Metastasis-Associated 1 (MTA1) Gene Expression Promotes Angiogenesis in Mouse Xenografts from Human Non-Small Cell Lung Cancer (NSCLC) Cells

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
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Background: This study aimed to investigate the effects of metastasis-associated 1 (MTA1) gene expression and gene silencing in human non-small cell lung cancer (NSCLC) cells *in vitro* and on angiogenesis in tumor xenografts *in vivo* in nude mice.

Material/Methods: Human H460 and H1299 NSCLC cell lines underwent transfection with lentiviral transfer plasmids (lenti) and short-interfering RNA (si-RNA) and included a control group, a lenti-MTA1 group, a lenti-si-MTA1 group, a lenti control group, and a si-RNA control group. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to detect MTA1 gene expression after cell transfection. MTA1 transfection was more effective in H460 cells, which were selected for further *in vivo* studies. Sixty Balb/c nude mice, containing human H460 cell tumor xenografts, included a control group (N=20), a lenti-MTA1 group (N=20), and a lenti-si-MTA1 group (N=20). Tumor tissue immunohistochemistry was used to detect the expression of MTA1 protein and microvessel density (MVD) using CD31. Western blot was used to quantify the expression of cyclooxygenase-2 (COX-2), angiopoietin 1/2 (Ang1/2), hypoxia-inducible factor 1-α (HIF-1α), and vascular endothelial growth factor (VEGF).

Results: MTA1 silencing with si-RNA significantly reduced the tumor growth rate in nude mice (p<0.01), reduced tumor MVD, and 70% of mice survived for more than 30 days. MTA1 overexpression resulted in the death of all mice at 30 days after tumor inoculation and upregulated the expression of COX-2, Ang1/2, HIF-1α and VEGF, which were down-regulated by MTA1 silencing.

Conclusions: MTA1 gene expression promoted angiogenesis in mouse xenografts from human NSCLC cells.

MeSH Keywords: Carcinoma, Non-Small-Cell Lung • Neovascularization, Physiologic • RNA, Small Interfering

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Background

Worldwide, lung cancer is the leading cause of cancer-related mortality, and non-small cell lung cancer (NCSLC), including adenocarcinoma and squamous cell carcinoma, accounts for 85–95% of all cases of primary lung cancer [1]. NCSLC is characterized by a high metastatic potential and resistance to therapy, and no effective treatment is available for late-stage NSCLC [2]. Patients with a diagnosis of NCSLC have a poor 5-year survival rate of approximately 15% [1]. Recent developments in targeted therapy strategies in NSCLC have shown advantages when compared with conventional radiotherapy and chemotherapy treatments, which have high toxicity. Therefore, there remains a need for continued research to identify new molecular targets for the treatment of NSCLC.

All malignant tumors, particularly more aggressive and rapidly growing tumors, rely on angiogenesis, or the formation of new vessels, to promote tumor growth. Therefore, one important strategy for lung cancer treatment is anti-angiogenic therapy [3]. Treatments that target angiogenesis-related signaling pathways or growth factors, including vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR), have now changed the approach to the treatment of NSCLC, to reduce new blood vessel formation in tumors. A significantly improved outcome for patients with cancer has been reported with the use of anti-angiogenic therapy, bevacizumab (Avastin) [4]. However, malignant tumors can develop resistance mechanisms, including metabolic and hypoxic adaptation, that reduces the efficacy of anti-angiogenic therapy [5,6]. Therefore, new biomarkers that target angiogenesis continue to be investigated to improve treatment outcomes in NCSLC.

The metastasis-associated gene 1 (MTA1) is a key gene that has a role in cancer metastasis. MTA1 is part of the nuclear remodeling and deacetylation (NuRD) complex that ubiquitously regulates the expression of genes that enhance the migration and invasion of malignant cells [7]. Upregulation of MTA1 has been reported in a variety of cancers, including colorectal carcinoma [8,9], gastric cancer [10], and laryngeal squamous carcinoma [11]. In lung cancer, MTA1 has recently been identified as a prognostic biomarker that is associated with the poor prognosis in patients with lung cancer [12,13]. Also, improved therapeutic outcome based on anti-MTA1 therapy has been reported [12,14].

