miR-125a-3p targets MTA1 to suppress NSCLC cell proliferation, migration, and invasion

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Abstract

Metastasis-associated gene 1 (MTA1) is associated with cell growth, metastasis, and survival in non-small-cell lung cancer (NSCLC). Several previous reports have demonstrated that microRNAs affect gene expression through interaction between their seed region and the 3′-untranslated region of the target mRNA, resulting in post-transcriptional regulation. The aim of this study was to identify miRNAs that suppress malignancy in NSCLC cells by targeting MTA1. Two human NSCLC cell lines were analyzed for the expression of MTA1 by quantitative RT-PCR and western blotting after transfection with MTA1 mimics. A luciferase reporter assay was established to test the direct connection between MTA1 and its upstream miRNAs. Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, 5-ethynyl-2′-deoxyuridine analysis, and colony formation assay. Cell migration and invasive capacity were evaluated by wound-healing assay and transwell assay. The miRNA/MTA1 axis was also probed by quantitative RT-PCR and western blotting in samples from eight NSCLC patients. Among the candidate miRNAs, miR-125a-3p was shown to post-transcriptionally regulate MTA1 in NSCLC cells. These data were reinforced by the luciferase reporter assay, in addition to the demonstration that MTA1 is inversely correlated with miR-125a-3p in NSCLC tissues. Furthermore, miR-125a-3p was found to inhibit NSCLC cell proliferation, migration, and invasion, through the same mechanisms of down-regulated MTA1. Our report demonstrates that miR-125a-3p inhibits the proliferation, migration, and invasion of NSCLC cells through down-regulation of MTA1, indicating the role of the miR-125a-3p/MTA1 axis in NSCLC, and may provide novel insight into the molecular mechanisms underpinning the disease and potential therapeutic targets.

Key words: miR-125a-3p, metastasis-associated gene 1 (MTA1), non-small-cell lung cancer, proliferation, invasion

Introduction

Lung cancer is the leading cause of cancer mortality worldwide [1], and 80%–85% of lung cancers are non-small-cell lung cancer (NSCLC) [2]. Approximately 56% of lung cancers are diagnosed at a distant stage, while only 15% of patients are diagnosed at a local stage [3]. The overall 5-year survival rate for NSCLC is as low as 17.1% [3]. Metastatic disease seriously impacts the treatment outcome and overall survival rate in NSCLC patients. Further insight into the molecular mechanisms underpinning metastasis is significantly important and may facilitate the identification of new molecular targets for the treatment of the disease.

Metastasis-associated gene 1 (MTA1) was discovered by screening a cDNA library from rat metastatic breast tumors [4]. It is one of the metastasis-related genes which is over-expressed in numerous...
cancers, including NSCLC, colorectal carcinoma, hepatocellular carcinoma (HCC), and breast cancer [5–9]. In the past decades, MTA1 has been confirmed to be a critical component of the nucleosome remodeling and histone deacetylase (NuRD) complex. It is a key regulator of the DNA damage response, and is involved in epithelial-mesenchymal transition (EMT) and inflammation in both transcription-dependent and -independent manners [10]. However, the mechanism of MTA1 deregulation remains to be fully elucidated in order to achieve MTA1-targeted therapies.

MicroRNAs (miRNAs) are a category of small non-coding RNA molecules with 18–23 nucleotides. miRNAs affect gene expression through interaction between their seed region and the 3'- or 5'-untranslated region (UTR) of the target mRNA [11], which leads to mRNA cleavage, translational repression or activation, and the formation of heterochromatin [12]. Numerous studies have shown that different miRNAs in cancer might function either as activators or suppressors of tumors through regulation of different targets. For instance, the miR-200 family was shown to abrogate the ability of cancer cells to undergo EMT [13]. Through modulation of the PTEN signaling pathway, up-regulation of miR-21 in NSCLC might promote the invasion and migration ability of cancer cells [14]. However, the relationship between MTA1 signaling and miRNA remains largely undetermined.

