Original Article

MTA1, an independent prognostic factor, promotes melanoma cell invasion by induction of EMT

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Abstract: Background: The prognosis of melanoma, the most aggressive skin cancer, remains unfavorable. Metastasis-associated gene 1 (MTA1) have been demonstrated as serving critical roles in various biological events including tumorigenesis. However, the clinical significance and biological function of MTA1 in melanoma remain to be clarified. Therefore, the purpose of our present study was to determine the role of MTA1 in melanoma. Methods: The mRNA and protein levels of MTA1 in melanoma tissues and cells were detected by qRT-PCR and western blot analysis. The clinical and prognostic significance of MTA1 in melanoma patients was also analyzed. Furthermore, the biological function of MTA1 on melanoma cell proliferation, cell cycle distribution, cell migration and invasion were explored through MTT assay, colony formation assay, flow cytometric analysis, wound healing assay and transwell assay in vitro. The expression of epithelial-mesenchymal transition (EMT)-related proteins in melanoma cells was detected through western blot analysis. Tumor xenograft model was established to determine the role of MTA1 in vivo. Results: We found that MTA1 was overexpressed in melanoma tissues and cell lines, and high expression was closely corrected with tumor thickness and TNM classification. Moreover, patients with high levels of MTA1 had poorer prognosis than those with lower MTA1 expression. Further functional experiments indicated that knocking down MTA1 expression significantly suppressed the melanoma cell proliferation, cell cycle progression, cell invasion in vitro and regulated EMT-associated proteins expression. In vivo, tumor volumes were also decreased significantly in MTA1 silenced group compared to the control. Conclusions: These data suggest that MTA1 serves an important role on melanomagenesis and progression, and would be not only a potential prognostic indicator but also a potential therapeutic strategy for melanoma.

Keywords: Melanoma, MTA1, epithelial to mesenchymal transition, proliferation, invasion, prognosis

Introduction

Melanoma is potentially the most aggressive type of skin cancer worldwide, which results in approximately 80% of the mortality associated with skin cancer [1]. Featured as a malignant disease of melanocytes which produce pigment, melanoma often occurs in fair-skinned Caucasians [2]. It has been found that men have more possibility to suffer from melanoma compared to women [3]. Despite current advances in surgical therapy, chemotherapy and molecular targeting therapy for melanoma, the overall 5-year survival rate for melanoma patients with distant metastasis still remains as low as 16% [4]. Although solar and solarium UV exposure represents the main cause of melanomagenesis [5], it is now promising the correlation also with aberrant accumulation of genetic mutations [6]. Thus, a well understanding of the molecular mechanisms underlying melanomagenesis may provide potential targets for its diagnosis and treatment.

Metastasis-associated gene 1 (MTA1), a subunit of the nuclear remodeling and deacetylation (NuRD) complex, represses the transcription of target genes through recruiting histone deacetylases onto the promoter regions of target genes and inducing histone deacetylation [7]. MTA1 has been implicated in the carcinogenesis and metastasis of a variety of human tumors, including breast cancer [8], nasopharyngeal carcinoma [9], hepatocellular carcinoma [10] and gastric cancer [11]. MTA1 was also found to promote the expression of Wnt1 and to be a critical upstream regulator of Wnt1 pathway in cancer cells [12]. However, the clinical
significance and biological function of MTA1 in melanoma remain largely unclear.

Therefore, in the present article, we aimed to elucidate the role of MTA1 in mediating tumor cell proliferation, cell cycle progression and cell invasion, as well as the underlying mechanisms in melanoma. Our findings suggested that MTA1 might offer an attractive new target for therapeutic intervention of melanoma in the near future.

**Materials and methods**

**Patients and tissue samples**

We obtained 81 melanoma tissues and 30 benign nevi tissues (collected as controls) from patients who had undergone operations between 2008 and 2010 at Department of Plastic Surgery, Zhongshan Hospital, Fudan University, P.R. China. None of the patients had received chemotherapy or radiotherapy prior to operations. Data from all participants were acquired from pathology reports, medical archives, and personal interviews with the participants. All tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. All of the patients had complete follow-up information until their deaths, which ranged from 3 to 60 months. Our study was approved by the Research Ethics Committee of Zhongshan Hospital of Fudan University, and written informed consent forms were acquired from all patients.

