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Original Article

Suppression of *MGAT3* expression and the epithelial–mesenchymal transition of lung cancer cells by *miR-188-5p*

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ABSTRACT

Background: To investigate the effect of *miR-188-5p* overexpression on the invasion and migration of cultured lung cancer cells, and on related cellular mechanisms that underlie epithelial mesenchymal transition (EMT).

Methods: Human lung cancer cell line 95D was transfected with *miR-188-5p* mimic. Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot were performed to quantify the expression levels of genes including *E-cadherin*, *Snail*, *α-SMA*, and *MGAT3*. Changes in cell motility, invasion and proliferation were studied using scratch migration assay, transwell invasion assay, and colony formation assay, respectively. The expression levels of EMT-related proteins and MGAT3 protein were also determined via immunofluorescent staining. The ability of *miR-188-5p* to regulate its target gene, *MGAT3*, was assessed using dual luciferase activity assay.

Results: Lung cancer cell line 95D showed the lowest *miR-188-5p* expression level thus was used in this study. Transfection with *miR-188-5p* mimic significantly suppressed migration, invasion and clonal formation potency of 95D cells. Dual luciferase activity assay implicated that *miR-188-5p* exerts its negative regulatory effect on *MGAT3* expression through recognizing the 3′ untranslated region (3′UTR) of the *MGAT3* gene. Over-expression of *miR-188-5p* in 95D cells also remarkably increased E-cadherin protein expression and decreased the expression levels of Snail and α-SMA, which suppressed the EMT process.

Conclusion: *MiR-188-5p* reduces the expression of *MGAT3* and inhibits the metastatic properties of a highly invasive lung cancer cell line, probably via targeted regulation of EMT process. Further research to explore the potential therapeutic value of *miR-188-5p*, both as a biomarker and as a drug candidate for the management of metastatic lung cancer may be warranted.

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At a glance of commentary

Scientific background on the subject

Lung cancer is among the most common causes of cancer deaths in the world. The high incidence of metastasis is a major contributor to the poor prognosis of patients. miR-188-5p was identified as a lung cancer suppressor, but its role in lung cancer metastasis is not fully understood.

What this study adds to the field

Over-expression of miR-188-5p suppressed the invasion and migration of cultured lung cancer cell line 95D. We observed that the expression of MGAT3, a key component of EMT, was down-regulated by miR-188-5p expression. Our study suggests value in further exploring the potential therapeutic value of miR-188-5p for metastatic lung cancer management.

Background

Lung cancer is among the most common causes of cancer deaths in the United States and around the world [1]. Although major progresses have been made in the development of targeted therapies, the overall survival rate is still unfavorable (5-year overall survival < 20%) [2]. The high incidence of metastasis is a major contributor to the poor prognosis for patients with lung cancer; in one study, > 90% patients with metastatic lung cancer had tumor lesions in the brain, bone or liver [3]. Studies have shown that epithelial–mesenchymal transition (EMT) is a critical cellular mechanism involved in tumor metastasis [4]. Inhibition of EMT process offers a potential therapeutic strategy for the management of tumor progression [5]. Indeed, identification of targets for the purpose of inhibiting EMT is a key active area of current cancer therapeutics research [6–8].
The gene *MGAT3* encodes the glycosyltransferase N-acetylglucosaminyltransferase-III (GnT-III), which has been suggested to be a metastasis suppressor that affects cell adhesion and migration. Transfection of the *MGAT3* gene into B16 mouse melanoma cells with high metastatic capacity has been shown to suppress lung metastasis *in vivo* [9], and increase glycosylation of E-cadherin *in vitro*. E-cadherin is a crucial cell adhesion molecule which is often altered in EMT. Glycosylated E-cadherin exhibited delayed turnover and increased cell–cell aggregation, which thereby might contribute to the suppression of metastasis [10]. A recent study found that *MGAT3* expression was dramatically decreased during EMT, and later recovered when cells returned to an epithelial-like phenotype. This change in *MGAT3* expression leads to a variation in the expression levels of the enzymatic product of GnT-III, and specific modifications of E-cadherin expression levels [11].