Here, the regulation of MTA1 expression by tumor suppressive miRNAs was characterized by using bioinformatics analysis. Based on two commonly used algorithms, Targetscan and microrna.org, complete complementary sequences to three miRNAs (miR-199a/b-5p and miR-125a-3p) were found in the 3'-UTR of MTA1 [11]. These three candidate miRNAs have been reported to be anti-oncogenically involved in carcinogenesis, invasion, and metastasis [9,15–19]. In this study, miR-125a-3p was found to be a regulator of MTA1 expression through interaction with its 3'-UTR. The miR-125a-3p-induced suppression of the motility and proliferation of NSCLC cells was completely or partially mediated by the silencing of MTA1 expression. Furthermore, MTA1 protein level was found to be inversely correlated with miR-125a-3p expression in NSCLC tissues.

Materials and Methods

Tissue sample

Eight pairs of primary NSCLC tissues and their corresponding normal lung tissue samples were obtained from three squamous cell lung carcinoma patients (one highly differentiated and two moderately differentiated) and five lung adenocarcinoma patients (one highly differentiated, two moderately differentiated, and two poorly differentiated). Consent forms were signed by all patients. The procedures were approved by the Clinical Research Ethics Committee of Nanfang Hospital. None of the patients had been treated with radiotherapy or chemotherapy prior to surgery. All samples were immediately placed in liquid nitrogen and stored at −80°C. Both normal and tumor tissues were verified by histological analysis.

Cell culture

HEK293T, SPC-A-1, and 95D cell lines were purchased from Shanghai Cell Bank of Chinese Academy of Science (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Gaithersburg, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). Cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C.

miRNA mimics and siRNAs

miRNA mimics, small interfering RNA (siRNA) against MTA1 (si-MTA1), and their negative controls were synthesized by GenePharma (Shanghai, China). The sequences were as follows: miR-199a-5p mimic: 5′-CCCAGUUUGCAUUCCGCUUC-3′; miR-199b-5p mimic: 5′-CCAGUGUUAGAUCUGUUC-3′; miR-125a-3p mimic: 5′-ACAGGUGAGUCUGGAGCC-3′; scrambled mimic: 5′-UUCUCGAACUGUCACUGTT-3′ (negative control for miRNA mimics); si-MTA1: 5′-CCAGCAUAGUGACUATTT-3′; and scrambled siRNA: 5′-GACCAGCAUGCUCAAGAUGGdTdT-3′ (negative control for si-MTA1) (Supplementary Table S1).

Transfection

Cells were seeded in 6-well plates and cultured for 18–24 h. Then, cells were transfected with miRNA mimics or siRNAs (100 μM) using Lipofectamine 3000 (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. Co-transfection of miR-125a-3p mimics and si-MTA1 in SPC-A-1 and 95D cells were performed according to the similar procedures. At 48 h post-transfection, the cells were harvested for further analysis.

Western blot analysis

Proteins were extracted from whole cell lysates and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to polyvinylidene fluoride membranes (Merck Millipore, Milford, USA). The following primary antibodies were used: mouse anti-MTA1 (1:1500; Abcam, Cambridge, USA) and mouse anti-β-actin (1:5000; Sigma, St Louis, USA). Membranes were incubated with the horseradish peroxidase-conjugated secondary antibodies (1:5000; Santa Cruz, Santa Cruz, USA). LuminataChemiluminescent Detection Substrates (Merck Millipore) were used to visualize the immunoreactive bands, and the density of each band was analyzed using ImageJ software.

