

Original Article

Tumor-suppressive effect of miR-148a on salivary adenoid cystic carcinoma via down-regulation of MTA2

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Abstract: The aim of this study was to investigate the effect on miR-148a of salivary adenoid cystic carcinoma cells (SACC) and to clarify the mechanism. Expression of miR-148a and MTA2 was analyzed by real-time PCR in SACC cancer tissues and SACC cell lines. Available software algorithms, luciferase reporter assay system, agarose gel electrophoresis, and Western blotting were used to explore the potential target genes of miR-148a in SACC. Real-time PCR showed that expression of miR-148a was significantly decreased in SACC cancer tissues compared with normal tissues. Northern blotting results revealed significant down-regulation in SACC cancer tissues compared with normal tissues. The SACC cell line ACC-M transfected with miR-148a mimics could increase the expression level of miR-148a effectively. Compared with the NC group, cell proliferation, invasion rate, and cell migration ability of the mimics group declined. MiRanda, Mirwalk, and Targetscan predicted that human MTA2 was a target gene of miR-148a. Luciferase reporter assay showed that there was a site of miR-148a in the MTA2 mRNA 3'-UTR region. Western blotting showed that overexpression of miR-148a inhibited expression of MTA2. Up-regulation of MTA2 expression transfected with miR-148a mimics promoted cell proliferation, migration, and invasion compared with the negative and miR-148a mimic groups. Expression of miR-148a was down-regulated in SACC tissues. Additionally, miR-148a negatively regulated cell proliferation, migration, and invasion by down-regulation of MTA2, which is a target gene of miR-148a. Therefore, miR-148a suppresses expression of the MTA2 gene and plays a role of tumor suppressor gene in SACC.

Keywords: Salivary adenoid cystic carcinoma, miR-148a, metastasis-associated gene 2 (MTA2), proliferation, metastasis

Introduction

Salivary gland adenoid cystic carcinoma (SACC) is a rare kind of tumor, accounting for approximately 1% of all malignant head and neck tumors, and 27% of malignant tumors of the salivary gland [1, 2]. Because of its growth pattern of invading nerves easily and high incidence of distant metastasis, it carries a bad prognosis for patients. The main therapies include surgery, radiotherapy, and chemotherapy with no molecular targeted drugs currently. The main site of metastasis is the lung [3-5], resulting in a high mortality rate. Although it is certain that many studies show aberrant expression of genes leading to SACC, precise mechanisms for SACC have not yet been identified.

microRNAs (miRNAs) are a kind of non-coding RNA with only about 20 nucleotides, regu-

lating target genes by binding to the 3'-UTR and inhibiting translation of target genes [6, 7]. Hundreds of miRNAs, related to many diseases, have been found in cells, involved in cell growth, apoptosis, and differentiation [8-10]. It has been confirmed that miR-148a is a member of the miR-148/152 family closely related to tumor growth and metastasis with tissues-specific expression and plays a crucial role in the occurrence, tumor development, and function of oncogenes or suppressor genes. Overexpression of MTA2 is closely related to tumor occurrence and development [11, 12].

In this study, we examined and analyzed the miR-148a and MTA2, exploring the roles of proliferation, apoptosis, invasiveness and migration. The results demonstrate that miR-148a negatively regulates MTA2, and is involved in proliferation and invasion-related processes.

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Materials and methods

Patients and tissue samples

Pathologically proven SACC clinical samples of 32 patients and normal tissues samples of 10 people were collected from 2010 to 2016 at Jining No. 1 People's Hospital. None of the patients received chemotherapy and radiation therapy before surgery. All people voluntarily joined this study with informed consent.

Cell culture

SACC-M cell lines were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China) and cultivated in RPMI-1640 (Invitrogen, Carlsbad, CA, USA), supplemented with 100 units/ml penicillin (Sigma-Aldrich, St. Louis, Mo, USA), 0.1 mg/ml streptomycin (Enpromise, Hangzhou, China), and 10% fetal bovine serum (FBS), with all cell lines being cultivated in a humidified incubator at 37°C and 5% CO₂.

