MicroRNA-484 promotes cell migration and invasion in non-small cell lung cancer

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Xiaoning Yang
wenzhou medical university

Junfeng Ma
wenzhou medical university

Fanghua Gong
wenzhou medical university

✉ gongwenheng@163.comCorresponding Author

Yu Liu
wenzhou medical university

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Abstract
Background Lung cancer is one of the most common causes of cancer-related deaths in the world, and non-small cell lung cancer (NSCLC) accounts for 85% of it. Studies have reported that microRNA-484 (miR-484) plays an important regulatory role in carcinogenesis and cancer development.

Methods 25 clinical NSCLC samples were collected for microRNA array. The function of miR-484 was investigated through Transwell and Migration assays. The expression levels of epithelial-mesenchymal transition (EMT) related factors were assessed by Western blot.

Results miR-484 was up-regulated in tissues from NSCLC patients relative to tumor-adjacent normal tissues. Knocking-down miR-484 expression in A549 cells significantly suppressed tumor cell invasion and migration, suppressed epithelial-mesenchymal transition (EMT) process by increasing the expression of E-cadherin, and decreasing the expression of N-cadherin, vimentin, and MMP2.

Upregulation of miR-484 expression in BEAS-2B normal lung epithelial cells significantly promoted tumor cell invasion and migration, decreased E-cadherin expression levels, and increased N-cadherin, vimentin, and MMP2 expression levels.

Conclusion miR-484 can promote tumor cell invasion and migration in NSCLC and may be a new target for NSCLC treatment.

Background
Lung cancer is one of the most common causes of cancer-related deaths in the world and can be divided into two major types: non-small cell lung cancer (NSCLC), which accounts for 85% of all lung cancers, and small cell lung cancer (SCLC), which accounts for 15% of all lung cancers [1–3]. In recent years, personalized treatments using clinical radiochemotherapy and based on genetic changes have brought significant improvements in lung cancer treatment. However, the 5-year survival rate of patients after surgery is still very low. This is mainly due to a lack of effective diagnostic tools at the early stages and a lack of effective treatment modalities at the advanced stages [4]. miRNAs are 21-25 bp-long endogenous noncoding small RNAs that can regulate target gene expression by binding to 3'-UTR regions [5]. When an miRNA is fully complementary to its target, it induces degradation of the target mRNA. When the miRNA is not fully complementary to its target mRNA, it inhibits its
translation, thereby negatively regulating the expression of target genes at the post-translational level [6, 7]. Studies have found that miRNA-21, miRNA-92a, miRNA-106a, and miRNA-205 are carcinogenic miRNAs that can regulate the expression of the tumor suppressor PTEN to promote the growth, metastasis, and apoptosis of NSCLC cells [8–11]. Similar to these miRNAs, miRNA-155 and miR-134/487b/655 target the suppressor of cytokine signaling 1 (SOCS1) and MAGI2 genes, activate downstream signaling pathways, and inhibit the expression of tumor suppressors, such as PTEN, to promote tumorigenesis [12, 13].

In contrast to the aforementioned miRNAs, miRNA-200c and miRNA-126 can inhibit the expression of their target genes ZEB1 and IRS-1 and the PI3K/AKT signaling pathway to induce apoptosis in tumor cells [14, 15]. Similarly, miRNA-223, miRNA-99a, miRNA-134, miRNA-218-5p, and miRNA-143 can regulate the expression of target genes and relevant signaling pathways to inhibit tumor cell proliferation, invasion, and migration [16–20]. In addition, studies have also found that differences in miRNA expression are not only present between lung cancer and healthy lung tissues, but also in the serum or plasma between lung cancer patients and healthy subjects [21].

