The present study aimed to evaluate the expression of miR-4284 in NSCLC tissues and cell lines and further explored the biological functions of miR-4284 in NSCLC progression. Several studies have reported on the enhancing effects of Ultrasound-targeted microbubble destruction (UTMD) on the anti-tumor action of miR-4284 inhibitor in non-small cell lung cancer cells.
UTMD on the functional role of miRNAs in disease progression, UTMD-mediated miR-767 inhibition has been indicated to result in a notable suppressive effect of tumor cell proliferation, migration and invasion, and UTMD has also been revealed to assist the exosome delivery of miR-21, serving a protective role in the heart (14,15). Therefore, the present study further compared the differences in biological functional changes between UTMD-mediated and conventional transfection of miR-4284 in NSCLC cells. The results may provide a theoretical basis for UTMD-mediated targeted therapy of miRNAs in NSCLC.

Materials and methods

NSCLC tissue collection. Between May 2017 and May 2019, a total of 65 patients with NSCLC underwent surgical tumor resection at Zibo Central Hospital (Shandong, China) and were diagnosed by histopathological examination. Cancer tissues and matched non-cancerous tissues were collected from each patient and all tissues were frozen in liquid nitrogen and stored at -80°C for further use. Patients who received preoperative treatment were excluded from the study. The protocols for tissue collection and analysis were approved by the Ethics Committee of Zibo Central Hospital (Shandong, China; approval no. 170239) and written informed consent was provided by the patients prior to sampling.

Cell culture and conventional cell transfection. NSCLC cell lines, including SK-MES-1, A549, NCI-H460 (RRID: CVCL_0459) and H522, and the normal lung cell line NHBE were obtained from the Cell Bank of the Chinese Academy of Sciences. All cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO₂ at 37°C.

In this study, cell transfection was used to achieve the regulation of miR-4284 in vitro. The cell lines A549 and H460 were selected to perform the transfection experiments due to significantly higher expression of miR-4284 in these two cell lines compared with that in the normal cells. Inhibitor negative control (NC) and miR-4284 inhibitor were synthesized from Gene Pharmaceuticals and transfected into A549 and H460 cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells treated with transfection reagents alone were set as a mock group. After 48 h of transfection, the cells were used for further analysis.

Preparation of microbubbles and cell transfection. The method of obtaining microbubbles was according to the protocol of a previous study (16). It was performed by sonication of 0.4 mg/ml 1,2-distearoyl-3-trimethylammonium propane (Avanti Polar Lipids, Inc.) with 1 mg/ml polyethyleneglycol-2000 stearate (Avanti Polar Lipids, Inc.), 2 mg/ml distearoyolphosphatidylcholine (Avanti Polar Lipids, Inc.) and perfluoropropane gas. miR-4284 inhibitor (5'-AUUGGGGUAGUGAGCCC-3') or non-targeting inhibitor-NC (5'-CAGUACUUUUGUGUAGUAA-3') was incubated with the microbubbles for 30 min at 37°C. According to the manufacturer's protocol, the mixtures were added to A549 and H460 cells and transfected with Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.).

RNA extraction and reverse transcription-quantitative PCR. Total RNA was isolated from fresh tissue samples and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reversed transcribed into single-stranded complementary DNA with the PrimeScript reverse transcriptase kit (Takara Bio, Inc.), according to the manufacturer's protocol. The expression levels of miR-4284 were determined by quantitative PCR with the SYBR-Green I Master Mix kit (Invitrogen; Thermo Fisher Scientific, Inc.) on a 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 10 min; followed by 40 cycles at 95°C for 20 sec, 60°C for 15 sec and 72°C for 20 sec. U6 was used as an endogenous control for miR-4284. The final expression value was calculated using the 2^ΔΔCq method (17). The sequences of primers used were as follows: miR-4284 forward, 5'-GCCGAGGGGCTCACATCACCC-3' and reverse, 5'-CTCAACTGGTGTCGTGGA-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCAGAATTTGCGT-3'.