Quantitative real-time reverse transcription-PCR

According to the standard protocol, total RNAs from normal SACC tissues, normal tissues, and cell lines, were prepared using Trizol (Invitrogen Life Technology, CA, USA). Total RNA including miRNA was extracted using the mirVana™ miRNA isolation kit and detected by UV absorbance (A260/280) and agarose gel. Prime-Script™ RT reagent Kit (Takara Bio, Japan) was used for reverse transcription. qRT-PCR reaction was performed in the ABI Prism 7500 PCR system (Applied Biosystems, Foster city, CA, USA) with SYBR Premix EX Taq (Takara Bio, Qtsu, Japan) following the standard instructions. In this study, U6 acted as the internal criterion, and 2^{-ΔΔct} method was taken to calculate relative expression of miR-148a. The primer sequences for MTA2 were (F) 5'-AGGATACCA-GGGCCCTAC-3' and (R) 5'-TGCAGGTAGAGGGA-CAGG. The primers sequences for GADPH were (F) 5'-GAAGGTGAAGGTCCGAGTC-3' and (R) 5'-GAAGATGGTATGGGATTTC-3'. Each PCR reaction was performed in three parallel wells.

Cell transfections

miR-148a, mir-148a inhibitor, miR-148a mimics and negative control (miR-NC) were purchased from GenePharma Co.Ltd (Shanghai,

China). With Lipofectamine 2000 (Invitrogen), the miR-148a mimics, mir-148a inhibitor, negative control (miR-NC) and MTA2 overexpression plasmid were transfected into cells. Cells were harvested and subjected to gene expression analyses, cell proliferation, cell migration, and invasion assays.

Cell migration and invasion assays

Both cell migration and cell invasion assays were performed in chambers with 8 μm pore filters. SACC-M cell lines were plated in this chamber. After cultivating for 48 hours, the cells were fixed with methanol, removed through the membrane at room temperature, and dyed with 0.1% crystal violet. Five select visual fields were used to count the cell number in a microscope. All the experiments were repeated three times independently.

Cell proliferation assay

Cell proliferation assays were carried out with CCK8 assay. 10⁴ transfected cells were plated into 96-well plates per well. The plates were incubated for 24 hours in a humidified chamber at 37°C and 5% CO₂. Then, cell absorption was measured by CCK8 kit (R&D System, M.N., USA) under the manufacturer's instructions. All the experiments were repeated three times independently.

Bioinformatics analyses and dual-luciferase assays

In order to target miR-148a, online software programs targetScan (<http://www.targetscan.org>), miRanda (<http://www.microrna.org>) and Mirwalk (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>) were used. According to the predicted results, miR-148a may be the target gene and was used to verify the biluciferase and function of the target gene. Because of 3'-UTR of the MTA2 was predicted containing miR-148a binding sites, we amplified it from human cDNA and cloned it into a dual-luciferase expression vector.

Northern blotting

According to the standard protocol, total RNAs from SACC tissues, normal tissues were extracted. The integrity of RNA was detected through the polyacrylamide gel electrophoresis RNA. RNAs were then transferred from the gel

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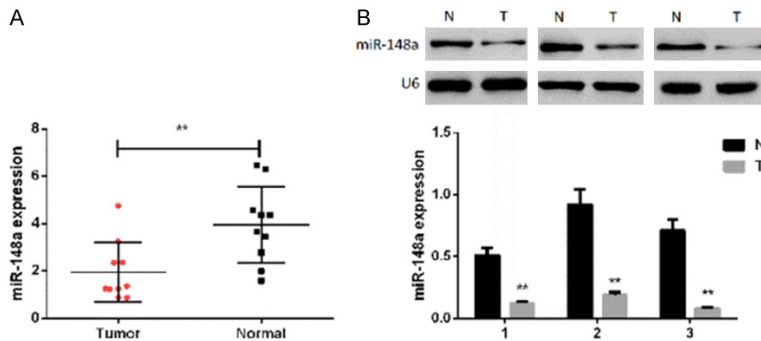


Figure 1. miR-148a is downregulated in SACC tissue samples compared with normal tissues. A: miR-148a expression levels detected by qRT-PCR in SACC tissue samples and normal tissues. B: Northern blotting analysis showed that miR-148a was significantly downregulated in SACC tissues compared with U6 as internal control and the experiment was conducted three times independently. ** $P < 0.01$ compared with the normal group.

