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ORIGINAL RESEARCH

LncRNA ADAMTS9-AS1 Restrains the Aggressive Traits of Breast Carcinoma Cells via Sponging miR-513a-5p

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Purpose: Long noncoding RNAs (lncRNAs) exert important functions in the progression of cancers. Currently, we aim to investigate the potential roles of lncRNA ADAM Metallopeptidase with Thrombospondin Type 1 Motif 9 Antisense RNA 1 (ADAMTS9-AS1) in breast carcinoma.

Materials and Methods: The expressions of ADAMTS9-AS1 and miR-513a-5p in breast carcinoma tissues and cell lines were detected using qRT-PCR. Cell Counting Kit-8 (CCK-8) and transwell assays were used to assess the viability and invasive ability of breast cancer cells. The direct interaction between ADAMTS9-AS1 and miR-513a-5p was predicted using bioinformatics tools. The target of miR-513a-5p, ZFP36 Ring Finger Protein (ZFP36) was validated by luciferase assay. The expression of ZFP36 was measured using Western blot assay. Breast cancer MDA-MB-231 cells growth in vivo was evaluated using xenograft tumor assay.

Results: ADAMTS9-AS1 was downregulated in breast cancer tissues as well as cell lines. Upregulation of ADAMTS9-AS1 suppressed the growth and invasiveness of breast carcinoma cells in vitro as well as inhibiting cellgrowth in vivo. Furthermore, ZFP36 was manifested as the target gene of miR-513a-5p and negatively modulated by ADAMTS9-AS1. In addition, overexpression of ADAMTS9-AS1 neutralized the promoting impact of miR-513a-5p on the aggressiveness of breast cancer cells.

Conclusion: In conclusion, lncRNA ADAMTS9-AS1 inhibited the aggressive phenotypes of breast carcinoma cells via sponging miR-513a-5p and regulating ZFP36.

Keywords: breast carcinoma, MiR-513a-5p, ZFP36, ADAMTS9-AS1

Introduction

Breast cancer was the third highest incidence cancer in 2017, with an estimated 1,960,681 cases and a high prevalence in females.[1] Breast cancer is characterized by its heterogeneity and is classified into different subtypes: Luminal A; Luminal B; human epidermal growth factor receptor-2 (Her-2) positive and triple negative breast cancer (TNBC), based on distinct gene expression signatures.[2] Most breast carcinomas are derived from the epithelium lining the ducts or lobules, and these cancers are classified as ductal or lobular carcinoma. Ductal carcinoma in situ (DCIS) is growth of low-grade cancerous or precancerous cells within the mammary duct without invasion of the surrounding tissue. In contrast, invasive ductal carcinoma (IDC) does not confine itself to the initial tissue compartment. TNBC is the most invasive and aggressive among the breast cancer subtypes.[3] So far there is no clinical therapy specifically for patients with TNBC.[4]

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Although the diagnostic tools and treatments for breast cancer have greatly improved, metastasis is the leading cause of death in patients with breast cancer.[5] Hence, blocking metastases is urgent for improving the clinical outcomes of patients. LncRNAs are types of noncoding RNAs with more than 200 nucleotides.[6,7] According to early investigations, dysregulated lncRNAs are closely relevant with the progression of various cancers.[8–11] For instance, a high level of lncRNA MIR31 Host Gene (MIR31HG) is a prognostic indicator and boosts lung adenocarcinoma cells proliferation.[12] LncRNA Prostate Cancer Associated Transcript 6 (PCAT6) accelerates the development and chemoresistance of cervical carcinoma cells by sponging miR-543.[13] LncRNA AGAP2 Antisense RNA 1 (AGAP2-AS1) heightens the viability and migration, and induces gemcitabine resistance through suppressing miR-497 in colorectal carcinoma.[14]

An earlier report demonstrates that ADAMTS9-AS1 is significantly downregulated in prostate cancer and plays a suppressive role in cell growth and proliferation.[15] ADAMTS9-AS1 suppresses colorectal cancer by inhibiting the Wnt/β-catenin signaling pathway and is a potential diagnostic biomarker.[16] Fan et al. identified the potential lncRNA signatures capable of predicting overall survival (OS) of patients with breast cancer. They concluded that in human breast carcinoma, higher expression of ADAMTS9-AS1 is associated with a better overall prognosis in patients.[17] Although previous investigation has clarified that ADAMTS9-AS1 has prognostic value in breast cancer, the role of ADAMTS9-AS1 in the aggressive process of breast carcinoma has not yet been fully revealed.