Cell invasion and migration are basic steps in NSCLC metastasis, and epithelial-mesenchymal transition (EMT) plays an important role in this process [22]. Changes in the expression of important molecules during EMT include decreased E-cadherin expression, which is the basis for EMT induction and a key step in tumor cell invasion. In addition, EMT also includes the upregulation of the expression of non-epithelial adhesion molecules N-cadherin and vimentin. At this point, the cytoskeleton of tumor cells undergoes changes in its characteristics, such as decreased adhesion, and cells tend to detach from the primary lesion to invade or migrate into surrounding tissues. In addition, during EMT, the secretion and activation of matrix metalloproteinases (MMPs) cleaves the extracellular matrix (ECM), and this plays an important role in cell invasion or migration [23].

Materials And Methods
Study subjects. Tissue samples from NSCLC patients were obtained from the 1st Affiliated Hospital of Wenzhou Medical College. All patients signed the informed consent form, and this study was approved by the Medical Ethics Committee of the hospital. Tissues were rapidly frozen in liquid
nitrigen after collection.

Cell culture. The two cell lines used in this study, the NSCLC cell line A549 and the normal lung epithelial cell line BEAS-2B, were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). A549 and BEAS-2B cells were cultured in DMEM basal media and F-12L basal media, respectively (Gibco, United States) with 10% FBS (Ausbian). All cells were cultured at 37 °C, 5% CO₂, and saturated humidity, and 1% penicillin-streptomycin was added (Invitrogen, Carlsbad, CA). Cells were passaged thrice until they were stable, and transfection was carried out when confluency was 75%.

Transfection. Lentivirus siRNA against miR-484 and miR-484 mimics lentivirus were purchased from Genechem. A549 and BEAS-2B cells were seeded in 6-well plates according to the manual and cultured until a confluency of 15–30% was reached before changing to a 1 mL system containing 3 µL of viruses for infection. After culturing for 72 h, the cells were passaged into a tissue culture flask for expansion culture for subsequent experiments.

Cell invasion assay. A serum-free cell suspension was prepared, and cell density was adjusted to 10⁵/well. Invasion chambers (Corning) were transferred to a fresh 24-well plate, and the culture medium in the upper chamber was carefully removed. Following that, 500 µL of cell suspension was added to the upper chamber, and 750 µL of culture medium containing 30% FBS was added to the lower chamber. After culturing in a 37 °C incubator for 32 h, Giemsa stain (Shanghai Dingguo Biotechnology Co., Ltd.) was added before photographs were taken under a microscope, and the number of invading cells was calculated for each group.

Cell migration assay. A serum-free cell suspension was prepared, and cell density was adjusted to 10⁵/well. The culture medium in the upper chamber was carefully removed. Following that, 100 µL of cell suspension was added to the upper chamber, and 600 µL of culture medium containing 30% FBS was added to the lower chamber. After culturing in a 37 °C incubator for 24 h, chambers that were swabbed clean were placed in 4% paraformaldehyde solution for 0.5 h of fixing. This was followed by Giemsa staining (Sigma) before photographs were taken under a microscope, and the number of
migrating cells was calculated for each group.

Western blotting. 10% SDS-PAGE was used to separate the proteins in the supernatant followed by transfer onto a PVDF membrane. Then, MMP2 (Abcam, 1:1000), E-cadherin (Abcam, 1:10,000), vimentin (Abcam, 1:1000), GAPDH (Abcam, 1:10,000), and N-cadherin (Cell Signaling Technology, 1:1000) primary antibodies were used for labeling before incubation with the corresponding horseradish peroxidase-conjugated secondary antibody. A gel imager and Quantity One analysis system were used for grayscale analysis of the results.

Statistical analysis. All data were expressed as means ± standard deviation. One-way ANOVA and t tests were used for comparisons of sample differences. All data were analyzed using GraphPad Prism 5.0 software. A difference of P < 0.05 was considered to be statistically significant.