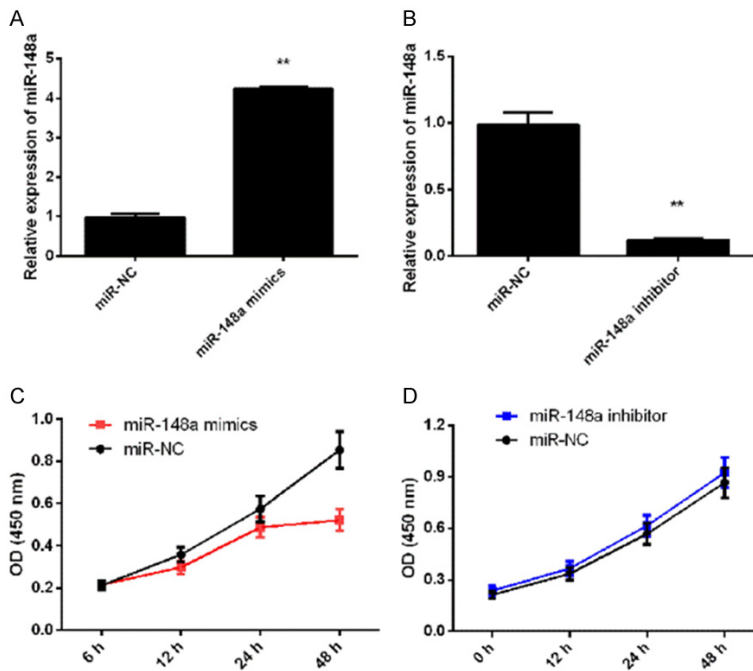


Figure 2. miR-148a suppresses cell proliferation. A: miR-148a expression level was measured in ACC-M cells transfected with miR-148a mimics by qRT-PCR. B: miR-148a expression level was measured in ACC-M cells transfected with miR-148a inhibitor through qRT-PCR. C: CCK8 assay indicated the viability of cells was suppressed transfected with miR-148a mimics. D: CCK8 assay indicated the viability of cells was promoted following transfected with miR-148a inhibitor. ** $P < 0.01$ compared with the miR-NC group.

to PVDF membranes. After transferring for 15 hours, vacuum drying treatment was used to dry the membranes for 2 hours at 80°C. Hybridization lasted 16 hours at 42°C and then the membranes were washed with SSC buffer. Finally ELC chemiluminescence reagent was used and the exposed images were captured and analyzed.

Western blotting

Cells were lysed in lysis buffer with 50 mM Tris-HCl at pH 7.6, 2 mM EDTA, 10% glycerol, 2% SDS and 5% β-mercaptoethanol. 10% SDS-PAGE was used to separate protein samples. Protein samples were then transferred to PVDF membranes. Membranes were blocked in the TBS buffer (50 mM Tris-HCl at pH 7.6 and 150 mM NaCl) with 5% non-fat dry milk for overnight at 4°C. Then membranes were incubated for 2 hours at 37°C with appropriate primary antibodies added into the TBS buffer. After that, the membranes were washed in TBST buffer (50 mM Tris-HCl at pH 7.6, 150 mM NaCl and 0.05% Tween-20). Then the membranes were incubated with secondary antibody. Using the same conditions, the membranes were washed in TBST buffer. Finally, ELC chemiluminescence reagent was used and the exposed images were captured and analyzed.

