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 cell lines, and then Cell Counting Kit-8 (CCK-8) test and 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 significant lower expression in NSCLC tissue samples than in adjacent normal ones. In comparison to patients with high expression of microRNA-488-3, patients with low expression of microRNA-488-3p 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 ability of NSCLC cells. Subsequently, a significantly increased expression of ADAM9 was found, which indicated a negative correlation between microRNA-488-3p and ADAM9. Overexpression of ADAM9 could counteract the impact of microRNA-488-3p on the 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-488-3p, ADAM9, Non-small cell lung cancer, Invasion and migration.

Introduction

Lung cancer is the most common cancer, accounting for 11.3% of all cancers worldwide. In the world among cancer-induced deaths, 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 females. There are many risk factors that may contribute to the occurrence of lung cancer, including smoking, long-term exposure to occupational hazards, carcinogens in food and genetic factors, among which smoking is the most dangerous. Among males, there are more cases of lung cancer than females. 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%. 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%. However, so far, there is currently no mature lung cancer screening program in the world. 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%. Therefore, it is of great significance to search for targets for accurate diagnosis, prognosis evaluation and treatment of NSCLC.
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. 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. 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. MicroRNA-488-3p, a member of the miRNA family, is located in the Xp38.13 region of the human chromosome and distributed in clusters. Some studies have revealed that microRNA-488-3p is abnormally expressed in a variety of solid malignancies, but its mechanism of action in NSCLC remains elusive. Here, 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.

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.

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, fetal bovine serum (FBS) were from Life Technologies (Gaithersburg, MD, USA). Cells were cultured with DMEM medium containing 10% FBS in an incubator with 5% CO2 at 37°C.

Transfection

Control (NC mimics) and microRNA-488-3p overexpression sequences (microRNA-488-3p mimics) were provided by Shanghai GenePharma Company (Shanghai, China). Cells were plated in a 6-well plate and grown to a cell density of 70%, and 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 3.0×10^3/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 lye 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’-CGGGGCCACGCUCAGUACAG-3’, R: 5’-CAGTGCGTGTCGTGGAGT-3’, U6: F: 5’-CTGCGAATGGCGTCATTAAATCAG-3’, R: 5’-CCGTCGGCATGATTAGCTCTAG-3’, ADAM9: F: 5’-GCTGTCTTGCCACAGACCCGGTATGTGGAG-3’, R: 5’-TGGAATATTAAAGAAGGCAGTTTCCCTTT-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 PRO-PREPTM protein lysate, shaken on ice for 30 min, and centrifuged at 14,000 × g for 15 min at 4°C. Total protein concentration was calculated by NS-CLCA Protein Assay Kit (Pierce, Rockford, IL, USA). Immunoblotting was carried out using specific antibody against ADAM9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with GAPDH as an internal reference control. Finally, the intensity of protein expression was determined using alpha SP image analysis software.

Dual-Luciferase Reporting Assay

HEK293T cells were seeded in 24-well plates and co-transfected with microRNA-488-3p mimics/NC and pMIR luciferase reporter plasmids. Prior to transfect, the plasmid was paired with the ADAM9 mutation binding site 3’-untranslated region (UTR) by insertion of other wild-type ADAM9. The mutation binding site was constructed into pMIR reporter plasmid was then introduced into HEK 293T cells using Lipofectamine 2000. After 48 h, ratios of firefly luciferase activity were measured for results (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analysis was conducted using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA). Univariate analysis was carried out using the χ²-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 method, and the intergroup curves were compared by the Log-rank test. Data were expressed as mean ± standard deviation, and p<0.05 was considered to be statistically significant.

Results

Downregulated MicroRNA-488-3p in NSCLC Tissues and Reduced Expression of MicroRNA-488-3p Connected with Advanced Clinical Procedure and Poor Patient Prognosis

In comparison to adjacent normal tissues, qPCR detected a significant reduction in microRNA-488-3p expression in NSCLC tissues (Figure 1A). Similarly, in NSCLC cell lines, especially in A549 and SPC-A1 cells, microRNA-488-3p also showed a lower expression than that in BEAS-2B cells (Figure 1B). According to microRNA-488-3p expression, the NSCLC tissue specimens were divided into high- and low-expression groups to explore the relation-ship 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 positive-ly 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 pro-gression 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-
ties were measured. Consequently, CCK-8 assay showed that overexpression of microRNA-488-3p markedly attenuated the proliferation capacity of NSCLC cells in comparison to the control group (Figure 2A), and same tendency was observed in cell invasion as well as migration abilities, which was indicated by transwell assay (Figure 2B).

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

**Table I.** Association of miRNA-488-3p expression with clinicopathologic characteristics of non-small cell lung cancer.

<table>
<thead>
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<th>Parameter</th>
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<td>24</td>
<td>13</td>
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<tr>
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</table>
MicroRNA-488-3p and non-small cell lung cancer

of ADAM9 after overexpression of microRNA-488-3p (Figure 3B). Meanwhile, Luciferase reporter assay confirmed that microRNA-488-3p could indeed combine with ADAM9 through specific sequences (Figure 3C and 3D). Moreover, either in the collected NSCLC tissues or in A549 and SPC-A1 cell lines, ADAM9 showed a higher expression than the corresponding normal ones (Figure 3E and 3F), which means, microRNA-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 microRNA-488-3p and ADAM9 overexpression vectors were performed to further understand the role of their interaction in NSCLC progression, and microRNA-488-3p transfection efficiency was verified by qPCR (Figure 4A). As a result, upregulation of ADAM9 counteracted the influence of overexpression of microRNA-488-3p alone on proliferation and metastasis of NSCLC cell, measured by CCK-8 and transwell assays (Figure 4B and 4C).

**Figure 2.** MiR-488-3p inhibits proliferation, invasion and migration of lung cancer cells. A, The CCK-8 assay detects the effect of overexpression of miR-488-3p on proliferation of lung cancer cells in A549 and SPC-A1 cell lines. B, The transwell migration and invasion assays detect the invasive ability of lung cancer cells after overexpression of miR-488-3p in A549 and SPC-A1 cell lines. Data are mean ± SD, *p<0.05.
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 of 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 is detected by qRT-PCR after co-transfection of miR-488-3p and ADAM9 overexpression vectors in A549 and SPC-A1 cell lines. B, 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'UTR 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 shows that miRNAs have different functions in different tumors, which are able to promote or inhibit the occurrence and development of tumors\(^16\)-\(^18\). MicroRNA-488-3p is one of the most popular molecular markers in recent years and has been proved to play a 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 its matching adjacent ones of 46 patients with NSCLC were detected. It was found that the tumor tissues contained higher microRNA-488-3p levels, which was 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 gene in NSCLC. Moreover, to further explore the influence of microRNA-488-3p on the biological 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 through complete complementary pairing\(^15\),\(^16\). In addition, the “seed sequence” of miRNA can also bind to the 3’-non-coding region of the target gene in an incomplete complementary form, ultimately achieving the purpose of regulating the expression of the target gene\(^16\). In this study, microRNA-488-3p target genes were further searched through bioinformatics analysis, and it was found that it may act on ADAM9. 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.

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Conflict of Interests

The authors declare that they have no conflict of interests.
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References