RNA preparation and quantitative real-time PCR analysis

Total RNAs were isolated from cells or tissues using Trizol reagent (Invitrogen). The TaqMan Reverse Transcription Kit (Takara, Dalian, China) was used to obtain cDNA for mRNA detection, whereas TaqMan MicroRNA Reverse Transcription Kit (Takara) was applied to reverse transcribe RNA for miRNA detection. For mRNA and miRNA, real-time PCR was performed using miRscript SYBR Green PCR Kit and SYBR Green PCR Kit (Takara), respectively, on a Roche LightCycler 480 platform (Roche, Basel, Switzerland). The PCR steps included enzyme activation for 10 min at 95°C followed by 40 cycles of 95°C for 15 s and annealing at 60°C for 60 s, according to manufacturer’s protocol. U6 and GAPDH were used as internal controls for miRNA and mRNA analysis, respectively. The primers for miRNAs and mRNAs are listed in Supplementary Table S2. Data were expressed as fold changes relative to GAPDH or U6 and calculated based on the following formula: RQ = 2−ΔΔCt.

Luciferase reporter assay

The pmirGLO vectors containing wild type or mutant putative miR-125a-3p binding site in human MTA1 3'-UTR were synthesized by GenePharma (Shanghai, China). HEK293T or SPC-A-1 cells were seeded into 24-well plates the day before transfection and then cotransfected with 50 ng of wild-type or mutant-type luciferase vector and 20 μM miR-125a-3p mimic or negative control. After 48 h,
luciferase activity was assayed by using the Dual-luciferase Reporter Assay System (Promega, Madison, USA).

Cell proliferation assay
Cells were seeded in 96-well plates at a density of $3 \times 10^3$ cells/well and cultured overnight. After transfection, cells were cultured for 1–5 days. On the indicated days, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml; Sigma) reagent was added to each well and incubated for 4 h. Then, the formazan was dissolved in 150 μl of dimethyl sulfoxide after the removal of the medium. Finally, the absorbance at 490 nm for each sample was measured using a microplate reader (ELx800; BioTek, Winooski, USA).

The 5-ethyl-2′-deoxyuridine (EdU) assay was performed using a Cell-Light™ EdU imaging detecting kit (RiboBio, Guangzhou, China) according to the manufacturer’s recommendations. Forty-eight hours after transfection, NSCLC cells (500 cells/well) were cultured in 6-well plates with DMEM containing 10% FBS. After 10–14 days, the cells were fixed in 4% methyl alcohol and stained with 0.1% crystal violet, and the number of colonies (≥50 cells/colony) was counted.

Invasion and migration assays
For the wound-healing assay, cells were seeded in 6-well plates (5 × 10^5 cells/well) and incubated for 24 h, and then transfected with miRNA mimics, scrambled mimic, si-MTA1, or scrambled siRNA. After 24 h culture in complete culture medium, a pipette tip was used to create a wound. The cells were then cultured in serum-free medium. The ability of wound healing was measured under a microscope (Olympus, Tokyo, Japan) at 0 and 48 h.

The invasion assay was performed in 24-well transwell plates. Briefly, cells (5 × 10^4 cells/well) were re-suspended in serum-free DMEM and grown in the upper chambers with Matrigel-coated membrane (BD Bioscience, San Jose, USA), and 500 μl of DMEM containing 10% FBS was added into the bottom of the chambers. Cells were allowed to migrate through the 8 μm polyethylene terephthalate membrane for 24 h. Cells passed through the membrane were fixed in 4% formaldehyde and stained with 0.1% crystal violet.

Statistical analysis
The data were expressed as the mean ± SD of three independent experiments. All statistical analyses were conducted using SPSS 14.0 statistics software package (SPSS Inc., Chicago, USA). A $P$-value of <0.05 was considered to be statistically significant.

Results
MTA1 is a direct target of miR-125a-3p
MTA1 was demonstrated to be an oncogene that plays a pivotal role in NSCLC. To gain further insight into which miRNAs among the three candidate miRNAs regulate the expression of MTA1, qRT-PCR and western blotting were performed to confirm if restoration of
miR-199a-5p, miR-199b-5p, or miR-125a-3p resulted in down-regulation of MTA1 in SPC-A-1 and 95D cells. It was found that none of the three miRNAs had any effect on MTA1 mRNA expression (Fig. 1A), whereas at the protein expression level, MTA1 was significantly repressed in miR-125a-3p transfected cells compared with mock, miR-control, miR-199a-5p, or miR-199b-5p transfected cells (Fig. 1B). These data suggest that miR-125a-3p down-regulated MTA1 expression at the post-transcriptional level.