Statistical analyses

SPSS software was used to analyze experiments data. The one-way analysis variance (ANOVA) statistically was used to analyze the difference between among groups. Student's t-tests were used to analyze the fluorescent value of the luciferase reporter assay. P value < 0.05 was considered to be statistically significant.

Results

miR-148a expression level is down-regulation in SACC tissues

To study and analyze the expression of miR-148a in SACC samples, a comparison of miR-148a between SACC tissues and normal tis-

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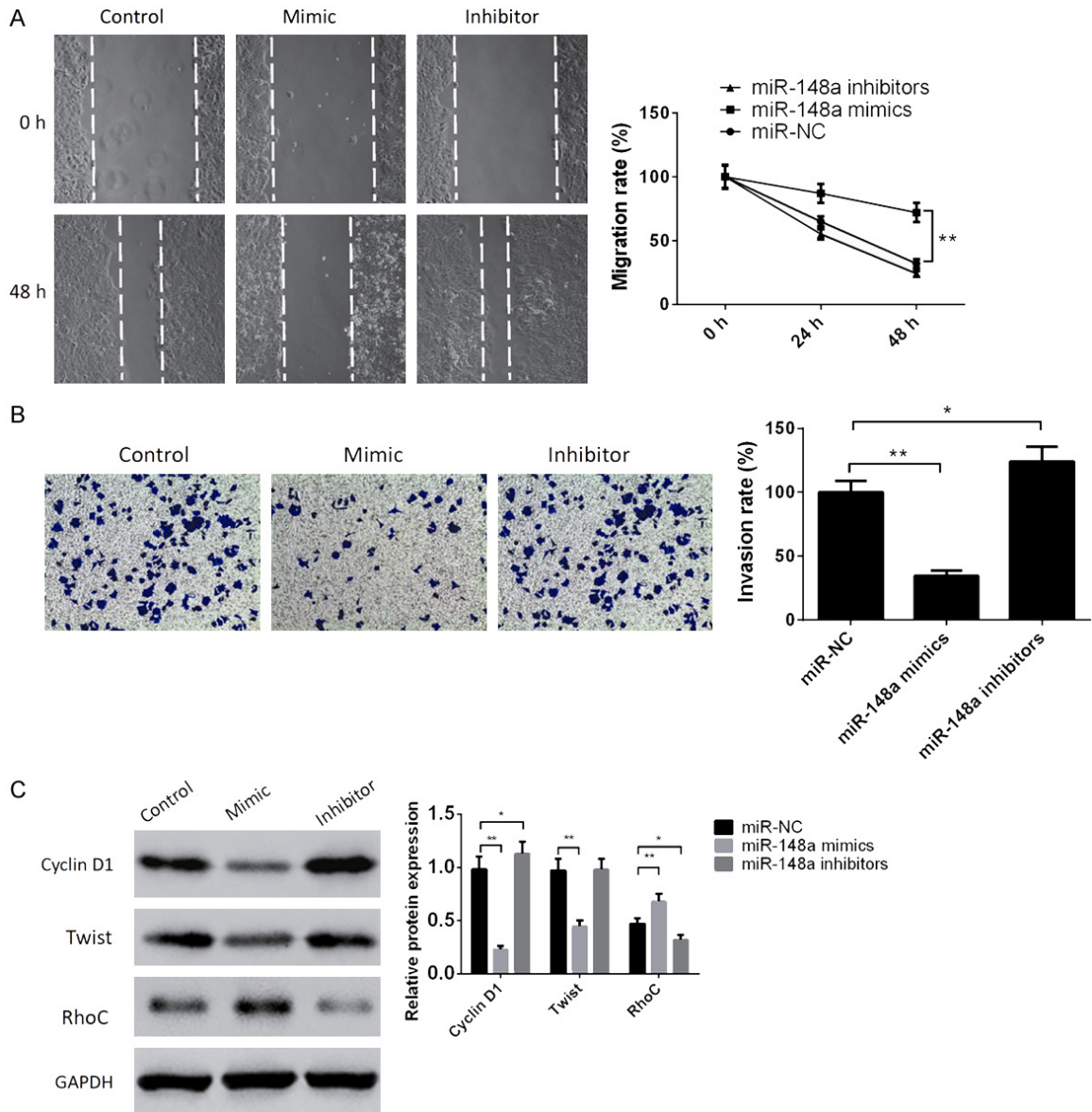