**Cell culture**

The normal human melanocyte (HM) cells were purchased from ScienCell (Carlsbad, CA) and maintained in melanocyte medium (ScienCell). Six human melanoma cell lines, including CHL-1, A375, SK-MEL-1, WM-35, WM-115 and UACC903, were purchased from American Type Culture Collection (Manassas, VA, USA), and cultured in DMEM medium (GIBCO-BRL; Invitrogen, Carlsbad, CA, USA) or RPMI-1640 medium (GIBCO-BRL; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified atmosphere containing 5% CO2 at 37°C. The medium was routinely changed 2-3 days after seeding.

Knockdown of MTA1 was induced by transfection with the MTA1 small interfering RNA (si-MTA1) using Lipofectamine 2000 (Invitrogen). si-MTA1 and negative control siRNA (si-NC) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequence of the si-MTA1 was 5’-GACCCTGCTGGCAGATAAA-3’.

**RNA extraction and qRT-PCR assay**

Total RNA was extracted from cells and specimens using Trizol reagent (Invitrogen). RNA concentration and purity were assessed on a Nanodrop spectrophotometer (Thermo Fisher Scientific). RNA was reverse transcribed into cDNA using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China), qRT-PCR was conducted to detect the mRNA level of MTA1 on a ABI 7500 fast realtime PCR system (Applied Biosystems, Foster City, CA) with SYBR Green Real-time PCR Master Mix (Takara). The relative MTA1 expression was expressed by the $2^{-ΔΔCt}$ method with GAPDH as the control [13, 14]. The primer sequences used in this study were as follows: GAPDH, forward: 5’-GGGCATCTTGCGCTACAC-3’ and reverse: 5’-GGTCCAGGGTTCTTACTCC-3’; MTA1, forward: 5’-CGCTGACCAGCATCATTGAT-3’ and reverse: 5’-TGGTTCGGATTTGGCCTTTGAT-3’. Each experiment was performed in triplicate.

**Protein isolation and western blot assay**

Cells were washed twice with cold PBS and lysed with iced RIPA lysis buffer (Beyotime, Beijing, China) containing 1% PMSF (KeyGen, Nanjin, China). After total protein detection using a BCA protein assay kit (Beyotime), protein lysates were separated on 10% SDS-PAGE, and transferred to nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 5% skim milk and 0.1% Tween-20 in Tris Buffer Saline (PBS) for 2 h, the membrane was incubated with the primary antibodies against MTA1 (Abcam, Cambridge, MA), E-cadherin, N-cadherin, Vimentin, and Snail (Cell Signaling Technology, Boston, MA, USA), GAPDH (KangChen, Shanghai, China) overnight at 4°C. The membranes were washed twice with PBS and probed with horseradish peroxidase-conjugated secondary antibodies. Signals were detected using enhanced chemiluminescence reagents, and the protein levels were normalized to GAPDH expression.
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**MTT assay**

Cells were seeded in 96-well plates (3000 cells/well). Cell proliferation was detected every 24 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution was added to each well at a final concentration of 0.5 mg/ml and incubated for 4 h. After incubation, formazan crystals were dissolved by addition of 150 μl DMSO per well. The absorbance at 570 nm was read on a microplate reader (Bio-Rad, Hercules, CA, USA).

**Colony formation assay**

For colony formation assay, 500 cells were seeded into each well of a six-well plate and cultured for 12 days. Colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 15 min. The colony formation was determined by counting the number of stained colonies. Triplicate wells were measured in each treatment group.

**Analysis of cell cycle by flow cytometry**

For cell cycle analysis, cells stained with propidium iodide (PI) were analyzed by flow cytometry (FACScan) using the Cycle TEST PLUS DNA reagent kit (BD Biosciences). The proportion of cells in the G0/G1, S, and G2/M phases were counted and compared. All samples were tested in triplicates.