In the current study, the dysregulated lncRNAs in breast carcinoma tissues were identified using The Cancer Genome Atlas (TCGA) database. LncRNA ADAMTS9-AS1, whose expression level was lower in breast carcinoma tissues compared with normal tissues, was selected as the object of our present study. Upregulation of ADAMTS9-AS1 suppressed the biological phenotypes of breast carcinoma cells. Furthermore, the functional relationship between ADAMTS9-AS1 and miR-513a-5p was illuminated. Our findings provide a new theoretical basis for the ADAMTS9-AS1/miR-513a-5p axis in the progression of breast carcinoma.

Materials and Methods

Breast Carcinoma Tissues

Fifty-six pairs of breast carcinoma tissues and adjacent samples were obtained from patients who underwent surgical resection for breast carcinoma at Qilu Hospital (Qingdao), Cheeloo College of Medicine, Shandong University. Collected tissue samples were stored in RNAlater (RNAlater™ Stabilization Solution, Thermo Fisher Scientific, Waltham, MA, USA) at −80°C.[18] Written informed consents were obtained from participants and this study was approved by the Ethics Committee of Qilu Hospital (Qingdao), Cheeloo College of Medicine, Shandong University. The clinicopathological characteristics are shown in the Table 1.

Cell Lines and Transfection

Breast carcinoma cell lines (Hs 578T, MDA-MB-231, MCF-7, MDA-MB-453) and the non-tumorigenic breast epithelial cell line, MCF-10A were purchased from ATCC. MDA-MB-231 was maintained in L-15 (Thermo Fisher Scientific, Waltham, MA, USA), MCF-10A was maintained in MEGM (Thermo Fisher Scientific), MCF-7 and Hs 578T was maintained in DMEM with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. The cDNA

Table 1 Characteristics of Patients with Breast Cancer in the Present Study

Variables	No. of Patients
Age (years)	
<50	17
≥50	39
Menopausal status	
Premenopause	16
Postmenopause	40
LNM	
Yes	34
No	22
Clinical stage	
I	18
II	26
III	12
Molecular subtypes	
Luminal A (ER+/PR+/HER2-)	9
Luminal B (ER+/PR+/HER2+)	16
HER2 positive (ER-/PR-/HER2+)	18
Triple negative (ER-/PR-/HER2-)	13
Pathological type	
IDC	39
DCIS	17

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor-2; LNM, lymph node metastasis; PR, progesterone receptor; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ.

sequences of ADAMTS9-AS1 or ZFP36 were subcloned into pcDNA3.1 vector (Thermo Fisher Scientific) to generate pcDNA-ADAMTS9-AS1 and pcDNA-ZFP36 overexpression plasmids. MiR-513a-5p mimics and negative control (miR-Ctrl) were purchased from RiboBio (Guangzhou, China). MiR-513a-5p mimics or pcDNA were transfected into cells using Lipofectamine 2000 kit (Thermo Fisher Scientific) for 24 hours.

qRT-PCR

Total RNAs were isolated using TRIzol kit (Thermo Fisher Scientific) and miRNAs were extracted by RNeasy/miRNeasy Mini Kit (Qiagen, Limburg, the Netherlands). The reverse transcription was carried out with Super Script First Strand cDNA System (Thermo Fisher Scientific). U6 and GAPDH served as an endogenous control. The primer sequences were shown as following: ADAMTS9-AS1 (Forward: 5′-CTCAGACCACAACTCTCCACCTTG-3′, reverse: 5′-CAGATGCTGCCTGGCTGATGG-3′); miR-513a-5p (Forward: 5′-TAAATTTCACCTTTCTGAGAAGG-3′, reverse: 5′-GCGAGCACAGAATTAATACGAC-3′); U6 (Forward: 5′-CTCGCTTCGGCAGCACA-3′, reverse: 5′-AACGCTTCACGAATTTGCGT-3′); GAPDH (Forward: 5′-GTGAAGGTCGGAGTCAAC-3′ reverse: 5′-GTTGAGGTCAATGAAGGG-3′). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative abundance of RNA genes compared with GAPDH or U6 expression.

Western Blot

Proteins were extracted using RIPA lysis buffer (Beyotime Biotechnology, Nanjing, China). 25 μg of lysate were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, USA). PVDF membrane was incubated with ZFP36 or β-Tubulin antibody (1:1000, Abcam, Cambridge, UK). Primary antibodies were labeled with HRP-conjugated secondary antibody (1:5000, Beyotime Biotechnology). Immunoblots were visualized by ECL kit (Millipore, Braunschweig, Germany).