Cell Counting Kit 8 (CCK-8) assay. After cell transfection, the effect of miR-4284 on the proliferation of A549 and H460 cells was detected using a CCK-8 assay. Cells were seeded into a 96-well plate (3x10^4 cells/well) and incubated for 0, 24, 48 or 72 h. Subsequently, 10 µl of CCK-8 reagent (Beyotime Institute of Biotechnology) was added to each well and the plates were further incubated for 2 h. Cell proliferation was quantified by determining the optical density at 450 nm using a microplate reader.

Transwell assay. Transwell chambers (Corning, Inc.) were applied in the present study for the measurement of cell Table I. Demographics and clinical characteristics of patients with NSCLC.

| Characteristics         | Patients with NSCLC (n=65) |
|-------------------------|-----------------------------|
| Age, years              | 59.8±13.9                   |
| Tumor size, cm          | 3.9±1.7                     |
| Sex                     |                             |
| Male                    | 41                          |
| Female                  | 24                          |
| Smoking status          |                             |
| Never                   | 22                          |
| Current/ever            | 43                          |
| Differentiation         |                             |
| Well/moderate           | 39                          |
| Poor                    | 26                          |
| TNM stage               |                             |
| I-II                    | 37                          |
| III-IV                  | 28                          |

Data are presented as the mean ± SD or count. TNM, tumor-node-metastasis; NSCLC, non-small cell lung cancer.
migration and invasion of NSCLC cells. Transwell chambers precoated with Matrigel (Corning, Inc.) were used for the invasion assay, while chambers without Matrigel coating were used for migration assay. The transfected cells were seeded into the upper chambers with serum-free medium at a density of $3 \times 10^5$ cells/chamber, while the lower chambers were filled with culture medium supplemented with 10% FBS as a chemoattractant. The cells that had transgressed through the filter/membrane to the lower chambers were stained after 48 h of incubation and were counted under an inverted microscope (Olympus Corp.).

Statistical analysis. Values are expressed as the mean ± standard deviation and were analyzed in SPSS 21.0 (IBM Corp.) and GraphPad Prism 7.0 (GraphPad Software, Inc.). A paired Student's t-test was used to compare the differences between two groups and one-way ANOVA followed by Tukey's test was used for differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-4284 in NSCLC tissues and cell lines. The expression level of miR-4284 was evaluated in 65 patients with NSCLC, including 41 males and 24 females with an average age of 59.8±13.9 years (Table I). The results indicated that the expression levels of miR-4284 in NSCLC tissues were significantly upregulated compared with those in matched normal tissues (P<0.001; Fig. 1A). Furthermore, compared with that in the normal NHBE cell line, miR-4284 expression levels were also observed to be increased in four NSCLC cell lines (SK-MES-1, A549, H460 and H522) (all P<0.001; Fig. 1B). In addition, the expression levels of miRNA-4284 were different among patients with different stages of TNM: The level of miR-4284 in patients with stage II was higher than that in patients with stage I and the level in patients with stage III was higher than that in patients with stage II. **P<0.01, ***P<0.001. NSCLC, non-small cell lung cancer; miR, microRNA.

miR-4284 inhibition suppresses cell proliferation, migration and invasion of NSCLC cells. Next, cell experiments were performed to determine the role of miR-4284 in NSCLC. The cell lines A549 and H460 were subjected to cell transfection, as they had significantly higher miR-4284 expression compared with that in the normal cell line NHBE, and among the four cells lines (SK-MES-1, A549, H460 and H522) the expression level of miR-4284 was higher in A549 and H460 cell lines. After transfection with inhibitor, the expression levels of miR-4284 in the A549 and H460 cell lines decreased compared with those in the corresponding negative controls (all P<0.01; Fig. 2A). A cell proliferation assay indicated that decreased expression of miR4284 inhibited cell proliferation (all P<0.05; Fig. 2B). In addition to cell proliferation, the regulatory effects of miR-4284 on the migration and invasion of the A549 and H460 cell lines were further analyzed, revealing that knockdown of miR-4284 inhibited cell migration and invasion (all P<0.05; Fig. 2C and D).