Figure 3. miR-148a suppresses cell migration and invasion. A: Image of wound healing assay on ACC-M cells indicated the invasion potential of SACC cells transfected with miR-148a inhibitor is highest among the NC groups, groups of cells transfected with mimics and groups of cells transfected with inhibitor. B: Transwell assay showed the invasion of cells was inhibited significantly transfected with miR-148a mimics. C: Western blotting of proteins of Cyclin D1, Twist and RhoC related to cell downstream proliferation, migration and invasion. Expression of Cyclin D1 protein and Twist increased and expression of RhoC decreased in group of miR-148a inhibitor. The experiments were repeated for three times. * $P < 0.05$, ** $P < 0.01$ compared with the miR-NC group.

sues was performed. qRT-PCR and Northern blotting indicated that the miR-148a expression level in SACC tissues was lower than the corresponding normal tissues acted as controls (Figure 1A, $P < 0.01$). Furthermore, Northern blotting analysis from indicated the same results (Figure 1B), showed that miR-148a acts as a tumor suppressor in SACC. Our findings show that the miR-148a was significantly de-

creased in SACC tissues compared with that in normal tissues of controls.

miR-148a suppresses cell proliferation

To analyze functions and roles of miR-148a in SACC cells, the SACC cell line ACC-M was transfected with miR-148a mimics (up-regulated miR-148a, Figure 2A), miR-148a inhibitor

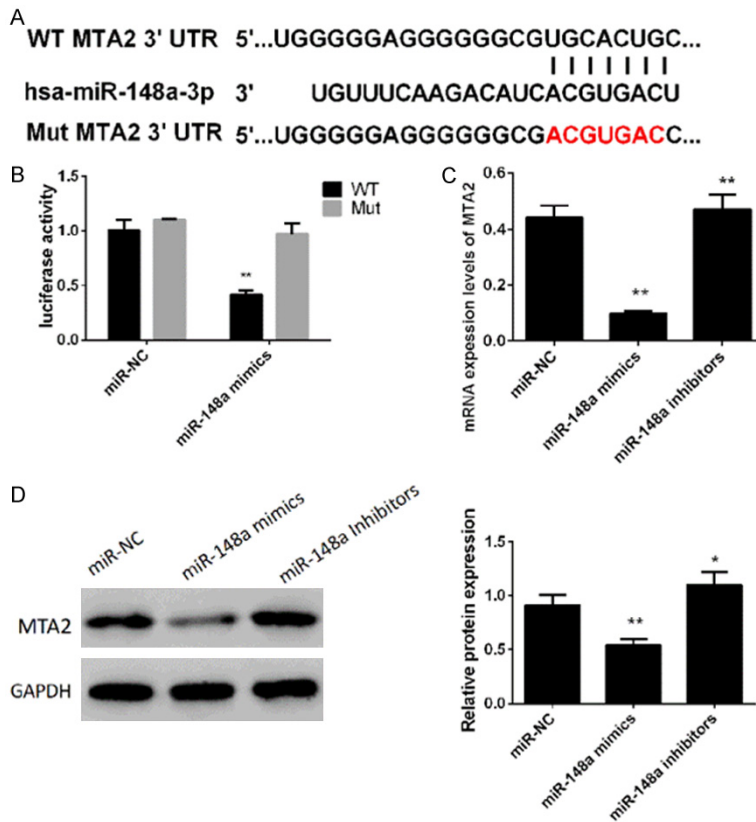


Figure 4. MTA2 was targeted by miR-148a expression. A: Binding sites of miR-148a predicted from the online software programs TargetScan. B: Dual-luciferase activity in MTA2 3'UTR including Mu and Wt pattern, with miR-148a. C: mRNA of MTA2 by RT-qPCR with miR-148a mimics, miR-148a inhibitor and miR-NC. D: Expression of MTA2 examined by Western blotting in cells transfected with miR-148a mimics, miR-148a inhibitor and miR-NC. * $P < 0.05$, ** $P < 0.01$ compared with the miR-NC group.