Cell Viability

100 μL cells (1×10^4) were cultured in 96-well plates. 10 μL of Cell Counting Kit-8 (CCK-8) reagent (Nanjing KeyGen Biotech, Nanjing, China) were added to each well at 24, 48, 72 or 96 h, respectively. After incubating for 2 h, the absorbance was assessed at 450 nm using a microplate reader. In colony formation, Hs 578T or MDA-MB-231 cells (5×10^2 cells/well) were cultured into 6-well plates for 14 days. After staining cells with 1% crystal violet in methanol, the number of colonies was counted.

Transwell Assay

200 μL of cells suspension (1×10^3) were cultured in Matrigel (BD Biosciences, San Jose, CA, USA) coated transwell inserts for 24 h. 800 μL of medium supplement with 10% FBS was plated into the lower chamber. After 24 h, invaded cells were dyed with 1% crystal violet and the invaded cells were counted.

Luciferase Reporter Gene Assay

The partial sequences of ADAMTS9-AS1 containing binding sites for miR-513a-5p or losing binding sites for miR-138-5p (ADAMTS9-AS1-wt or ADAMTS9-AS1-mut) were inserted into pmirGLO plasmid (Promega, Madison, WI, USA). Furthermore, the wt 3′-UTR or the mut 3′-UTR of ZFP36 (ZFP36-wt or ZFP36-mut) were cloned into pmirGLO plasmid. Hs 578T or MDA-MB-231 cells were co-transfected with ADAMTS9-AS1 ZFP36 or pmirGLO plasmids combination with miR-513a-5p mimics. At 48 h post-transfection, the relative luciferase activity was determined using a luciferase reporter assay system.

Xenograft Assay

MDA-MB-231 cells were transfected with ADAMTS9-AS1 or pcDNA3.1 vector. 100 μL of 2×10^6 MDA-MB-231 cell suspension were inoculated subcutaneously into BALC/c nude mice (n=5 in each group). The length and width of xenograft tumor were measured every 3 days. Tumor volume = length×width^2/2. At 35 days after implantation, mice were sacrificed. Tumors were isolated and weighed. Animal experiments were approved by the Committee for Animal Research of Qilu Hospital (Qingdao), Cheeloo College of Medicine, Shandong University. All procedures involving experimental animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985).

Statistical Analysis

Measurement results were expressed as the mean ± standard deviation (SD). All experiments were repeated three times and all data in this study were analyzed using GraphPad Prism 7.0. Comparison analysis was carried out using Student's *t*-test or one-way ANOVA with post hoc Tukey's Multiple Comparison. Pearson correlation analyses were used to investigate the correlation between

ADAMTS9-AS1 and miR-513a-5p expression. $P<0.05$ was considered to be statistically significant.

Results

ADAMTS9-AS1 Was Downregulated in Breast Carcinoma

High throughput sequencing is increasingly being widely used and it has been utilized as an important tool for cancer diagnosis, cancer grading and prognosis prediction. In this study, the lncRNAs expression profiles in breast carcinoma tissues and normal tissues were comprehensively analyzed using The Cancer Genome Atlas Program (TCGA). 841 downregulated lncRNAs and 300 upregulated lncRNAs were found between breast carcinoma and normal tissues (fold change >1 and *P*-value <0.05) (Figure 1A and B). We focused on ADAMTS9-AS1, which was one of the downregulated lncRNAs in breast carcinoma tissues when compared with normal tissues (fold change=−3.3, $P<0.001$, Figure 1C). Although the prognostic value that ADAMTS9-AS1 has in breast cancer has been identified, the role of ADAMTS9-AS1 in the growth and invasion of breast carcinoma is not clear. The Kaplan-Meier curve showed that ADAMTS9-AS1 was linked to overall survival (OS) in patients with breast cancer. Patients with low ADAMTS9-AS1 expression had worse OS than those with high expression (Figure 1D). Next, the result of qRT-PCR assay indicated that ADAMTS9-AS1 was strikingly downregulated in breast carcinoma tissues in contrast to that in adjacent samples (Figure 1E). Besides, the expressions of ADAMTS9-AS1 in the breast cancer cell lines (MDA-MB-231, Hs 578T, MCF-7 and MDA-MB-453) and non-tumorigenic breast epithelial cell line, MCF-10A were detected via qRT-PCR. The level of ADAMTS9-AS1 in breast carcinoma cell lines was lower than that in MCF-10A (Figure 1F). After confirming the lower expression of ADAMTS9-AS1 in breast cancer cell lines, we analyzed the invasion ability of four cell lines in vitro. We found that Hs 578T and MDA-MB-231 had more distinct invasion capacities than MCF-7 and MDA-MB-453 cell lines (data not shown). To seek the role of ADAMTS9-AS1 in breast carcinoma cells growth, human triple negative breast cancer MDA-MB-231 and Hs 578T cells were transfected with pcDNA3.1-ADAMTS9-AS1 (Figure 1G). The CCK-8 assay showed that MDA-MB-231 and Hs 578T cells proliferation were suppressed after upregulation of ADAMTS9-AS1 (Figure 1H). The same trends were also obtained in the colony formation assay (Figure 1I). In addition, upregulated ADAMTS9-AS1 decreased the invasion of breast carcinoma cells (Figure 1J).