Therefore, this study aimed to investigate the effects of metastasis-associated 1 (MTA1) gene expression and gene silencing in human non-small cell lung cancer (NSCLC) cells *in vitro* and on angiogenesis in tumor xenografts *in vivo* in nude mice.

Material and Methods

Cell culture conditions

Human non-small cell lung cancer (NSCLC) cell lines H460 and H1299 were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and were cultured in RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptavidin (100 μg/ml), and maintained in 37°C and 5% CO_2. Cells in the logarithmic growth phase (80% confluence) were used for the experiments.

Plasmid construction and cell transfection

Human H460 and H1299 NSCLC cell lines underwent transfection with lentiviral transfer plasmids (lenti) and short-interfering RNA (si-RNA) and were randomly assigned into a control group, a lenti-MTA1 group (MTA1 group), a lenti-si-MTA1 group (si-RNA group), a lenti-control (NC) group, and a si-RNA control group. The lenti-MTA1, lenti-si-MTA1, and lenti-control vectors were purchased from Shanghai GenePharma Co, Ltd. (Shanghai, China). The sequence of the MTA1 si-RNA was: 5'-GACCACCGACAGATACGTG-3'.

Transfection was mediated by lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) (Cat no. 11668-019). A scrambled si-RNA (5'-GACGACGATAAGGGATCCTGA-3') without homology with the mammalian mRNA sequences, was cloned into the lentivirus vector (GeneChem Co., Ltd. Shanghai, China) as the control si-RNA. Cells were all transfected with 3 μg of plasmid, or empty lentivirus vector, using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The total RNA in the cells was extracted using the TRIzol kit (Takara, Dalian, China). The reverse transcription kit (Applied Biosystems, Waltham, MA, USA) was used to transcribe cDNA, followed by transcription using a reverse transcription kit (Applied Biosystems, Waltham, MA, USA). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using a Mastercycler nexus X2 (Eppendorf, Hamburg, Germany) using the following conditions: 95°C 15 min, 95°C 15 s, 60°C 30 s, 55°C 60 s (35 cycles). Data were processed using the $2^{-\Delta\Delta Ct}$ method and the relative expression levels were calculated using GAPDH as an internal reference.

The primer sequences were as follows:
MTA1 forward: 5'-ACGCAACCCTGTCAGTCTG-3';
MTA1 reverse: 5'-GGGCAGGTCCACCATTTCC-3';
GAPDH forward: 5'-AGCCCATCACCATCTTCCAG-3';
GAPDH reverse: 5'-CCTGCTTCACCACCTTCTTG-3'.

The mouse animal model

Animal experiments were conducted following the guidelines from the National Institutes of Health (NIH) (NIH Pub. No. 85-23, revised 1996) with the Jinan Pengyue Experimental Animal Breeding Co., Ltd. (license number SCXK 20140007 assigned to Dr. Lu). The experimental protocols were reviewed and approved by the Affiliated Yantai Yuhuangding Hospital of the Qingdao University Animal Care and Use Committee. Sixty Balb/c nude mice that were six weeks of age (mean weight, 20±2 gm) were randomly divided into three groups, containing human H460 cell tumor xenografts, including a control group (N=20), a lenti-MTA1 group (N=20), and a lenti-si-MTA1 group (N=20).

H460 cells that were transfected with MTA1 overexpression vectors, were suspected in 0.2 mL PBS and were subcutaneously injected into the mice, and H460 cell transfected with MTA1 si-RNA were also suspended in PBS (3×10^6 cells/ml) applied to the left armpit of the nude mice. Equivalent amounts of untreated cells were also injected as a control. After five days, the mice were observed daily and survival was noted for each group. The tumor size was measured with a Vernier caliper every 2–3 days. The changes in tumor volume within 20 days were observed. The average volume of the tumors in each group was calculated as, volume (mm^3)=(length×width^2)/2. After 20 days, 10 nude mice were randomly selected from each group and anesthetized with 0.3% sodium pentobarbital (45 mg/kg). Tumor weight was measured. The remaining mice were continuously observed for 80 days and the survival rates was noted for each group.