MicroRNAs (miRs) are a group of small non-coding RNA molecules associated with the post-transcriptional down regulation of specific genes by turning off corresponding protein expression and promoting mRNA degradation [12]. Since their initial discovery, the roles of miR molecules in tumor biology have been extensively investigated, especially those with a prospect to be used as a pre-diagnosis biomarkers and/or those as a potential therapeutic targets or disease management tools [13]. In particular, the involvement of microRNA (such as miR-145) in lung cancer metastasis has been well characterized [14]. During tumor EMT, miRs were found to perform critical functions, some of which serve as triggers for lung cancer metastasis [15] whilst others act to inhibit metastasis via down-regulating the expression of tumor-related gene [16]. Among the various miR molecules identified to be tumor-associated, *miR*-188 has been previously characterized as a tumor suppressor for non-small cell lung cancer (NSCLC) both *in vitro* in lung cancer cell lines and *in vivo* in a murine lung cancer model. MiR-188 over-expression was found to suppress the proliferation and migration of NSCLC cells, as well as promote cell apoptosis [17]. However, whether or not *miR-188-5p* is involved in the EMT of lung cancer remains unclear.

In this study, we examine the role of *miR-188-5p* in mediating lung cancer cell migration and invasion using a cell culture model. Our results show that over-expression of *miR-188-5p* significantly suppressed the invasion and migration of cultured lung cancer cell line 95D. Mechanistic studies show that *miR-188-5p* strongly suppressed *MGAT3* gene expression, which is associated with an impaired EMT process in tumor cells. To the best of our knowledge, our results provide the first direct evidence for the mechanism of lung cancer inhibition by *miR-188-5p*. In addition, our findings support the potential use of *miR-188-5p* as a biomarker for lung cancer metastasis and underline its role as a potential therapeutic target against metastatic lung cancer.

Materials and methods

Cell culture and transfection

Different lung cancer cell lines such as A549, 95D, H446, H460 were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were kept in RPMI-1640 medium (Gibco, NY, US) containing 10% fetal bovine serum (FBS) and were incubated in a 37 °C chamber with 5% CO_2 perfusion.

Cell transfection was performed using the cationic liposome method and the Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, US) following the manufacturer's instructions. In brief, cells at log-phase were rinsed twice with phosphate buffered saline (PBS), and were treated with 1 mL trypsin. Cell suspensions were prepared, enumerated and adjusted to 5×10^5 cells/mL. Two-milliliter cell suspension was added to a 6-well plate and incubated. Oligonucleotide sequences for *miR-188-5p* mimic (forward, 5′-CAUCC CUUGC AUGGU GGAGG G-3'; reverse, 5′-CUCCA CCAUG CAAGG GAUGU U-3′) and controlled negative control (NC) sequence (forward, 5′-UUCUC CGAAC GUGUC ACGUT T-3'; reverse, 5′-ACGUG ACACG UUCGG AGAATT-3′) were synthesized by GenePharma (Shanghai, China). These were prepared into 20 μM working solution in diethyl pyrocarbonate (DEPC)-

treated water. Twenty-four hours before transfection, 4–5 × 10^5 cells per well were seeded onto 6-well plate. These were cultured using 2 mL antibiotic-free complete medium till reaching 70–90% confluence within 24 h. Cells were left to rest for 4–6 h after transfection (with 50 pmol mimic per 10,000 cells) before being switched to fresh serum-containing medium and used for further experiments.

Scratch assay

Cells were seeded onto 6-well plate at a density of 5 × 10^5 cells per well. When cells reached 90% confluence, serum-free culture medium containing 1 μg/mL mitomycin C was added and incubated for 1 h. Sterilized pipette tip was used to plot three parallel lines to equally divide each well. Floating cells were removed by rinsing with PBS and serum-free culture medium was added for continuous incubation in a 37 °C incubator with 5% CO_2. The migration of cell was observed and images obtained at 0 h and 24 h. The width of scratch and the migration distance were used as markers to evaluate migration. These were measured under AE31 phase-contrast microscope (Motic, Xiamen, China). Each group (*miR-188-5p* mimic transfected group and Negative Control group) had three wells and the experiment was performed in triplicate.

Transwell assay for cell invasion

Transwell chamber was used to examine the migration efficiency of *miR-188-5p* transfected cells. Approximately 2 × 10^4 cells in serum-free medium were seeded onto the upper chamber (pore size 8.0 μm) which was pre-coated with Matrigel (BD Biosciences, US). The lower chamber contained in RPMI-1640 medium with 30% FBS. The chamber was incubated in a 24-well plate for 24 h, and excess cells were rinsed using PBS. Cells were fixed and stained with 0.5% crystal violet for 5 min. Cells undergoing invasion were imaged and counted under an inverted microscope (AE31 Motic, Xiamen, China).