**Wound healing assay**

To determine cell motility, cells (5×10^5 cells/well) were plated in 6-well plates and cultured until they reached 80-90% confluence. A diametric scratch was carefully made using a 200-μl sterile plastic tip. Cells were photographed every 24 h.
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**Transwell assay**

As for migration assay, $5 \times 10^4$ cells in serum-free medium were added to the upper chamber of an insert with $8 \mu$m pore (Millipore). As for invasion assay, $1 \times 10^5$ cells in serum-free medium were added to the upper chamber of an insert coated with Matrigel (Sigma-Aldrich). The lower chamber was filled with medium containing 10% FBS. After incubation for 24 h, cells remaining on the upper surface were removed. Cells that had migrated or invaded through the membrane were fixed and stained with 0.1% crystal violet, imaged, and counted under a microscope. Experiments were repeated in triplicate.

**In vivo tumor xenograft assay**

A total of 16 male BALB/c nude mice (8 weeks) were purchased from Shanghai Laboratory Animals Center (Shanghai, China), and maintained under specific pathogen-free (SPF) condition. All animal protocols were approved by the Institutional Animal Care and Use Committees of Zhongshan Hospital, Fudan University. Efforts were made to reduce suffering in the animals [15].

To determine the effect of MTA1 on the tumorigenesis of melanoma cells in vivo, nude mice in the MTA1 silenced group (n=8) were injected subcutaneously in the dorsal flank with $5 \times 10^6$ melanoma cells stably transfected with si-MTA1. In addition, in the control group (n=8), nude mice were injected with $5 \times 10^6$ melanoma cells stably transfected with si-NC. Once palpable tumors developed, the volume of tumor was measured with a caliper every 4 days, using the formula $V (mm^3) = 0.5 \times$ length (mm)$^3 \times$ width (mm)$^2$. Survival time was also recorded. Nude mice were euthanized, if not died, on 45 days after tumor implantation. Tumor weight was also recorded when mice died.

**Statistical analysis**

All data are presented as the mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was tested using SPSS 17.0 software (IBM, Chicago, IL, USA) and Graphpad Prism (Graphpad Software, San Diego, CA, USA). The correlation between MTA1 mRNA expression and clinicopathological features of melanoma patients was evaluated by the $\chi^2$ test. Differences between groups were determined by the Student’s t-test (two-tailed). Kaplan-Meier survival curves were generated and compared by log-rank analysis. A two-sided $P$ value of less than 0.05 was regarded significant.

**Results**

**MTA1 is upregulated in melanoma and is associated with poor prognosis**

The level of MTA1 was detected in 81 melanoma tissues and 30 nevi tissues through qRT-PCR, and normalized to GAPDH. The results

<table>
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**Table 1. Relationship between MTA1 expression and clinicopathological characteristics of melanoma patients (n=81)**
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exhibited that MTA1 expression was noticeably overexpressed in melanoma tissues, compared to nevi tissues ($P<0.001$, Figure 1A).

To understand the significance of MTA1 overexpression in melanoma, we analyzed the potential associations between MTA1 expression and melanoma patients’ clinicopathological features. Clinicopathological features of melanoma patients were recorded in Table 1. Noticeably, high MTA1 expression was closely correlated with tumor thickness ($P=0.041$) and TNM classification ($P=0.003$). However, MTA1 expression was not correlated with other variables such as age ($P=0.364$), gender ($P=0.797$), family history ($P=0.593$), tumor site ($P=0.164$) and histologic type ($P=0.270$) in melanoma patients.

Kaplan-Meier survival analysis and log-rank tests were carried out to detect the correlation between MTA1 expression and the prognosis of melanoma patients after surgery. From the Kaplan-Meier survival curves, we found that
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Patients with high MTA1 expression had a more unfavorable prognosis than those with low MTA1 expression ($P=0.010$, Figure 1B).

Consistent with the findings in clinical specimens, qRT-PCR and western blot data further demonstrated that MTA1 was also overexpressed in melanoma cell lines including CHL-1, A375, SK-MEL-1, WM-35, WM-115 and UACC903, and showed the highest expression level in SK-MEL-1 cells, when compared with the normal melanocyte (HM) cells (Figure 1C and 1D). Thus we selected SK-MEL-1 cells for further analysis.