Immunohistochemistry

Tumor tissues were fixed with 4% paraformaldehyde (Beijing Suolebao Biotechnology Co., Ltd.), sectioned at 4 μm, deparaffinized and hydrated using a routine procedure, followed by endogenous peroxidase inactivation and antigen recovery. The MTA1 primary antibody (1: 200) (ab71153, Abcam, Shanghai) was then added and incubated on the tissue sections overnight at 4°C. After washing, a biotin-labeled MTA1 secondary antibody (1: 800) was added using a Histostain-Plus immunohistochemistry kit, horse-radish peroxidase (HRP) broad spectrum (85-9043) (Thermo Fisher, Waltham, MA, USA). The 3,3'-diaminobenzidine (DAB) substrate was added, followed by hematoxylin counterstaining. Tissues were then washed, mounted, and examined by light microscopy (Olympus BX51, Olympus Japan). Immunostaining scores were based on the degree of staining based on five randomly chosen field using the following criteria: 0 – negative, 1 – light yellow, 2 – light brown, 3 – dark brown. The negative immunostaining was given a score of 1 point (0–25%), 2 points (26–50%), 3 points (51–75%), and 4 points (76–100%). Final scores were obtained by adding the scores of the degree of immunostaining and the negative range.

Microvessel density (MVD)

The microvessel density (MVD) was determined based on the protocol described by Weidner et al. [15]. MVD was calculated using CD31 immunohistochemistry for endothelial cells. The high vascular density area (hot-spot) was calculated by light microscopy at a magnification of ×100, and the MVD of five fields was counted at a field magnification of ×400. The mean value was used as the MVD value of the tumor.

Western blot

Protein lysates were used to extract the proteins in the tumor tissues. Protein concentrations were determined by the bicinchoninic acid (BCA) colorimetric protein assay method. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to resolve the proteins, followed by transfer to polyvinylidene difluoride (PVDF) membranes (Merck, Darmstadt, Germany) at 80 V for 30 min. Membranes were blocked with 5% nonfat dried milk powder in Tris-buffered saline (TBS) and Tween 20 (TBST) solution for 1 hr. The primary antibodies included a rabbit polyclonal anti-COX-2 antibody (1: 1000) (ab102005, Abcam) an anti-Ang1 antibody (1: 1000) (Ab183701, Abcam), an anti-Ang2 antibody (1: 500) (ab8452, Abcam), an anti-HIF-1α antibody (1: 1000) (ab82832, Abcam), and anti-VEGF antibody (1: 2000) (ab39256, Abcam), which were incubated on the membranes overnight at 4°C. The membranes were incubated with the horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1: 2000) (Proteintech, Chicago, Ill, USA) for 1 h. The enhanced chemiluminescence (ECL) system was used to detect the signals on the membranes. The protein expression level was standardized with GAPDH, and quantification of band intensity was performed by Image J software (National Institutes of Health, USA).