Results

miR-484 is upregulated in NSCLC tissues. Quality tests were first carried out on the 25 collected clinical samples, and samples that conformed to miRNA array test requirements were selected. The test result showed that a total of 12 samples conformed to requirements, of which there were 3 each of stage IA, IB, and IIIA, and of paracancerous tissues. The miRNA array data showed that, compared with normal lung tissues, there were 26, 23, and 15 miRNAs that were significantly upregulated in stage IA, IB, and IIIA lung cancer tissues (P<0.05, Fig. 1A; Table 1). Among these miRNAs, the expressions of miR-484 in stage IA, IB, and IIIA NSCLC tissues were higher than in paracancerous tissues by 1.72- (P>0.05), 1.88- (P<0.05), and 1.9-fold (P<0.05) (Fig. 1B).

Underexpression of miR-484 inhibits A549 cell migration and invasion. Lentiviral infection of A549 lung cancer cells was carried out to knock-down miR-484 expression. The results showed that, compared with cells that were infected with control virus (NC-inhibitor), the expression level of miR-484 was reduced by 99% in A549 cells that were transfected with miR-484 inhibitor (P<0.001, Fig. 2A). After lentiviral knock-down of miR-484 expression, the invasion and metastasis capabilities of A549 tumor cells were significantly decreased: the numbers of invading and metastatic cells were decreased by 3.14-fold (P<0.001, Fig. 2B) and 2.3-fold (P<0.001, Fig. 2C), respectively.

Overexpression of miR-484 promotes BEAS-2B cell migration and invasion. To further validate the
inhibitory effects of miR-484 on tumor cell invasion and migration, we overexpressed miR-484 in BEAS-2B normal lung epithelial cells. After BEAS-2B cells were transfected with miR-484 mimic or NC-mimic, real-time PCR showed that the miR-484 expression level in miR-484 mimic-transfected cells was significantly higher than that in the NC-mimic group (P<0.001, Fig. 3A). In addition, the invasion and metastasis capabilities of miR-484-overexpressing BEAS-2B cells were significantly increased: the numbers of invading and metastatic cells were increased by 1.38-fold (P<0.001, Fig. 3B) and 1.68-fold (P<0.001, Fig. 3C), respectively.

EMT-related factors contribute to miR-484-mediated cell migration and invasion. We examined the expression status of EMT-related proteins in order to determine the importance of EMT in tumor cell invasion and migration. Fig. 4A shows that after miR-484 expression was silenced in A549 cells, E-cadherin expression was increased by 1.17-fold (P<0.05), while N-cadherin, vimentin, and MMP2 expression were significantly decreased by 0.78 (P<0.01), 1.47- (P<0.05), and 1.15-fold (P<0.05) in the miR-484 inhibitor group compared with the control group. Following that, we observed the expression status of the aforementioned proteins in BEAS-2B cells after miR-484 overexpression (Fig. 4B). E-cadherin expression was decreased by 2.12-fold (P<0.001), while N-cadherin, vimentin, and MMP2 expressions were increased by 1.27- (P<0.05), 2.1- (P<0.01), and 2.39-fold (P<0.01) in the miR-484 mimic group compared with the control group. This shows that miR-484 can regulate EMT to promote cell invasion and migration.

Discussion

Studies have reported that some tissue-specific miRNAs, circulating miRNAs, and miRNA-derived exosomes can act as potential diagnostic and treatment biomarkers in NSCLC patients [24]. In this study, we tested and analyzed clinical samples and found that miR-484 was significantly upregulated in stage IB and IIIB tumor tissues. This shows that miR-484 may participate in NSCLC development. It is worth noting that miR-484 expression differs in different cancers: in liver cancer, miR-484 expression is significantly upregulated, but it is significantly decreased in gastric cancer [25, 26]. This shows that miR-484 has diverse roles that are determined by the type of tumor, miRNA localization, and its target genes. However, the specific regulatory mechanisms of miR-484 in NSCLC are still
unclear and require further study.