(down-regulated miR-148a, **Figure 2B**), and miR-NC (negative control) respectively. CCK8 assay was used to measure the effect of miR-148a on proliferation of ACC-M cells (**Figure 2C, 2D**). As shown in **Figure 2C**, overexpression of miR-148a (the ACC-M cells transfected with miR-148a mimics) suppressed significantly the growth rate of cells at 24 hours post transfection compared with miR-NC ($P < 0.01$). In contrast, when miR-148a was transfected with miR-148a inhibitor, cell proliferation was observed that it was promoted significantly at 24 hours post transfection compared with miR-NC ($P < 0.01$). The results indicate that overexpression of miR-148a suppresses cell proliferation and growth by CCK8 assay.

miR-148a suppresses cell migration and invasiveness

To analyze the effect of miR-148a on cell migration, scratch wounding-healing assay was per-

formed. Similar to experiments grouping designing of CCK8 assay, groups of SACC cell lines ACC-M were transfected with miR-148a mimics, miR-148a inhibitor, and miR-NC, respectively. As shown in **Figure 3A**, wound healing was significantly reduced after 48 hour incubation in the miR-148a mimics group, compared with the miR-NC group ($P < 0.01$). The rate of wound healing was decreased significantly in the miR-148a mimics group and promoted significantly in the miR-148a inhibitor group, compared with the miR-NC group ($P < 0.01$). Also, transwell assay was performed to analyze cell line migration further. Overexpression of miR-148a (the ACC-M cells transfected with miR-148a mimics) inhibited significantly the invasion of cells (**Figure 3B**, $P < 0.01$), while in the miR-148a inhibitor group, invasion was increased (**Figure 3B**, $P < 0.05$). In Western blotting analysis, Cyclin D1, Twist and RhoC antibodies were used to detect and analyze cell proliferation

and apoptosis. As shown in **Figure 3C**, in the group overexpressing miR-148a (the ACC-M cells transfected with miR-148a mimics), expression of RhoC was increased, and expression of Cyclin D1 and Twist was decreased compared with the miR-NC group. In the group of ACC-M cells transfected with miR-148a mimics, expression of RhoC was dewas increased compared with the miR-NC group. The results indicate that miR-148a regulated cell proliferation, migration, and invasion by regulating the expression of proteins of Cyclin D1, Twist, and RhoC.

MTA2 is a molecular target of miR-148a regulation

Potential mRNA targets of miR-148a were searched from the online software programs MiRanda, Mirwalk, and Targetscan (**Figure 4A**). In order to determine whether MTA2 was regulated by miR-148a, 3'UTR of the MTA2 gene