Statistical methods

Statistical analysis was performed using SPSS version 19.0 statistical software. The results were expressed as the mean ± standard deviation (SD). The t-test was used to compare data between two groups. One-way analysis of variance (ANOVA) was used to analyze the data between multiple groups. The survival curves were analyzed by regression curve-fitting. Follow-up analysis was performed using the least significant difference (LSD) test. The difference was statistically significant at $p < 0.05$.
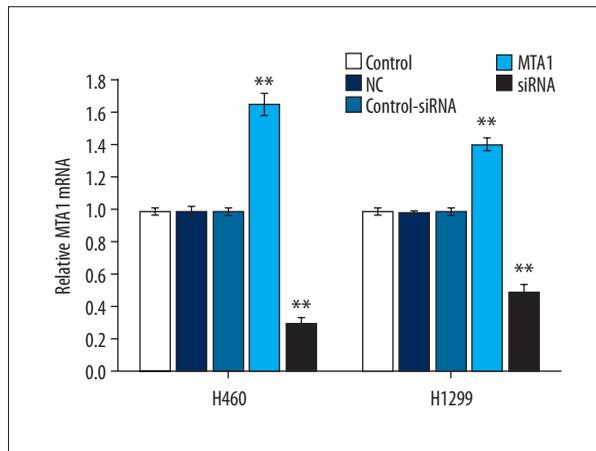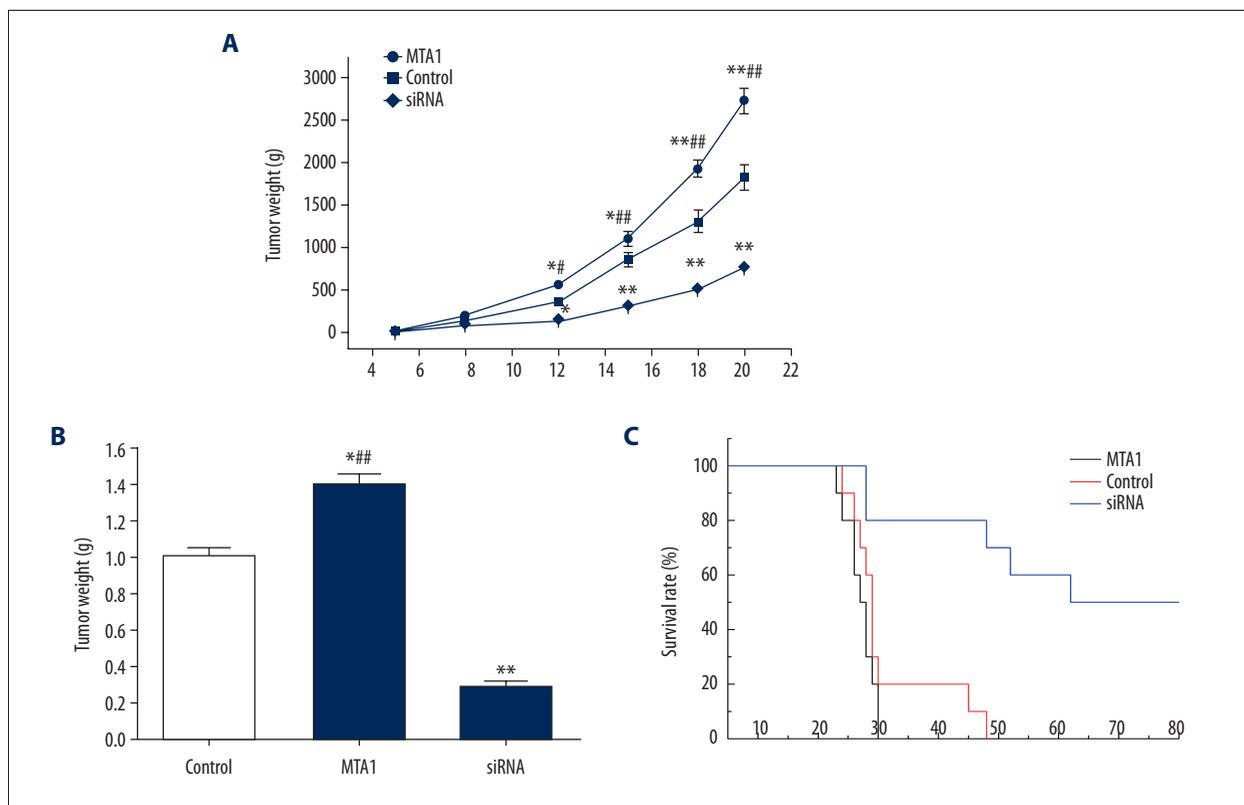
Figure 1. Metastasis-associated 1 (MTA1) gene transfection promoted the expression levels of the MTA1 gene in H460 and H1299 human non-small cell lung cancer (NSCLC) cells *in vitro*, as shown by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Compared with the control group. ** p<0.01.

Results

Overexpression of MTA1 in non-small cell lung cancer (NSCLC) cells *in vitro*

To investigate the role of MTA1 in non-small cell lung cancer (NSCLC), human NSCLC cells with upregulation or downregulation of MTA1 were developed and verified by quantitative reverse transcription polymerase chain reaction (qRT-PCR) for expression of MTA1 (Figure 1). Compared with the control group, MTA1 si-RNA transfection resulted in significant down-regulation of MTA1 expression (p<0.01), while transfection of the MTA1 overexpression vector led to significant upregulation of MTA1 mRNA (p<0.01). Compared with the control group, there was no significant MTA alteration in NC and control si-RNA groups, compared with the control group (p>0.05). Because MTA1 transfection was more effective in H460 cells than that in H1299 cells, H460 cells were selected to further investigate the effect of MTA1 expression in the tumor vasculature *in vivo*.