Previous studies have reported that miR-484 directly targets MAP2, which causes downstream ERK1/2 signal transduction to activate cancer stem cell characteristics and promote glioma tumorigenesis [27]. miR-484 can also inhibit Apf-1 expression to promote NSCLC proliferation and inhibit apoptosis in tumor cells to promote NSCLC development [28]. In contrast, miR-484 overexpression in cervical cancer cells targets ZEB1 and SMAD2 expression to inhibit proliferation and simultaneously increase apoptosis and inhibit cell migration, invasion, and EMT [29]. In addition, some studies have reported that certain lncRNAs can regulate miR-484 to affect tumorigenesis. One example is lncRNA ZFAS1, which downregulates miR-484 expression to promote proliferation and invasion of colorectal cancer cells. Another example is lncRNA H19, which negatively regulates miR-484 to promote EMT in lung cancer cells [30, 31]. In this study, we examined the effects of miR-484 on invasion and migration in NSCLC cells. The experimental results showed that not only can miR-484 promote A549 invasion and migration, but it can also promote invasion and migration in BEAS-2B cells. This shows that miR-484 can act as a proto-oncogene to promote NSCLC tumorigenesis. Finally, we focused on explaining how miR-484 regulates invasion and migration at the molecular level. The results showed that miR-484 can regulate EMT-related proteins to promote invasion and metastasis.

In summary, we found that miR-484 is overexpressed in NSCLC tissues and that it can regulate EMT-related proteins to promote invasion and metastasis in NSCLC cells. However, further study is required to determine the target genes and specific molecular mechanisms by which miR-484 acts. In future studies, we will explore the molecular mechanisms of miR-484 action to provide a theoretical basis for its potential clinical applications.

Declarations

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
FHG and YL designed the study, XNY wrote the manuscript and conducted experiments. JFM designed and helped conduct the experiments. All the authors have reviewed the manuscript before submission and have approved the final manuscript.

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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Table
Table 1. Upregulated miRNA expression in NSCLC tissues (P < 0.05).