UTMD enhances the cell transfection efficiency of miR-4284. UTMD is able to improve the transfection efficiency of foreign genes into target tissues and organs (6). The effect of UTMD on

Figure 1. Expression of miR-4284 in NSCLC tissues and cell lines. (A) The expression of miR-4284 was increased in NSCLC vs. normal tissues. ***P<0.001 vs. normal tissues. (B) The expression level of miR-4284 was increased in four NSCLC cell lines compared with that in a normal lung cell line. ***P<0.001 vs. NHBE. (C) The expression level of miR-4284 exhibited differences among patients with different TNM stages: The level of miR-4284 in patients with stage II was higher than that in patients with stage I and the level in patients with stage III was higher than that in patients with stage II. **P<0.01, ***P<0.001. NSCLC, non-small cell lung cancer; miR, microRNA.
the transfection efficiency of miR-4284 was thus investigated. In A549 and H460 cells, UTMD significantly increased the inhibitory effects of miR-4284 inhibitor on miR-4284 in both A549 and H460 cells, which manifested as markedly decreased miR-4284 expression levels induced by UTMD-mediated miR-4284 inhibitor compared with the inhibition achieved by miR-4284 inhibitor alone (all P<0.05; Fig. 3).

**Effects of UTMD-mediated miR-4284 transfection on NSCLC cell proliferation, migration and invasion.** As presented in Fig. 4A, UTMD-mediated knockdown of miR-4284 significantly inhibited the proliferation of A549 and H460 cells compared with that in the mock group. As expected, further inhibition of cell proliferation was observed in cells co-transfected with UTMD and miR-4284 inhibitor compared with that in cells transfected with miR-4284 inhibitor alone (all P<0.05). Similarly, further experiments suggested that cell migration and invasion were significantly reduced after in vitro transfection with UTMD-mediated miR-4284 inhibitor compared with those in the mock group and the miR-4284 inhibitor group (all P<0.05; Fig. 4B and C).

**Discussion**

Lung cancer is the most common cause of cancer-related death worldwide (18). The number of patients with NSCLC accounts for 85% of all patients with lung cancer and the 5-year overall survival rate is only 15% (19). NSCLC is the leading cause of malignancy-associated mortality worldwide (20). Lung cancer is a molecularly heterogeneous disease with differences in the growth rate, invasive ability, sensitivity to drugs and prognosis of the tumor during its progression (21). Thus, the heterogeneity provides ample opportunity for multiple treatment approaches and target pathways. MiRNAs are small non-coding RNAs that regulate gene expression at the post-transcriptional level, and increasing evidence suggests that miRNAs are involved in carcinogenesis and the development of human cancers (22,23). For instance, in colorectal cancer (CRC), studies have indicated the tumor suppressor function of miR-421; therefore, the miR-421/matting-type locus (MAT1) axis is expected to be one of the targets for CRC targeted therapy (24). miR-4319 is able to inhibit the proliferation of CRC by targeting ankyrin repeat and BTB/POZ domain containing protein 1 and miR-4319 may become a meaningful treatment for CRC (25). Yang et al (26) confirmed that miR-497 may be used as a biomarker for cancer diagnosis and prognosis and is a promising therapeutic target for future clinical applications. Ji et al (27) studied the effect of UTMD of miR-133a on breast cancer treatment and determined that it inhibited tumor growth and improved the survival rate in a breast cancer model of mice, which may indicate the safety and effectiveness of the UTMD method for
miRNA delivery in the regulation of tumorigenesis. However, the role of miR-4284 in NSCLC has remained elusive.

