting the translation of the target gene[16,18]. In this study, in microRNA-488-3p target gene were further searched through bioinformatics analysis and it was found that it may act on ADAM9 to exert its effects on NSCLC progression. It was reported that dysregulation of ADAM9 expression is closely relevant to the occurrence of various tumors, and its high expression could accelerate the invasion and metastasis of cancer cells[22,23]. Consistent to this, ADAM9 was found to be remarkably increased in the tumor tissues of NSCLC patients. Additionally, it was found that overexpression of microRNA-488-3p remarkably up-regulated ADAM9 mRNA and protein expressions, while overexpression of ADAM9 counteracted the effect of overexpression of microRNA-488-3p on proliferation and invasion and migration of NSCLC cells. The above observations suggested that microRNA-488-3p may inhibit the biological function of NSCLC cells through negatively modulating ADAM9, and thus regulate the malignant progression of NSCLC.

Conclusions

We indicated that microRNA-488-3p expression was remarkably associated with the incidence of lymph node or distant metastasis, and prognosis of patients with NSCLC, which may be involved in the malignant progression of NSCLC by modulating ADAM9.

Funding Acknowledgements

National Youth Science Foundation (No. 81702252); Healthy and Planning Commission Youth Program of Shanghai (No. 20164Y0160); ShengKang developing Program of Shanghai (SHDC12019X11); Healthy and Planning Commission Program of Shanghai (No. 201840188); Shanghai Science of Foundation (18ZR1413000).

Conflict of Interests

The authors declare that they have no conflict of interests.

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