Knockdown of MTA1 inhibits melanoma cell proliferation through suppressing the cell cycle progression in vitro

In order to identify the potential role of MTA1 on melanoma cells proliferation, MTA1 siRNA was transfected into SK-MEL-1 cells. qRT-PCR validation revealed that MTA1 expression was remarkably reduced after transfection with si-MTA1 (Figure 2A and 2B). Then MTT assay showed that knockdown of MTA1 expression evidently inhibited cell proliferation in SK-MEL-1 cells compared with control cells (Figure 2C). Similarly, the result of colony-formation assay revealed that clonogenic survival was evidently reduced following inhibition of MTA1 in SK-MEL-1 cells (Figure 2D).

The cell cycle of these cells was detected through flow cytometric analysis. As demonstrated in Figure 2E, transfection of si-MTA1 into SK-MEL-1 cells resulted in a marked increase in the percentage of cells in the G1/G0 phase compared with controls, and a concomitant reduction of cell numbers in S phase (all $P<0.05$). Accordingly, the results indicated that downregulation of MTA1 can suppress the cell cycle progression and inhibit the proliferation of melanoma cells.

Figure 3. Knockdown of MTA1 inhibits melanoma cell migration and invasion in vitro. A. Wound healing assay was performed to detect the migratory capacity of SK-MEL-1 cells transfected with si-MTA1 or si-NC. B. Transwell assay was performed to evaluate the migratory and invasive capacities of SK-MEL-1 cells transfected with si-MTA1 or si-NC. Data are presented as mean ± SD. *$P<0.05$, ***$P<0.001$ vs. control.
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Knockdown of MTA1 inhibits EMT in melanoma cells. Representative photographs and quantification of protein expression including Vimentin, E-cadherin, Snail and N-cadherin in SK-MEL-1 cells transfected with si-MTA1 or si-NC were shown. GAPDH levels were used as an internal control. Data are presented as mean ± SD. ***P<0.001 vs. control.

Figure 5. Knockdown of MTA1 inhibits the growth of melanoma in vivo. A. Nude mice were subcutaneously implanted with SK-MEL-1 cells stably transfected with si-MTA1 or si-NC, respectively. Tumor growth curves upon implantation were plotted. B. The survival curves were plotted. C. On 45 days after implantation, the nude mice in each group were sacrificed, and the melanoma xenograft was obtained. D. The tumor weight was calculated and compared. Data are represented as mean ± SD. ***P<0.001 vs. control.

Knockdown of MTA1 inhibits melanoma cell migration and invasion in vitro

To better know the biological significance of MTA1 in melanoma, we detected the effect of MTA1 on migration and invasion of melanoma cells. Wound healing assay revealed that the mobility of SK-MEL-1 cells evidently decelerated in rate in within 48 hours than controls (Figure 3A). Transwell assays showed that
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treatment with MTA1 caused a significant reduction of migratory and invasive capacities in SK-MEL-1 cells (Figure 3B). Taken together, downregulation of MTA1 suppresses the migration and invasion of melanoma.

**Knockdown of MTA1 inhibits EMT in melanoma cells**

Since epithelial-mesenchymal transition (EMT) is a critical step in the process of cancer metastasis, thus we validated the hypothesis that downregulation of MTA1 inhibited melanoma cell migration and invasion via blocking EMT. Western blot analysis showed that transfection of si-MTA1 into SK-MEL-1 cells blocked EMT progression through upregulation of E-cadherin and downregulation of Vimentin, Snail and N-cadherin in SK-MEL-1 cells (all P<0.05, Figure 4). These results demonstrated that MTA1 silencing could reverse EMT in melanoma cells.

**Knockdown of MTA1 inhibits the growth of melanoma in vivo**

Finally, the effect of MTA1 on melanoma growth in vivo was investigated. Nude mice were subcutaneously implanted with SK-MEL-1 cells transfected with si-MTA1 or si-NC, respectively. The xenografts of melanoma cells grew gradually after implantation. As exhibited in Figure 5A, tumors derived from the MTA1 silenced group grew at a much slower rate than controls at the indicated time. In the control group, six mice died from the 34th to the 45th days after implantation; however, only two mice died in the MTA1 silenced group, indicating that MTA1 silencing protected nude mice from death caused by downregulation of MTA1 in SK-MEL-1 cells (P=0.041, Figure 5B). On the 45th day after implantation, all mice if not died were sacrificed. As indicated in Figure 5C, the melanoma xenografts were finally obtained, and the tumor was 1.85 fold smaller in the MTA1 silenced group compared to the control group (P<0.001, Figure 5D).