Colony formation assay

Cells were digested by trypsin and were centrifuged for 3 min, and re-suspended into 1 mL complete medium. Cells were then enumerated and seeded onto 35-mm culture dish (300 cells per dish) and incubated. When visible colonies were observed, cells were rinsed twice with PBS, and fixed in 4% paraformaldehyde for 20 min at room temperature (RT). Cells were then washed twice with PBS and stained with Wright-Giemsa dye for 5 min. Images were obtained under a microscope, and cell clone was defined as a clone with > 50 cells. Clonal formation rate = (clone number/number of inoculated cells) × 100%.

Dual luciferase activity assay

95D cells were cultured till 90% confluence. These were rinsed twice with PBS, and digested with 0.25% trypsin. Cells were collected and centrifuged for 3 min. One-milliliter complete medium was added for enumeration. The cells were seeded onto 12-well plate with three wells for each group and incubated. After 24 h, reporter gene plasmid (pmirGLO, Item nr E133A, Promega, Wisconsin, USA), *miR-188-5p* mimic (forward, 5′-CAUCC CUUGC AUGGU GGAGG G-3'; reverse, 5′-CUCCA CCAUG CAAGG GAUGU U-3′), or NC sequence (forward, 5′-UUCUC CGAAC GUGUC ACGUT T-3'; reverse, 5′-ACGUG ACACG UUCGG AGAATT-3′) was used for transfecting cultured cells. Twenty-four hours after transfection, cells were collected by centrifugation, mixed with 0.5 mL lysis buffer and incubated for 15 min at RT. 50 μL IARII buffer was added into each tube. After adding 10 μL test sample, firefly luciferase activity was measured (E1910, Promega, US). By adding 50 μL Stop Glo reagent, Renilla luciferase activity was measured. The ratio of firefly and renilla luciferase activity was then calculated as the internally calibrated activity of the reporter.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cultured cells were lysed and RNA was extracted using extraction kit (TIANGEN, Beijing, China). cDNA was synthesized using a reverse transcription kit following the instructions in the manual (BioTeke, Beijing, China). Real-time PCR analysis was performed using the specific primers listed in Table 1. PCR conditions were: denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s

Table 1 qRT-PCR primer sequences.

Name	Sequence (5′-3′)	Tm (°C)
miR-188-5p F	CGATATTCATCCCTTGCATGGT	61.4
miR-188-5p R	GTGCAGGGTCCGAGGTATTC	59.2
U6–F	CTCGCTTCGGCAGCACA	60.4
U6- R	AACGCTTCACGAATTTGCGT	59.7
MGAT3 F	CCGCAGGATGAAGATGAGAC	57.6
MGAT3 R	AGTGGGAGTAGAGTGGGGTA	53.3
E-cadherin F	CAGGTCTCCTCTTGGCTCTG	57
E-cadherin R	GACCGGTGCAATCTTCAAAA	58.4
α-SMA F	CCTGAAGAGCATCCCACCCT	61.2
α-SMA R	ACCATCTCCAGAGTCCAGCACG	63.7
snail F	CCATTTCTGTGGAGGGAGGG	61.4
snail R	CCAGTGAGTCTGTCAGCCTTTGT	60.9
β-actin F	CTTAGTTGCGTTACACCCTTTCTTG	62
β-actin R	CTGTCACCTTCACCGTTCCAGTTT	64.4