ADAMTS9-AS1 is Negatively Correlated with miR-513a-5p in Breast Carcinoma

LncRNAs may contain one or more miRNA response elements and can act as endogenous miRNA "sponges", which contributes to the downregulation of intracellular miRNA. This is also called the "competing endogenous RNA (ceRNA)" mechanism. The ceRNA mechanism plays a crucial role in cancer progression.[19,20] LncRNASNP and miRDB were utilized to seek the putative target miRNAs of ADAMTS9-AS1 (Figure 2A). The binding sites between ADAMTS9-AS1 and miR-513a-5p are shown in Figure 2B. The results of luciferase report gene assay indicated that transfection of miR-513a-5p mimics lessened the luciferase activities in MDA-MB-231 and Hs 578T cells transfected with pmirGLO plasmid containing ADAMTS9-AS1-wt whereas there was no effect in the cells transfected with plasmid carrying ADAMTS9-AS1-mut (Figure 2C). Next, the result of qRT-PCR test indicated that miR-513a-5p level was distinctly higher in breast carcinoma tissues compared to that in adjacent samples (Figure 2D). Meanwhile, the results of qRT-PCR showed that miR-513a-5p level was negative related with the level of ADAMTS9-AS1 in breast carcinoma tissues (Figure 2E). Finally, we proved that miR-513a-5p expression was decreased in MDA-MB-231 and Hs 578T cells after ADAMTS9-AS1 treatment compared with vector group (Figure 2F).

ADAMTS9-AS1 Affects the Aggressive Traits of Breast Carcinoma via Sponging miR-513a-5p

Hs 578T and MDA-MB-231 cells were transfected with miR-513a-5p alone or co-transfected miR-513a-5p mimics in combination with ADAMTS9-AS1 plasmid, and the levels of miR-513a-5p were determined by qRT-PCR test. As shown in Figure 3A, the expression of miR-513a-5p in mimics transfected MDA-MB-231 and Hs 578T cells was significantly raised, however its level was decreased by ADAMTS9-AS1. CCK-8 results indicated that Hs 578T and MDA-MB-231 cells viability was raised in miR-513a-5p mimics group whereas the effect of miR-513a-5p mimics was removed by ADAMTS9-AS1 (Figure 3B). Consistently, colony formation assay displayed that