In the present study, it was determined that miR-4284 was aberrantly expressed in NSCLC. Previous studies have indicated that the expression of miR-4284 was altered in various diseases, suggesting that miR-4284 may be involved in their pathogenesis (12,28). For instance, Liu et al (29) reported that bone marrow-derived mesenchymal stem cells
(BMSCs) had a stronger ability to inhibit osteoclastogenesis through the miR-4284/C-X-C motif chemokine ligand 5 axis, which provided a novel perspective on pathological osteogenesis mechanisms of BMSCs. miR-4284 may serve as a tissue and prognostic biomarker for diffuse large B-cell lymphoma (30). In gastric cancer, the expression level of miR-4284 was significantly upregulated, which indicated that it may represent a novel diagnostic and prognostic biomarker for this tumor type (11). In the present study, the expression levels of miR-4284 was revealed to be upregulated in NSCLC tumors and in the A549 and H460 cell lines as compared with those in the corresponding normal control group. In addition, the expression level of miR-4284 exhibited a gradual enhancement with the increase of the TNM stage and was the highest in tumors from stage III patients. Furthermore, cell proliferation assays suggested that the proliferation ability of NSCLC cells decreased after miR-4284 silencing. Cell migration and invasion experiments were further performed, further demonstrating inhibitory effects of miR-4284: When the expression level of miR-4284 decreased, the cell migration and invasion ability declined. Therefore, miR-4284 may have an oncogenic role in NSCLC.

UTMD is a promising targeted gene delivery method that has been successfully applied in the treatment of numerous diseases over the past decade (31). As a potential drug/gene delivery system, UTMD may be used to improve the permeability of biological barriers and enhance the therapeutic effect of tumors (32). For instance, UTMD technology is able to significantly facilitate the co-transmission of gemcitabine and miR-21i and thus provides a promising strategy for the effective treatment of pancreatic cancer (33). In the study of Yang et al (34), miR-let-7b was transfected into ovarian cancer stem cells by flow cytometry, and the results indicated that miR-let-7b transfection efficiency using UTMD was significantly higher. In NSCLC, the expression level of miR-767 could be successfully downregulated by miR-767 inhibitor and UTMD further enhanced the transfection efficiency of miR-767 inhibitor, while downregulation of miR-767 was indicated to inhibit the proliferation, migration and invasion of tumor cells (15). In the present study, UTMD improved the transfection efficiency of a miR-4284 inhibitor in NSCLC cells. UTMD-mediated transfection further promoted the decrease in the proliferation, migration and invasion ability of NSCLC cells induced by miR-4284 inhibitor. This was due to UTMD enhancing the transfection efficiency of the inhibitor of miR-4284 in vitro, which thereby enhanced the efficacy of miR-4284 inhibition to impair the progression of NSCLC.

In conclusion, the present study indicated that the expression of miR-4284 was increased in NSCLC tissues and cell lines compared with that in the corresponding normal controls. Decreased expression of miR-4284 was able to inhibit tumor cell proliferation, migration and invasion. In addition, UTMD enhanced the transfection efficiency of miR-4284 inhibitors, leading to more significant inhibition of the biological function of NSCLC cells. Therefore, miR-4284 may be a potential therapeutic target for NSCLC and UTMD-mediated delivery of inhibitors of miR-4284 may be a promising therapeutic strategy for NSCLC. There are still certain limitations to the present study, such as the lack of elucidation of the detailed mechanism of the role of miR-4284 in NSCLC pathogenesis.

In addition, the present study provided in vitro results and it is esteemed that the effects of UTMD introduction methods in vivo and their detailed molecular mechanisms will be further addressed in depth in future studies.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

PT and WD designed and conceived the study, conducted the clinical studies, analyzed the clinical data and wrote the manuscript. YW conducted the cell experiments and analyzed the cell experimental data. All authors read and approved the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from each patient and the experimental procedures were approved by the Ethics Committee of Zibo Central Hospital (Shandong, China; approval no. 170239).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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