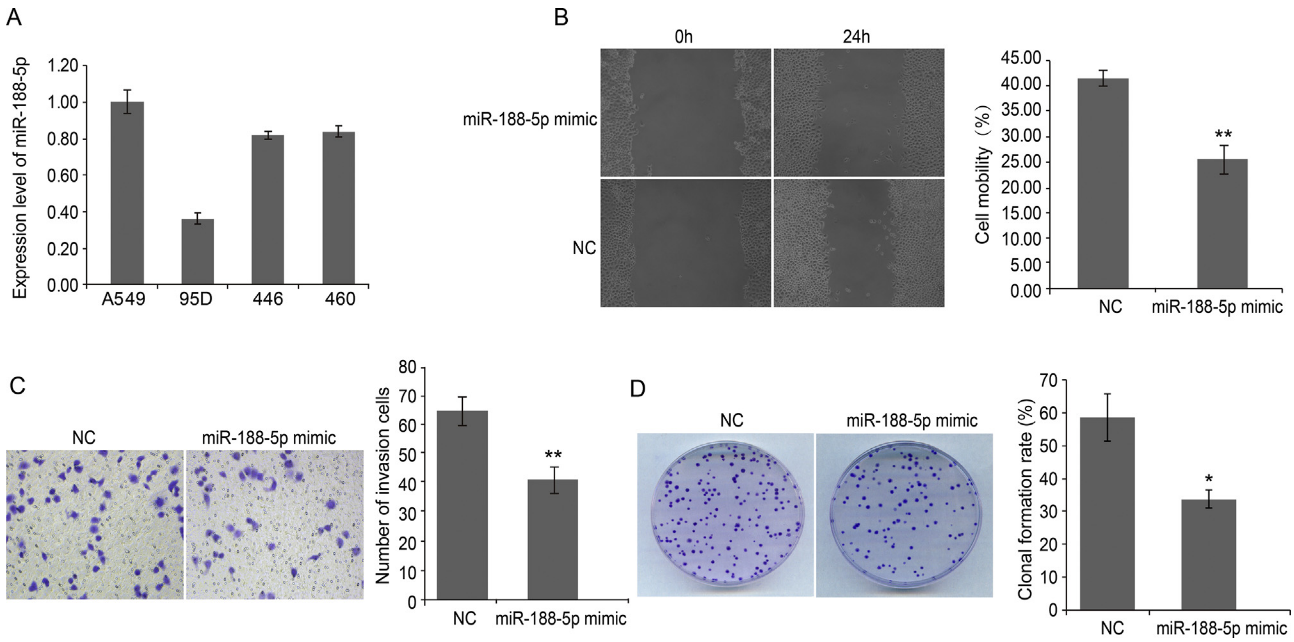

Fig. 1 ***miR-188-5p* mediates invasion and migration of lung cancer cells.** (A) Relative expression level of *miR-188-5p* (with *U6* as endogenous control) across four human lung cancer cell lines (n = 3, one-way ANOVA). (B) Representative images of scratch assay showing cell migration (left panels) and statistical analysis of cell mobility after *miR-188-5p* mimic transfection (right panel, n = 3, Student's t-test, $^{**}p < 0.01$). (C) Transwell assay for cell invasion (left panels) and quantification of invasion cells (right panel, n = 3, Student's t-test, $^{**}p < 0.01$). (D) Representative images of colony formation assay after *miR-188-5p* mimic transfection (left panels), and comparison of clonal formation rate (right panel, n = 3, Student's t-test, $^{*}p < 0.05$).

each, annealing at 60 °C for 20 s, followed by elongation at 72 °C for 30s. Relative expression level of target gene was calculated against *beta-actin* gene, or *U6* (endogenous control for miR) using 2-ΔΔCt approach. The amplified products were quantitated with SYBR Green fluorescence (Solarbio, Beijing, China).

Western blotting

Total proteins were extracted using RIPA lysis buffer. Total proteins were quantified using BCA kit (Wanleibio, Shenyang, China). Proteins were separated by SDS-PAGE, and were transferred onto PVDF membrane (Millipore, Billerica, MA, US). The membrane was blocked in defatted milk powder, and was incubated at 4 °C overnight in primary antibody (mouse anti-E-cadherin at 1:500; rabbit anti-α-SMA at 1:500; goat anti-Snail 1:400 and goat anti-MGAT3 1:200; Wanleibio, Shenyang, China). On the following day, the membrane was rinsed in PBS, and was incubated in secondary antibody (goat anti-mouse IgG-HRP 1:5000; goat anti-rabbit IgG-HRP 1:5000; donkey anti-goat IgG-HRP 1:5000; Wanleibio, Shenyang, China) for 1 h at room temperature. ECL chromogenic substrate (BD Bioscience, US) was added for development, and was imaged using a gel imaging system. Relative protein expression was quantified against the beta-actin band.