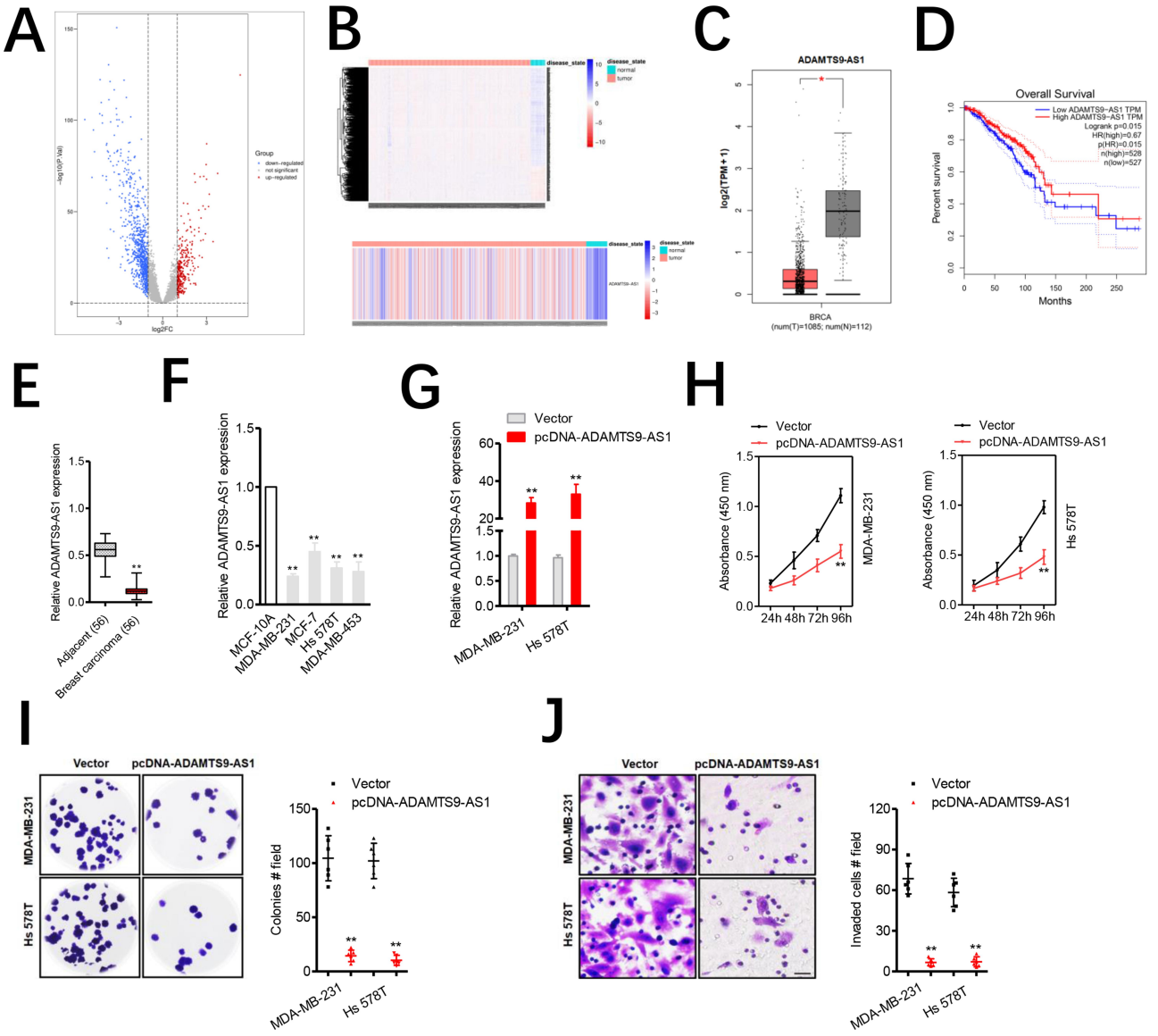

Figure 1 LncRNA ADAMTS9-AS1 was down-expressed in breast carcinoma tissues and cells. (**A**) Volcano plot showed that the upregulated lncRNAs and downregulated lncRNAs in breast carcinoma tissues, compared with normal tissues. (**B**) Clustering map showed that lncRNA ADAMTS9-AS1 was screened out from all differentially expressed lncRNAs in breast carcinoma tissues. (**C**) The expressions of ADAMTS9-AS1 in TCGA breast cancer samples were analyzed through GEPIA (http://gepia.cancer-pku.cn/index.html). *P<0.05 compared with normal (N). (**D**) Kaplan-Meier curves for overall survival rate of patients with breast cancer by ADAMTS9-AS1 expression in tumors. (**E**) The qRT-PCR results showed that the expression of ADAMTS9-AS1 was remarkably lower in breast carcinoma tissues (n=56) compared with adjacent tissues (n=56). **P<0.01 compared with adjacent. (**F**) The qRT-PCR results showed the endogenous expression of ADAMTS9-AS1 in MCF-10A and breast carcinoma cell lines. **P<0.01 compared with MCF-10A. (**G**) MDA-MB-231 and Hs 578T cells were transfected with vector or pcDNA-ADAMTS9-AS1. The levels of ADAMTS9-AS1 were analyzed using qRT-PCR assay. (**H**) The proliferation of MDA-MB-231 and Hs 578T cells were detected using CCK-8. (**I**) Colony formation analysis using pcDNA-ADAMTS9-AS1 transfected MDA-MB-231 and Hs 578T cells. (**J**) The invasion abilities of MDA-MB-231 and Hs 578T cells were measured using transwell assay. **P<0.01 compared with vector.

transfection of miR-513a-5p mimics markedly promoted the colony forming of breast cancer cells whereas co-transfection with ADAMTS9-AS1 neutralized the impact of miR-513a-5p (Figure 3C). Transwell assay results showed that MDA-MB-231 and Hs 578T cells invasion was markedly stronger in miR-513a-5p-mimics group and was repressed by ADAMTS9-AS1 (Figure 3D). Finally, Kaplan-Meier plotter (http://kmplot.com/analysis/) was employed to predict the prognostic values of miR-513a-5p in breast cancer. We observed that patients with higher miR-513a-5p expression had worse OS than those with lower expression of miR-513a-5p (Figure 3E).