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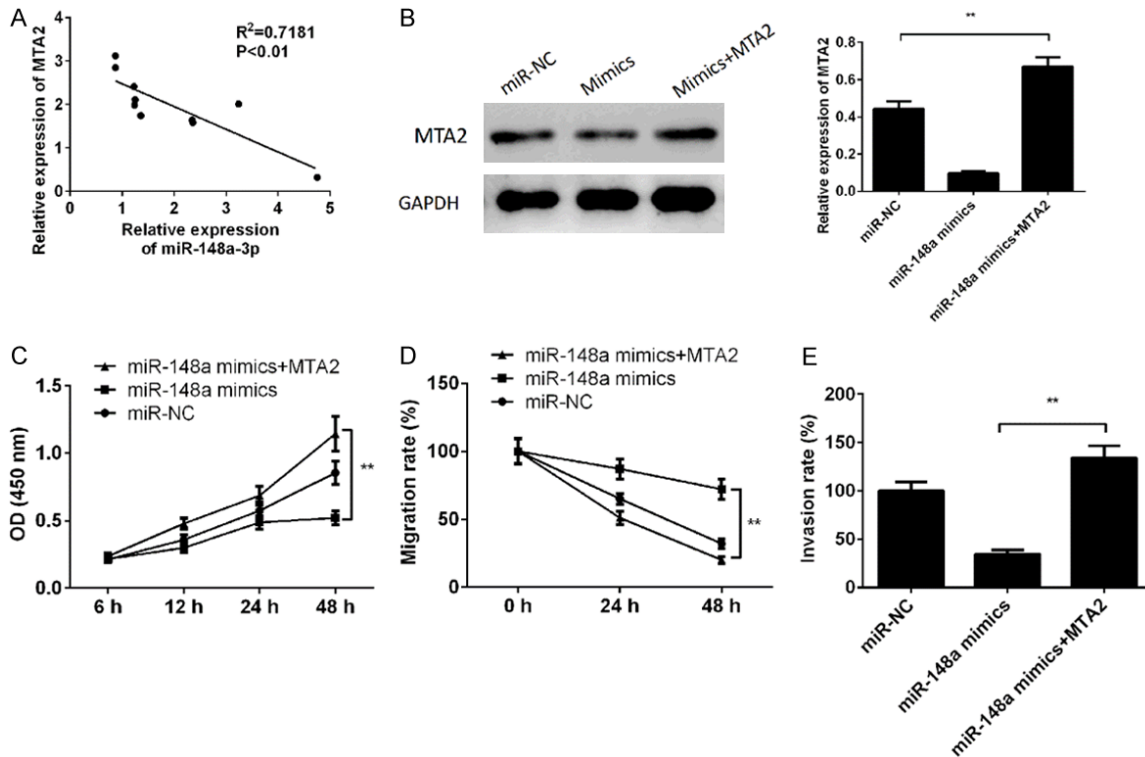


Figure 5. Effect of MTA2 in SACC cells with transfected miR-148a mimics. A: There was a negative correlation between miR-148a and MTA2. B: Western blotting was used to detect the expression level of MTA2. C-E: Cell proliferation, migration, and invasion were measured by CCK8 assay, transwell assay, and wound healing assay. * $P < 0.05$, ** $P < 0.01$ compared with the miR-148a mimics group.

with both wild-type and mutant miR-148a target sequences were cloned into the luciferase vectors respectively. Groups of HEK293T cell were transfected with 3'UTR of the MTA2 vectors both wild-type (Wt) and mutant miR-148a (Mu) respectively. Co-transfection with miR-148a, the luciferase activity of the wild-type 3'UTR reporter gene decreased significantly ($P < 0.01$), while luciferase activity of the mutant-type 3'UTR reporter gene was not affected (Figure 4B). RT-PCR and Western blotting showed that the MTA2 expression increased transfected with miR-148a inhibitor, compared with the group of miR-NC. Additionally, expression decreased when transfected with miR-148a mimics, compared with the group of miR-NC (Figure 4C, 4D, $P < 0.05$).

Overexpression of MTA2 in the mimics group reversed the suppressive effects on the growth proliferation and invasion

MTA2 was predicted to be targeted by miR-148a and there was a negative correlation between miR-148a and MTA2 (Figure 5A). To

investigate whether the suppressive effects of miR-148a on the proliferation, migration, and invasion were mediated via MTA2 down-regulation, MTA2 was overexpressed in cells transfected with miR-148a mimics. Western blotting indicated that the overexpression of MTA2 was successful (Figure 5B). CCK8 assay, scratch wounding-healing assay, and trans-well invasion assay were performed to analyze the effect on cells. Up-regulation the MTA2 expression level not only increased the proliferation of cells transfected with miR-148a mimics, but also rescued their migration and invasion capacities (Figure 5C-E, $P < 0.01$). Taken altogether, the results suggest that miR-148a exerts suppressive effects via targeting gene of MTA2, and that MTA2 overexpression can reverse the effect on the growth, migration, and invasion.