Immunofluorescent staining

Cells were seeded on coverslips and grown until reaching 50–70% confluence. The culture medium was then removed and the cells were rinsed with PBS three times before fixation with in 4% paraformaldehyde for 15 min. After rinsing with PBS (three times, 5 min each), the cells were incubated with 0.1% Triton X-100 at RT for 30 min, followed by three washes of PBS (5 min each). Cells on slides were blocked with 1% BSA for 15 min at RT. Primary antibody (mouse anti-E-cadherin at 1:200; goat anti-Snail at 1:100; rabbit anti-α-SMA at 1:200; and goat anti-MGAT3 at 1:50) was added for overnight incubation at 4 °C. On the following day, excess antibody was removed by rinsing with PBS, and fluorescent -labelled secondary antibody (1:300) was added and incubated at RT for 1 h and subsequently stained with DAPI. Coverslips were mounted and images were obtained under a laser scanning confocal microscope BX3 (Olympus, Tokyo, Japan).

Statistical analysis

All data are presented as mean ± standard deviation. Parametric data were compared by Student's *t*-test while one-way ANOVA with Dunnett's *post hoc* analysis was used for multiple comparisons. $p < 0.05$ was considered indicative of statistically significant between-group difference. All statistical analyses were performed using SPSS software (ver.18) (IBM, US).