Figure 2. Metastasis-associated 1 (MTA1) gene expression promoted tumor growth in mouse tumor xenografts *in vivo*. (**A**) The profile of tumor size in mice after transfection with the metastasis-associated 1 (MTA1) gene or MTA1 short-interfering RNA (si-RNA) after 20 days (n=10). (**B**) The graph of tumor weight in control, MTA1, and MTA si-RNA groups. At the end of the experiment the tumors were excised and immediately weighed. (**C**) The survival profile of mice treated with MTA1 or MTA1 si-RNA vectors at 80 days. * p<0.05, ** p<0.01 *vs.* the control group; # p<0.05, ## p<0.01 *vs.* the MTA1 short-interfering RNA (si-RNA) group.
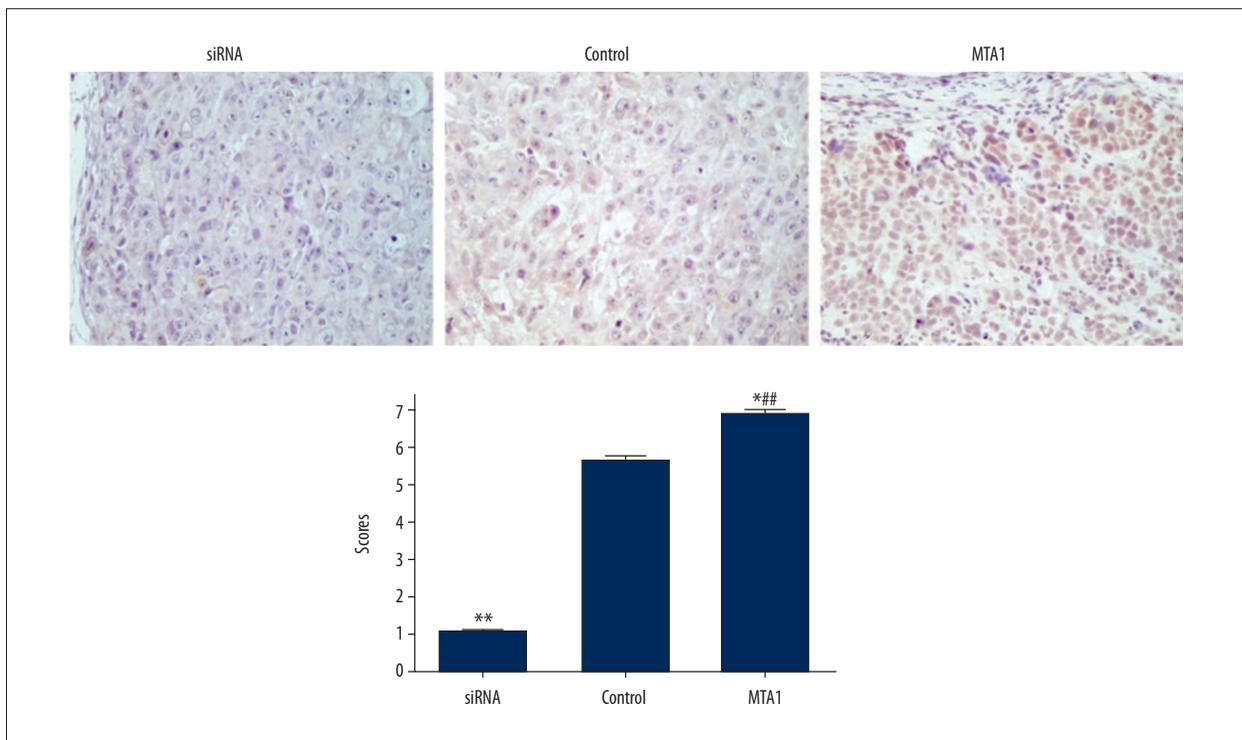
Figure 3. Photomicrographs of the immunohistochemistry for the expression of the metastasis-associated 1 (MTA1) protein in the tumor xenografts of control mice and mice transfected with the MTA1 gene or MTA1 short-interfering RNA (si-RNA). * $p < 0.05$, ** $p < 0.01$ *vs.* the control group; ## $p < 0.01$ *vs.* the MTA1 short-interfering RNA (si-RNA) group.