| Group | miR name       | miR seq                                      | log2 (fold change) |
|-------|----------------|----------------------------------------------|--------------------|
| I A vs AP | hsa-miR-589-5p_R-1 | TGAGAACCACGTCTGCTCTGTA                      | 1.16               |
|       | hsa-miR-29b-3p R-3 | TAGACCAATTGGAATACGAT                        | 1.57               |
|       | hsa-miR-629-3p   | GTTCTTCCAGCTGAGCTGTCA                      | 2.85               |
|       | hsa-miR-200c-3p  | TAATACTGCGGATGATAGG                         | 1.04               |
|       | pal-miR-9298-5p R-5 | AGACATGAACTATTTT                          | 1.92               |
|       | hsa-miR-199b-3p R-1 | ACAGAATTCATCTCCCTGATT                     | 0.53               |
|       | PC-5p-582-5p     | GTTCGTCAGATCTCCCTGATT                     | 2.53               |
|       | hsa-miR-1460-5p R+1 | TGAGAACTGACTCTAGCTAG                      | 0.71               |
|       | hsa-miR-10399-5p R+1 | AATTAACAGATTTGCTACAGAG                    | 1.62               |
|       | hsa-miR-212-5p   | ACCCTTGCTCAGACTGCTACT                     | 1.19               |
|       | hsa-miR-548v     | AGCTACAGTTACCTTTGAGA                      | 1.81               |
|       | pal-miR-9298-p3 1ss1CG | GTGCTGATTTCAACCAGTATT                  | 1.61               |
|       | hsa-miR-155-5p R-1 | TTAATCTGCTACTAGG                         | 1.79               |
|       | hsa-miR-210-5p R-1 | AGACCCCTGCACTAGACGAC                      | 2.15               |
|       | mmu-miR-146a-5p R+1_1ss22TA | TGAGAACTGATTCCTAGG                    | 1.27               |
|       | eca-miR-1543-p3 1ss1GA | ATCTCTAGCTGGTGCAAAACATT                | 1.90               |
|       | hsa-miR-182-3p L+2_1R-1 | TTTAGAGACCGGCTTTCCTT                      | 0.93               |
|       | hsa-miR-10303 L+1 | AAAATCTCCGGGCTGACCTT                      | 0.87               |
|       | hsa-miR-543     | AAAATTTGACAGACTCCGAC                      | 2.01               |
|       | hsa-miR-148a-5pv | TCCCTGTCCTCCAGGAGCTT                      | 1.17               |
|       | efu-miR-9298-p3 1ss18CT | CACAGATGAGAATCTTCTTT                   | 2.34               |
|       | bta-miR-150     | TCTCCTTACACGTGATGTT                       | 1.30               |
|       | hsa-miR-766-3p   | ACTCAGGAGCGAGAGCTTT                      | 1.51               |
|       | hsa-miR-182-3p   | TTTCTTGGAAGCTGGAGG                        | 2.32               |
|       | sha-miR-21 L+2_1R-3 | AATAGGCTTACAGACTGAC                   | 2.53               |
|       | hsa-miR-128-1_5p R-2 | CGGGGCCTGAGACTGCTGTA                     | 2.21               |
|       | hsa-miR-141-5p   | CACTTTCAGTACGTTGTTA                      | 1.47               |
|       | hsa-miR-550a-3p  | TTGTTTACCTCAGGCTGAC                      | 1.80               |
|       | hsa-miR-589-5p R-1 | TGAGAAACAGCTGCTGTTA                   | 1.06               |
|       | hsa-miR-484     | ACCGGTCTTGCAGCTTACT                      | 1.29               |
|       | hsa-miR-224-5p L-1_2R-1 | CAGTACAGTGTTGCTGGTT                     | 0.91               |
|       | mmu-miR-339-5p 1ss23GT | TCCCTGCTCCAGGAGCTTT                      | 1.17               |
|       | bta-miR-339a R+1 1ss22CA | TAGTTTGACAGACTGATGTT                  | 1.50               |
|       | hsa-miR-1304-3p 1ss13CA | TCTCTGAGAACGCTTGTTG                  | 2.99               |
|       | hsa-miR-10309-3p L+1_1ss22CT | TCTCTGAGAACGCTTGTTG                | 1.92               |
|       | hsa-miR-580-3p   | TGAGAACTGACTGCTGTTA                      | 1.32               |
|       | PC-5p-70713_38  | AGCCTGGAAGCTGAGCCTGAG                     | inf                |
|       | hsa-miR-550a-5p R-2 | AGTGCTCTGAGGGGATAGG                    | 1.98               |
|       | hsa-miR-181b-5p  | AACCATCATTCTGCTGCTGCT                     | 1.04               |
|       | hsa-miR-449c-p3  | CAGTCTCGAGCTGAGCCTGCT                    | inf                |
|       | hsa-miR-182-5p   | TTTGGCAATGGTAACTCGAC                      | 2.87               |
|       | hsa-miR-570-3p   | CAAAACACGTCTCAGGACTT                      | 3.02               |
|       | hsa-miR-7-5p     | TGGAAAGACTGTTATTTTGTTG                   | 2.39               |
|       | hsa-miR-548av-5p R+4 | AAAAGTACCTTGGAGTATTG                    | 1.06               |
|       | hsa-miR-4470 1ss21A1 | TGGCAAGCGCTTGGAGCTGAG                    | inf                |
|       | hsa-miR-182-3p L+2R-1 | GGGTTTCTAGCTGAGCTGAG                   | inf                |
|       | hsa-miR-132-5p   | ACCGTGCTTACAGCTGAG                      | 1.46               |
|       | hsa-miR-664a-3p  | TATATGCTTACATCGAGAGGATAGG                | 0.59               |
|       | sha-miR-21 L+2_1R-3 | AAAATGCTTACAGCTGAGAG                   | 2.98               |
|       | hsa-miR-28-5p R-1 | AAGGAGCTCATGCTATT                        | 0.68               |
|       | hsa-miR-974-5p   | TGAGTTGTTGTTGTTGTT                      | 1.10               |
|       | hsa-miR-301a-5p  | CGCTGAGCTTATTTGCTCTGAGA                  | 0.92               |
|       | hsa-miR-21-3p    | CAACACGCTGCTGAGCCTG                     | 2.87               |
|       | hsa-miR-301a-5p  | CGCTGAGCTTATTTGCTCTGAGA                  | 1.52               |
|       | hsa-miR-502-5p L+1R+2 | AATCCCTGCTTCTGCTGCTGAG                   | 1.22               |
|       | PC-3p-35609_123  | AGATCGGCTTGGAGCCTA                      | 2.83               |
|       | hsa-miR-92b-5p R+2 | AGGGACGAGCCGGTGCTGAGTT                   | 2.17               |
|       | hsa-miR-92b-5p   | TATTGAGACTGCTGCTGAGAG                   | 1.77               |
|       | hsa-miR-651b-5p R-1 | TTTGGAGTATCGAGCTGAGAG                   | 1.98               |
### Figures