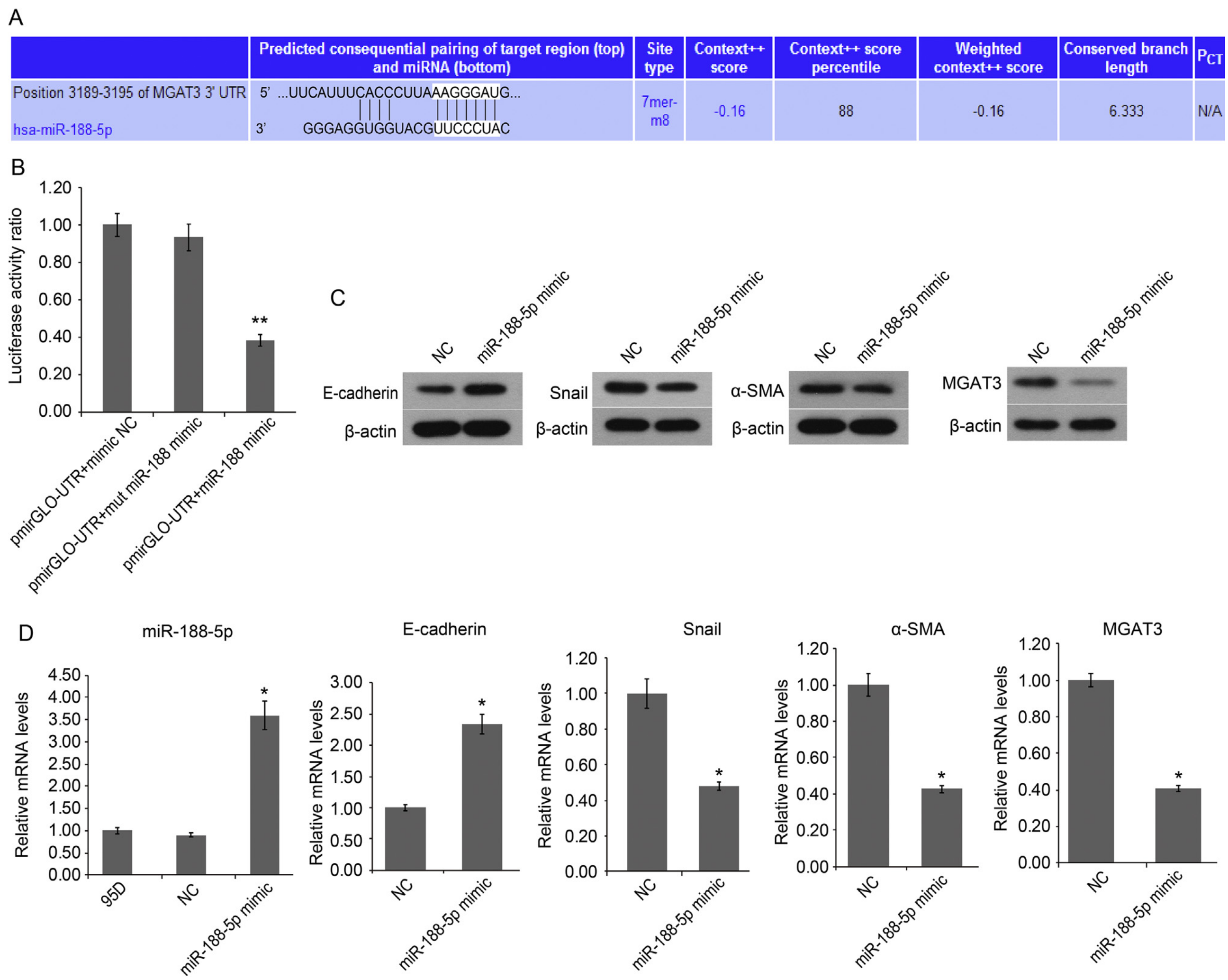

Fig. 2 ***miR-188-5p* targets *MGAT3* gene to mediate EMT of lung cancer cells** (A) Predicted binding sequence between *miR-188-5p* and 3′UTR of *MGAT3* gene; (B) Relative luciferase activity ratio after transfection with *miR-188-5p* mimic (n = 3, one-way ANOVA, $^{**}p < 0.01$); (C) Western blotting of E-cadherin, Snail, α-SMA, and MGAT3 proteins after transfection with *miR-188-5p* mimic. (D) Quantification of mRNA transcript for *miR-188-5p, E-cadherin, Snail, α-SMA* and *MGAT3* genes (n = 3, Student's t-test, $^{*}p < 0.05$).

Results

miR-188-5p *negatively regulates migration and invasion of lung cancer cells*

We first evaluated the expression levels of miR-188-5p in the commonly used lung cancer cell lines such as A549, 95D, H446, H460. qRT-PCR results showed that 95D cell line had the lowest level of *miR-188-5p* expression [Fig. 1A]. Hence, we selected 95D cell line as the *in vitro* model for further assays. After transfection with *miR-188-5p* mimic, 95D cells showed significantly lower migration rate [Fig. 1B]. Transwell assay revealed that the number of invading cells in the *miR-188-5p* mimic transfected group was significantly lower than that in the *miR-188-5p* NC group [Fig. 1C]. Taken together, *miR-188-5p* can inhibit both migration and invasion of lung cancer cells *in vitro*. Furthermore, results of colony formation assay showed that *miR-188-5p* effectively reduced the proliferation of cancer cells [Fig. 1D]. To summarize, these observations strongly support the role of *miR-188-5p* as a tumor suppressor, possibly acting on more than one aspect of tumor development.

Transfection with miR-188-5p *mimic regulates the expression of* MGAT3

Potential binding sites for *miR-188-5p* on *MGAT3* were predicted. The online prediction tools (Targetscan and miRDB) in combination with target sequence database suggested a strong binding site of *miR-188-5p* on *MGAT3* [Fig. 2A], which was later substantiated by dual luciferase activity assay. Using a reporter luciferase gene (in the background of pmirGLO) fused to the 3′UTR of *MGAT3*, we found remarkably decreased