Overexpression of MTA1 promoted tumor growth

As shown in Figure 2A and 2B, MTA1 silencing significantly inhibited tumor growth in nude mice ($p < 0.01$). Survival analysis indicated that all of the mice transfected with MTA1 died after 30 days, whereas the survival rate of mice in the si-RNA group was >70% (Figure 2C), indicating the tumor-promoting effects of MTA1.

### MTA1 expression in tumor tissues promoted tumor angiogenesis

Immunohistochemical analysis of MTA1 expression in tumors (Figure 3) showed that MTA1 expression was significantly increased in the MTA1 overexpression group when compared with the si-RNA group ($p < 0.01$). The expression level of MTA1 in the control group was significantly lower when compared with that in the MTA1 group and higher than that in the si-RNA group ($p < 0.05$). Further analysis of tumor microvascular density (MVD) findings (Figure 4) showed that MVD values in the MTA1 group were significantly increased when compared with the control and si-RNA groups ($p < 0.05$), while MVD values in the si-RNA group were significantly lower compared with those in the control group ($p < 0.01$).

### Overexpression of MTA1 promoted the expression of angiogenesis-related proteins

The protein expression of cyclooxygenase-2 (COX-2), angiopoietin 1/2 (Ang1/2), hypoxia-inducible factor 1-α (HIF-1α), and vascular endothelial growth factor (VEGF) in the tumor was quantified by Western blot (Figure 5). The expression of these proteins in the si-RNA group was significantly lower compared with the control group ($p < 0.01$). In contrast, the expression of these proteins in the MTA1 group was significantly increased when compared with the control group ($p < 0.05$). The expression level of Ang1 in the MTA1 group was about 4.7 times that in the si-RNA group and 1.4 times that in the control group. The expression level of VEGF in the control group was about 3 times that in the si-RNA group and 0.7 times that in the MTA1 group.