One putative miR-125a-3p-binding site within the 3′-UTR of MTA1 was predicted by Targetscan and the microrna.org database (Fig. 1C). To prove the direct interaction between MTA1 and miR-125a-3p, we performed luciferase reporter assay in HEK293T and SPC-A-1 cells co-transfected with MTA1 3′-UTR reporter plasmid or its mutant and miR-125a-3p mimic or negative control. The luciferase reporter activity analysis showed that ectopic expression of miR-125a-3p significantly suppressed the luciferase activity of the wild-type vector but not the mutant reporter. These results were reproducible in both HEK293T and SPC-A-1 cells (Fig. 1D) and suggested that miR-125a-3p could bind directly to specific site in the 3′-UTR of MTA1 mRNA.

Knockdown of MTA1 by siRNA elicits similar responses as re-expression of miR-125a-3p

To determine the functional relationship between miR-125a-3p and MTA1 in the proliferation, migration and invasion of NSCLC cell lines, SPC-A-1 and 95D cells were transfected with si-MTA1 plasmid. Results showed that both mRNA and protein levels of MTA1 were greatly decreased (Fig. 2) after transfection. A similar reduction in MTA1 protein expression but not mRNA expression was also observed in cells over-expressing miR-125a-3p (Fig. 2). This was in good agreement with the above results.

Knockdown of MTA1 by siRNA led to decreased cell viability and colony formation rate, which mimicked the inhibitory effect of miR-125a-3p on cell proliferation and further enhanced the effect of miR-125a-3p on suppressing proliferation (Fig. 3A,B). These results were confirmed in the EdU cell proliferation assay (Fig. 3C).

As observed in the wound-healing and transwell assays with or without Matrigel, ectopic expression of miR-125a-3p and knockdown of MTA1 had almost exactly the same inhibitory effect on cell migration and invasion. Notably, the migration and invasion ability of SPC-A-1 and 95D cells co-transfected with miR-125a-3p mimic and si-MTA1 was almost entirely similar to that of cells over-expressing miR-125a-3p or with decreased MTA1 expression (Fig. 4). These observations suggest that down-regulation of miR-125a-3p may stimulate proliferation, migration, and invasion by promoting the expression of MTA1.

Inverse relationship between the expression of MTA1 protein and miR-125a-3p in NSCLC tissues

Owing to the direct negative regulation of MTA1 by miR-125a-3p in vitro, we speculated that an inverse relationship between miR-125a-3p and MTA1 protein expression may exist in NSCLC clinical samples. Therefore, the expression level of miR-125a-3p and MTA1 protein in eight pairs of NSCLC and matched adjacent normal tissues was examined by qRT-PCR and western blot analysis (Fig. 5A,B). Spearman’s correlation analysis revealed a significant negative correlation between the expression of miR-125a-3p and MTA1 in NSCLC tissues ($r = -0.762, P = 0.028$, Fig. 5C), indicating that miR-125a-3p down-regulation was significantly associated with higher MTA1 protein level.
Discussion

MTA1 has been demonstrated to be a metastasis-promoting gene that is over-expressed in various human cancers including NSCLC [20–24]. In previous studies, Sasaki et al. [23] reported that MTA1 expression is associated with an invasive and metastatic phenotype based on microarray data. Most previous studies have focused on the role of MTA1 in tumor progression and clinical outcome of patients with NSCLC. The underlying molecular mechanisms of biological function remain to be fully determined. A single miRNA can target numerous mRNAs [25], whereas the 3′-UTR of a particular mRNA can be bound by multiple miRNAs [26]. This suggests that miRNAs are central regulators of cancer. Xia et al. [27] recently