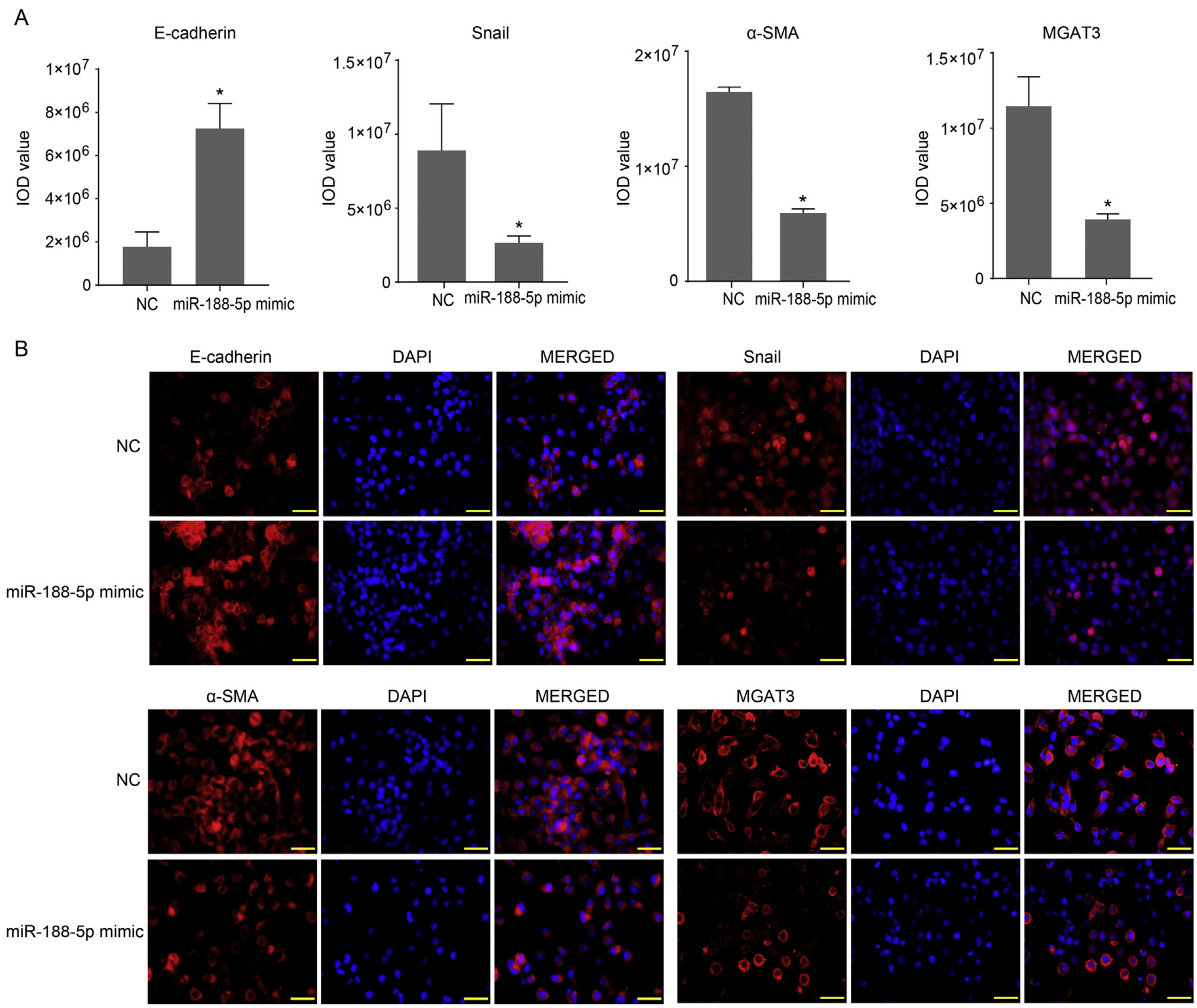

Fig. 3 **Expression of EMT -related proteins in 95D cells** (A) Fluorescent intensity (IOD values) for EMT-related proteins including E-cadherin, Snail, α-SMA, and MGAT3 proteins in 95D cells after transfection with *miR-188-5p* mimic or NC sequence (n = 3, Student's t-test, $*p < 0.05$). (B) Representative immunofluorescent-stained images of EMT and MGAT3 proteins in cultured 95D cells.

luciferase activity ratio after transfection with *miR-188-5p* mimic. However, no significant changes in luciferase activity was observed with the use of mimic NC sequence or a mutant form of 3′UTR sequence [Fig. 2B]. These data suggest that *miR-188-5p* may directly regulate the expression of *MGAT3*.

Transfection with miR-188-5p *mimic regulates the expression of EMT-related proteins via transcriptional mechanisms*

We investigated the effect of *miR-188-5p* on the expression of major proteins involved in the EMT process. Western blotting revealed elevated expression levels of E-cadherin and depressed levels of Snail, α-SMA, and MGAT3 proteins in 95D cells transfected with *miR-188-5p* mimic [Fig. 2C]. Further, qRT-PCR results also confirmed that *miR-188-5p* mimic transfection remarkably, at mRNA level, increased the expression levels of *E-cadherin*, and decreased the expressions of *Snail, α-SMA*, and *MGAT3* gene transcripts [Fig. 2D]. These results collectively showed that *miR-188-5p* inhibited EMT process in lung cancer cells.

We subjected 95D cells to immunofluorescent staining and examined the expressions of EMT-related proteins after transfection with *miR-188-5p* mimic. Consistent with the results of qRT-PCR, 95D cells with *miR-188-5p* over-expression showed prominent up-regulation of E-cadherin. Further, we observed decreased expressions of Snail, α-SMA and MGAT3 proteins [Fig. 3].