**Discussion**

Melanoma is a multifactorial disease caused by multiple environmental factors and a variety of susceptibility genes. Although surgical removal is considered as the primary treatment for melanoma patients, this kind of cancer still accounts for approximately 80% of all skin cancer-associated deaths because of aggressive metastasis. Metastasis, which is featured as a particularly ominous sign, leads to most of the morbidity and mortality cases in melanoma [16]. Cancer metastasis is a complicated process that involves multiple molecular and cellular events. Thus, to clearly understand the molecular mechanisms by which metastasis occurs is one of the most crucial issues in melanoma research.

MTA1, originally discovered by screening a cDNA library from rat metastatic breast tumors [17], is expressed physiologically at low levels in all normal murine tissue, except the testis [18]. MTA1 have been demonstrated to be widely overexpressed and serve critical roles in many types of human carcinomas through modulating tumor progression, metastasis and chemosensitivity. Although a previous study had evaluated the therapeutic value of MTA1 in the B16F10 melanoma cell line with the C57BL/6 mouse model [19], the current article provides the first evidence of the clinical significance of MTA1 expression in human melanoma and its possible role in the regulation of melanoma.

In the present study, we confirmed that MTA1 expression is aberrantly overexpressed and closely correlated with aggressive malignant phenotypes in melanoma. Melanoma patients with high MTA1 expression had unfavorable prognostic outcomes. Song and colleagues also found that MTA1 protein overexpression correlated with remarkably worsened 5-year survival for patients with esophageal carcinoma [20]. To further elucidate mechanisms underlying the biological function of MTA1, we knockeddown MTA1 in melanoma cells and found that knockdown of MTA1 inhibits melanoma cell proliferation and invasion in vitro, as well as tumor growth in vivo. Moreover, our study suggests that MTA1 exerts its metastasis-promoting function through regulating EMT. Collectively, our current findings and those described in previous studies suggest that MTA1 expression might play an important role in human melanoma biological processes.

Epithelial-mesenchymal transition (EMT), which enables epithelial cells to acquire invasive mesenchymal phenotype, is attracting more and more attention as a pivotal mechanism for the initial step of metastasis [21, 22]. A large
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amount of evidences support the critical role of EMT in the modulation of tissue regeneration, embryonic development, inflammatory response, and tumor invasion [23]. EMT is a complicated and reversible event which involves the acquisition of a mesenchymal phenotype and loss of epithelial cell adhesion to the basement membrane [24]. In the current study, we showed that decreased expression of MTA1 suppresses EMT through decreasing the expression of the mesenchymal markers N-cadherin, Snail, and vimentin, and increasing the expression of the epithelial marker E-cadherin in melanoma cells along with the impairment of cell invasion. A study by Xu et al. demonstrated that MTA1 could promote EMT through repressing the promoter activity of E-cadherin in malignant pleural mesothelioma cells [25]. Also, Tuncay reported that MTA1 expression is associated with metastasis and EMT in colorectal cancer cells [26].

Several limitations in the current article should be considered. First, the sample size for the clinical validation was relatively small, which might lead to significant fluctuations in statistics. Prospective studies with a larger cohort of samples would be of great desirable for further confirmation of the clinical significance of MTA1 in melanoma. Next, the survival data are missing mainly due to the fact that most patients were lost after they were discharged from our hospital. The prognostic value of MTA1 in melanoma requires further verification.

In conclusion, our findings provide evidence that MTA1 is significantly increased in melanoma tissues and cell lines, and loss of MTA1 markedly represses the proliferation, migration and invasion of melanoma cells, and inhibits EMT program, indicating that MTA1 can serve as the potential target for the diagnosis and treatment of melanoma, but further studies are still needed to elucidate the exact molecular mechanisms of MTA1 acting on melanoma.

Disclosure of conflict of interest

None.

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References

[13] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time
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