Figure 3. miR-125a-3p suppresses the proliferation of NSCLC cells by inhibiting MTA1. (A) MTT assay, (B) colony formation assay, and (C) EdU assay were performed in SPC-A-1 and 95D cells co-transfected with miR-125a-3p or miR-NC and si-MTA1 or scrambled siRNA. *P < 0.05, **P < 0.01, ***P < 0.001. N.S., non-significant.
verified that miR-30c could directly target MTA1 in A549 cells. In this study, we performed bioinformatics and biological functional analyses and identified miR-125a-3p as another upstream regulator of MTA1 gene.

The miR-125a family members identified by miRNA microarray analysis include miR-125a-3p and miR-125a-5p, which are derived from 3′ and 5′ ends of pre-miR-125a, respectively [9]. miR-125a has been shown to directly bind to the 3′-UTR the ESRRα transcription factor and repress its expression, resulting in reduced cell proliferation and increased apoptosis in oral squamous cell carcinoma [28]. The loss of miR-125a leads to up-regulation of MMP11 and VEGF-A, thus contributing to increased proliferation, invasion, and metastasis of HCC cells [29]. Meanwhile, it has been demonstrated that miR-125a correlates with the suppression of cell growth by decreasing HuR protein level in breast carcinoma cells [30]. miR-125a-5p might be oncogenic or tumor suppressive depending on specific context. Some studies have proven the oncogenic potential of miR-125a-5p. For instance, forced expression of miR-125a-5p could reduce the expression of TP53 to enhance NPC cell migration and invasion [31]. It could also down-regulate P53, which consequently promotes apoptosis and enhances cell growth and invasion in multiple myeloma cells [32]. It has also been reported that miR-125a-5p is a tumor suppressor in multiple cancers through directly inhibiting the expression of numerous genes like HDAC4, E2F3, Sirtuin7, p53, and TAZ [33–37].

Figure 4. Knockdown of MTA1 mimicked the inhibitory effect of miR-125a-3p on NSCLC cells migration and invasion. Representative images of (A) wound-healing assay (magnification, ×100) and (B) transwell assay (magnification, ×200) and quantification of cell migration and invasion. *P < 0.05, **P < 0.01, ***P < 0.001. N.S., non-significant.
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Figure 5. The expression of miR-125a-3p is inversely correlated with the expression of the MTA1 protein. (A) The related expression of miR-125a-3p in eight paired tissues was presented. NM, normal. (B) Expression of MTA1 in eight pairs of NSCLC and the corresponding adjacent normal tissues was determined by western blotting. (C) Spearman’s correlation revealed an inverse relationship between miR-125a-3p expression and the MTA1 protein level in NSCLC tissues ($r = -0.762, P = 0.028$).

Unlike miR-125a-3p, miR-125a-3p has been commonly identified as a tumor suppressive miRNA which is down-regulated in various human malignancies. In lung cancer cells, it has been suggested that miR-125a-3p decreases the expression of RhoA, a regulator of actin in the cytoskeleton, which therefore represses cell migration [38]. Moreover, over-expression of miR-125a-3p has been shown to result in down-regulation of Fyn and Fyn-downstream genes including FAK, paxillin, and Akt, as well as inhibition of proliferation and migration in prostate cancer cells [39].

In summary, the up-regulation of miR-125a-3p expression was found to result in the down-regulation of MTA1 expression at the post-transcriptional level, and the anti-oncogenic properties of miR-125a-3p in NSCLC were shown to be, at least in part, due to its inhibitory effect on the expression of MTA1. Our results might provide a partial explanation for the up-regulation of MTA1 expression in NSCLC cells, which has been confirmed to contribute to lung carcinogenesis.

Supplementary Data

Supplementary data is available at ABBS online.

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