Discussion

To our knowledge, this is the first study that has shown that expression of the metastasis-associated 1 (MTA1) gene might have potential as a biomarker for non-small cell lung cancer (NSCLC), demonstrated by increased tumor growth following MTA1 overexpression and decreased tumor growth following MTA1 silencing. Survival rates of tumor-bearing mice were also
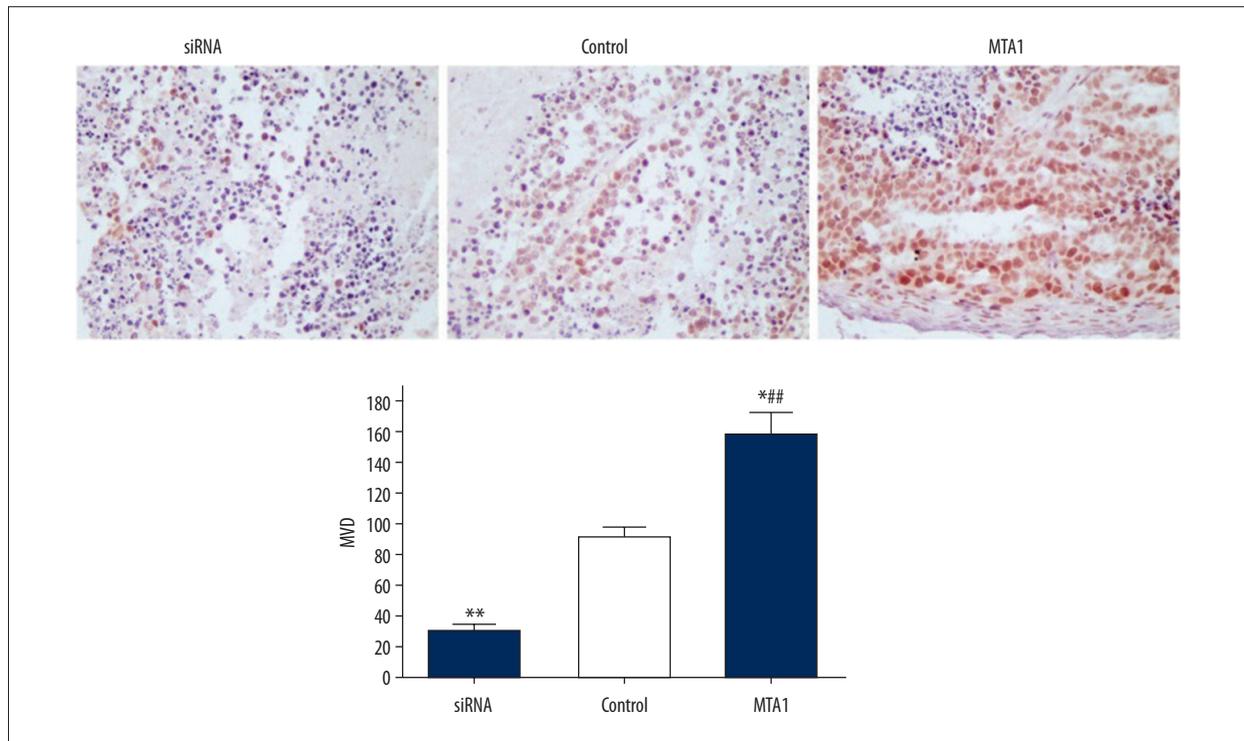
Figure 4. Photomicrographs of the immunohistochemistry for the expression of CD31 showed increased angiogenesis following transfection with the metastasis-associated 1 (MTA1) gene. CD31 staining (brown color) was used to calculate the microvascular density (MVD). * p<0.05, ** p<0.01 *vs.* the control group; ## p<0.01 *vs.* the MTA1 short-interfering RNA (si-RNA) group.
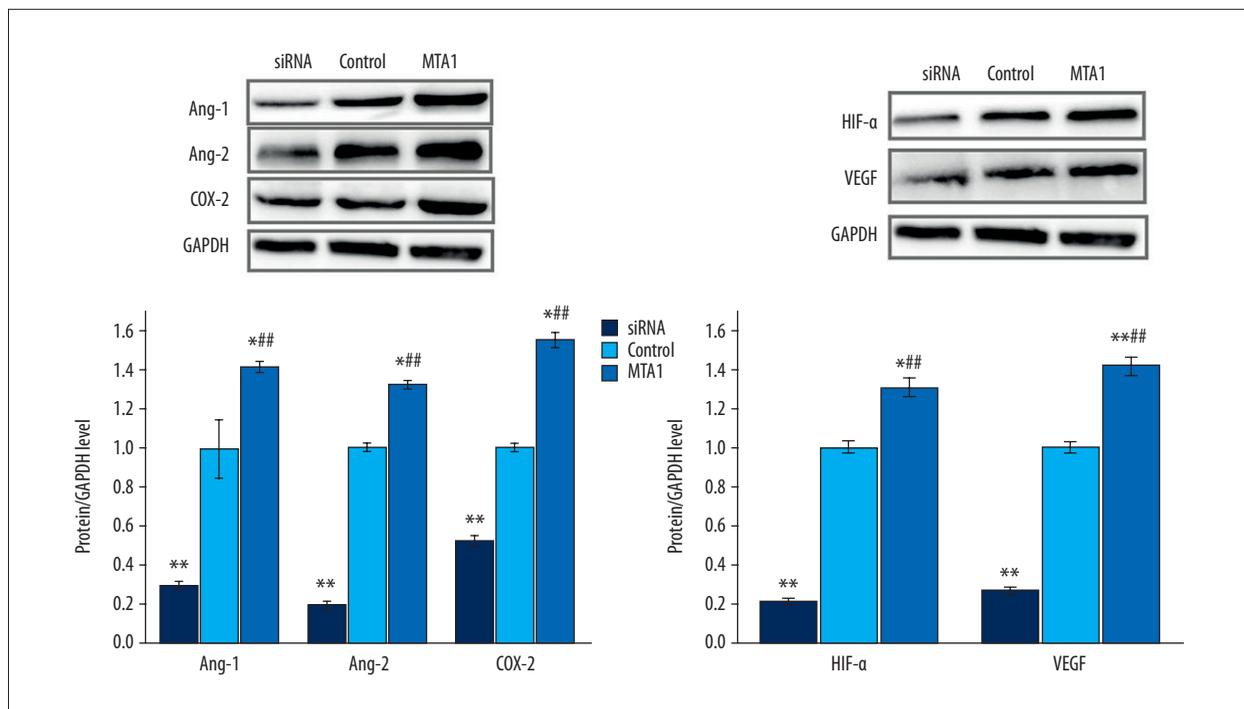
Figure 5. Western blot analysis of the expression levels of cyclooxygenase-2 (COX-2), angiopoietin 1/2 (Ang1/2), hypoxia-inducible factor 1-α (HIF-1α), and vascular endothelial growth factor (VEGF). * p<0.05, ** p<0.01 *vs.* control group; # p<0.05, ## p<0.01 *vs.* the metastasis-associated 1 (MTA1) short-interfering RNA (si-RNA) group.