#### Figure A

A bar chart showing the number of miRNAs. The x-axis represents different groups, and the y-axis represents the number of miRNAs. The chart shows a comparison of miRNA counts across various samples.

#### Figure B

The color scale represents the expression levels of miRNAs. The table below includes the groups, their expression levels (up/down), log2 fold changes, and p-values.

| Group | up/down | log2(fold_change) | P value |
|-------|---------|-------------------|---------|
| 1N    |         |                   |         |
| 2N    |         |                   |         |
| 3N    |         |                   |         |
| I B 1 |         |                   |         |
| I B 2 |         |                   |         |
| I B 3 |         |                   |         |
| 1N    |         |                   |         |
| 2N    |         |                   |         |
| 3N    |         |                   |         |
| IIIA 1|         |                   |         |
| IIIA 2|         |                   |         |
| IIIA 3|         |                   |         |
miR-484 expression is higher in NSCLC tissues. A: Number of differentially expressed miRNAs in tumor tissues from patients with different cancer stages as compared with paracancerous tissues. B: miR-484 is overexpressed in tumor tissues. The color table represents the relative expression ratio of every miRNA after standardization (red: high expression level; blue: low expression level).

|          | Change | Ratio 1 | Ratio 2 |
|----------|--------|---------|---------|
| I A_vs AP_ | up     | 0.78    | 0.155   |
| I B_vs AP_ | up     | 0.9135  | 0.01737 |
| IIIA_vs AP_ | up    | 0.9277  | 0.02759 |

Figure 1
Underexpression of miR-484 inhibits A549 cell migration and invasion. A: miR-484 expression status after transfection of A549 lung cancer cells with inhibitors. B-C: Underexpression of miR-484 not only inhibits A549 cell invasion but also A549 cell migration. Cell-permeable staining and optical microscopy were used to count metastatic cells in different fields (magnification: 200×), ***P < 0.001.
Overexpression of miR-484 promotes BEAS-2B cell migration and invasion. A: miR-484 expression status after transfection of BEAS-2B normal lung epithelial cells with miR-484-overexpressing virus. B-C: Overexpression of miR-484 not only promotes BEAS-2B cell invasion but also BEAS-2B cell migration. Cell-permeable staining and optical microscopy were used to count migrated cells in different fields (magnification: 200×), ***P < 0.001.
EMT-related factors contribute to miR-484-mediated cell migration and invasion. A- B: miR-484 inhibitor/miR-484 mimics were used to treat A549 and BEAS-2B cells for 72 h. Western blotting was used to analyze the expression levels of E-cadherin, N-cadherin, vimentin, and MMP2. *P <0.05; ** P <0.01; *** P <0.001.