Discussion

In sharp contrast to mature somatic cells, tumor cells exhibit unlimited potential for proliferation, ectopic seeding, and unregulated growth capacity, all of which required for tumor metastasis. A complex regulatory network governs the process of tumor metastasis, among which EMT is one of the critical processes that endows cells at the primary cancer site the ability to migrate to distant sites for the formation of secondary lesions [18]. Dysregulation of effective surveillance for tumor metastasis plays a crucial role in oncogenesis. This study found that overexpression of *miR-188-5p* suppressed the migration of cultured lung cancer cells via inhibition of EMT related gene expression. These results are consistent with those of previous studies that revealed the involvement of multiple miR molecules in tumor cell EMT and metastasis [19]. We believe that the findings presented in this study provide the theoretic foundation for developing novel therapeutic options for the management of metastatic lung cancer.

In lung cancer patients, *miR*-188 has been identified as a circulating marker for disease progression, as *miR-188* level showed a direct correlation with patient survival [20]. In this study, we further investigated the molecular mechanisms by which *miR-188-5p* mediates tumor cell biology. Our results supported that *miR-188-5p* inhibited EMT process. After *miR-188-5p* over-expression, we found consistent results such as upregulation of *E-cadherin* and down-regulation of *Snail* and *α-SMA* genes. The interplay between E-cadherin and miR molecules during cancer progression has been previously reported in the literature [21]. Similar miR-directed modulation was also demonstrated for Snail signaling [22] and for α-SMA modulation [23]. In addition, the luciferase assay augmented the notion that *MGAT3* gene expression is inhibited by *miR-188-5p*. As a glycosyltransferase gene, *MGAT3* modulates various cellular events in addition to the regulation of carbohydrate chain function on cell surface. MGAT3 protein was shown to exhibit a strong correlation with tumor metastasis [24]. To the best of our knowledge, no studies have demonstrated that any miR is involved in the post-transcriptional regulation of *MGAT3* gene. A previous study has shown that *MGAT3* can affect the function of E-cadherin proteins during EMT process [11]. The regulatory relationship between E-cadherin and MGAT3 appears to be bidirectional, as E-cadherin has also been shown to regulate *MGAT3* gene transcription [25]. However, while the previous results have shown a decrease in *MGAT3* expression during EMT [11], the results from the present study suggested that low MGAT3 levels were associated with high E-cadherin levels, which leads to suppressed EMT. Our results serve to elaborate the regulatory network for the *MGAT3* gene and provide preliminary insights into the mechanism underlying *miR-188-5p*-induced tumor suppression.

Additional sequence analysis and luciferase reporter-based assays are required to thoroughly investigate the mechanism underlying the regulation of EMT-related genes by *miR-188-5p* and the role of *MGAT3* in this process. Overexpression of *miR-188-5p* did not completely inhibit the migration and invasion of the 95D lung cancer cells, indicating the complex nature of the cognate regulatory mechanisms underlying the process. Many miRNAs have been shown to be involved in the EMT of lung cancer cells, such as the *miRNA-200* family [26]. Next generation sequencing analysis has shown dysregulation of 39 miRNA in NSCLC, where 28 were upregulated and 11 were downregulated [20]. We therefore cannot rule out the possibility that *miR-188-5p* may also directly modulate the expression of other genes associated with EMT process. These questions may be addressed by additional bioinformatics analysis and experimental confirmation such as the luciferase reporter system used in the present study. In addition, comparison of the effects of these different miRNAs as well as studying the possible synergistic effects of their combinatorial applications could be of interest for future research and possibly clinical studies.

Another limitation of the present study is that the effect of *miR-188-5p* overexpression was only studied in 95D cells, which are known to be highly invasive and has been shown in the present study to express a low level of *miR-188-5p*. Further studies that examine the effect of overexpression of *miR-188-5p* on the EMT process in other lung cancer cell lines, as well as inhibition of *miR-188-5p* expression in lung cancer cells with high levels of *miR-188-5p* could offer a more comprehensive understanding of the roles of *miR-188-5p* in lung cancer metastasis.

In summary, our results collectively present a working model wherein *miR-188-5p* suppresses the EMT activity and contributes to reduce tumor cell migration and invasion. This was an *in vitro* study that was inspired by a human study that showed a correlation between *miR-188-5p* and lung cancer metastasis [17,20]; future experiments using animal models are required to further validate the role of *miR-188-5p* in mediating tumor progression and metastasis. These should provide more information regarding metastatic regulation by miR, and help identify therapeutic targets for the management of metastatic lung cancer.

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Conflicts of interest

Authors declare no conflicts of interest.

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