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affected by the level of MTA1 expression in xenograft tumors. These data support that inhibition of the development of tumor microvasculature impedes tumor growth [6]. Currently, cytotoxic drugs such as docetaxel and paclitaxel are first-line therapies for NSCLC. However, due to the difficulty in treatment of chemoresistant cancers, anti-angiogenic targeted therapies are increasingly used alone or in combination with traditional chemotherapy drugs to improve patient survival, and the enhanced benefit has been demonstrated in several clinical trials [3,6]. Existing anti-angiogenic agents include antibody-based agents that target receptors of vascular endothelial growth factor (VEGF) receptors and tyrosine kinases, but these agents are not free from toxicity, which may include proteinuria, hypertension, impaired wound healing, and hemorrhage. Compared to VEGF and epidermal growth factor receptor (EGFR), the expression of MTA1 is more restricted to tumors [7]. It is possible that inhibition of MTA1 may have less toxic anti-angiogenic effects, but this requires further study.

In the present study, an RNA inference approach, based on short-interfering RNA (si-RNA), was used to downregulate MTA1 expression, an approach that is supported by previous studies [12,14]. An alternative approach for MTA1 inhibition is through the use of MTA1 antagonists. Dhar et al. recently demonstrated that naturally occurring compounds, such as pterostilbene and resveratrol, can exert chemopreventive and therapeutic effects in prostate cancer by targeting MTA1 [16]. However, these compounds remain to be tested in the treatment of NSCLC. In light of the marked anti-angiogenic efficacy induced by MTA1 downregulation, further studies are warranted to investigate the therapeutic potential of these drugs to improve the clinical outcome for patients with NSCLC.

The findings of the present study showed that in human NSCLC mouse xenografts, in addition to the inhibition of blood vessel formation, MTA1 silencing reduced the expression of cyclooxygenase-2 (COX-2), angiopoietin 1/2 (Ang1/2), hypoxia-inducible factor 1-α (HIF-1α), and vascular endothelial growth factor (VEGF). The upregulation of these angiogenic genes is also recognized to be biomarkers of lung cancer [17–20]. This finding supports the findings from previous studies that crosstalk between HIF-1α, VEGF, and MTA1 exist [21,22]. Notably, adaptation to hypoxia, which is characteristic of HIF-1α upregulation, is an important mechanism by which tumors gain resistance to anti-angiogenic therapies [5]. By reducing both angiogenesis and the expression of HIF-1α, a therapeutic approach that uses inhibition of MTA1 might potentially overcome resistance to anti-angiogenic therapy.

Conclusions

Silencing of the metastasis-associated 1 (MTA1) gene was effective in inhibiting cell growth and proliferation of human non-small cell lung carcinoma (NSCLC) cell growth *in vitro* and improved the survival of xenograft tumor-bearing mice *in vivo*. In tumor xenografts, blood vessel density, expression levels of cyclooxygenase-2 (COX-2), angiopoietin 1/2 (Ang1/2), hypoxia-inducible factor 1-α (HIF-1α), and vascular endothelial growth factor (VEGF) levels were reduced by short-interfering RNA (si-RNA) MTA1 transfection. These preliminary findings support the need for further clinical studies on the anti-tumor effects of suppression of MTA1 gene expression and its potential as a targeted therapy in patients with malignancy, including NCSLC.

Conflict of interest

None.

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