Discussion

MicroRNAs (miRNA) are not only engaged in regulation of signaling molecules through repression of gene splicing, but also associated

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with cell proliferation, differentiation, and apoptosis. According to predictions, although miRNA genes account for only 1% of all eukaryotic genes, they regulate 10 to 30 percent of the whole genome [13]. Because miRNA plays a negative regulatory factor in regulation of gene expression, overexpression of oncogene miRNAs can result in tumor occurrence by inducing cell proliferation and inhibiting apoptosis. Reduced expression of tumor suppressor gene miRNAs can achieve the same effect. Abnormal expression of various tumor-related genes can result in the occurrence of SACC and is closely related to the prognosis. It has been reported that the expression level of metallothionein in transitivity SACC is significantly higher than non-metastatic [14]. The expression of some kinds of certain miRNA is up-regulated or down-regulated, and expression abnormalities have tissue-specific effects related to the type of cancers [15]. In recent years, more and more studies have found that abnormal expression of miRNAs is related to the occurrence and development of SACC. Although studies have shown that miR-181a can inhibit proliferation, migration, and invasion in SACC, as well as pulmonary metastasis of SACC [16], studies on miRNAs, including the miR-148a, in SACC are few.

Many studies have shown that miR-148a is the member of the miR-148/152 family closely related to tumor growth and metastasis and plays a crucial role of the occurrence, development of tumor. miR-148a is down-regulated in breast cancer [17] with the exact mechanism being uncertain. It is also decreased in liver cancer cells acting as a tumor suppressor. In gastric cancer and cell lines, miR-148a is down-regulated, playing a role in the proliferation, invasion, and metastasis of tumor cells by regulating different target genes and signal pathways downstream [18, 19]. Different from breast cancer, liver cancer, and gastric cancer, miR-148a is up-regulated in human osteosarcoma, with the exact mechanism uncertain until now [20].

In this study, miR-148a expression pattern in SACC was investigated. Expression of miR-148a was lower in SACC tissues than in normal tissues. Additionally, overexpression of miR-148a suppressed cell proliferation, migration, and invasion in SACC cells transfected with miR-148a mimics, miR-148a inhibitor, and miR-C, through comparison of expression level

between normal tissue and SACC tissues. Assays included the CCK8 assay, wound healing assay, and transwell assay to detect cell viability and invasion as major characteristics of tumor cells. Western blotting was used to analyze related proteins in cell proliferation and invasion and the result confirmed traits of miR-148a in SACC. The MTA2 gene encodes a protein that has been identified as a component of NuRD, a nucleosome remodeling deacetylase complex identified in the nucleus of human cells. It has been reported that the aberrant expression of MTA2 in many tumors, including ovarian, lung, and pancreatic cancer [12, 21, 22]. The MTA2 gene was found that it was targeted by miR-148a, from the online software programs. Co-transfection with miR-148a, the luciferase activity of the wild-type 3'UTR reporter gene was decreased significantly, while the luciferase activity of the mutant-type 3'UTR reporter gene was not affected. Both the result and the results of expression of MTA2 reduced in Western blotting proved that MTA2 was the target gene of miR-148a. To find out if the effect on proliferation, invasion, and migration in SACC through the MTA2 pathway, cell proliferation assays, cell invasion assays and cell migration assays were performed and the results indicated that overexpression of MTA2 in cells transfected with miR-148a mimics greatly promoted cell proliferation, migration and invasion. In contrast, overexpression of miR-148a suppressed cell proliferation, migration, and invasion significantly. Furthermore, Western blotting also proved the result. In conclusion, the tumor-suppressive effect of miR-148a on salivary adenoid cystic carcinoma was via down-regulation of MTA2. Our findings may be a better understanding of the SACC and develop new therapeutic methods to SACC.

Disclosure of conflict of interest

None.

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