ZFP36 is the Target Gene of miR-513a-5p

MiRNAs negatively regulate gene expression by binding to the 3′-untranslated region (3′-UTR) of target mRNA

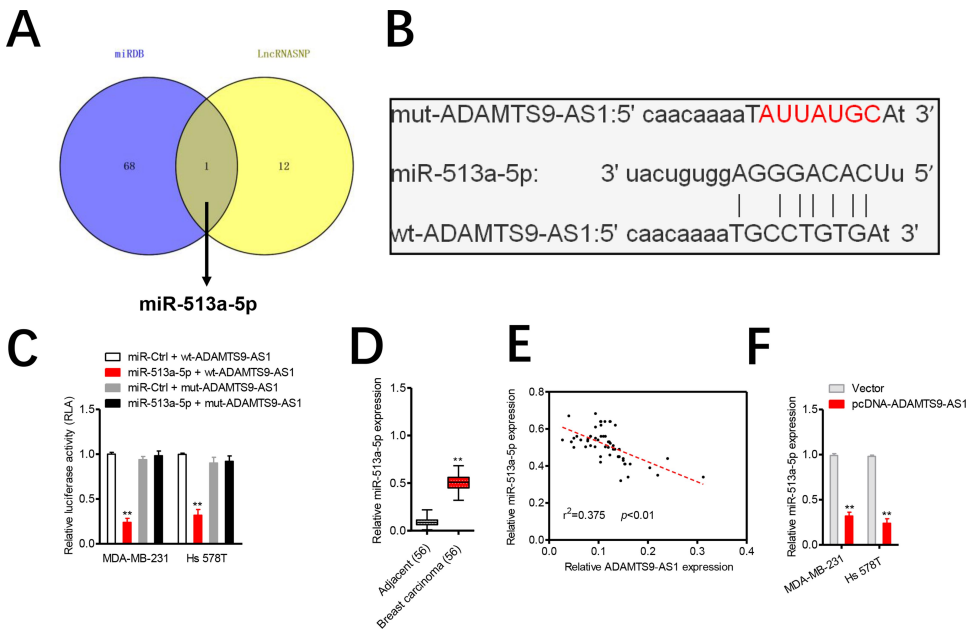

Figure 2 The negative correlation between miR-513a-5p and lncRNA ADAMTS9-AS1. (**A**) miR-513a-5p was predicted by LncRNASNP and miRDB to be the potential target of ADAMTS9-AS1. (**B**) The predicted miR-513a-5p binding sites of ADAMTS9-AS1 (ADAMTS9-AS1-wt) and the designed mutated sequence (ADAMTS9-AS1-mut) were indicated. (**C**) miR-513a-5p mimics in combination with ADAMTS9-AS1-wt or ADAMTS9-AS1-mut were co-transfected into breast cancer cells. The luciferase activity was detected. ***P*<0.01 compared with miR-Ctrl. (**D**) The results of qRT-PCR showed that miR-513a-5p was expressed significantly higher in tumor tissues than in adjacent tissues. ***P*<0.01 compared with adjacent. (**E**) The expression of miR-513a-5p was negatively correlated with the expression of ADAMTS9-AS1 in breast carcinoma tissues. (**F**) The results of qRT-PCR showed that the expression of miR-513a-5p was decreased after the transfection of pcDNA-ADAMTS9-AS1. ***P*<0.01 compared with vector.

molecules. TargetScan, miRDB, miRTarBase and TarBase v.8 were selected to find the potential target genes of miR-513a-5p (Figure 4A). We found that two genes (ZFP36 and MAP1B) were downregulated in breast cancer after checking TCGA (Figure 4B). We focused on ZFP36 in breast cancer owing to an earlier report which showed that the higher expression of ZFP36 is associated with lower grade and a better prognosis in breast carcinoma.[21] The relative luciferase activity in MDA-MB-231 and Hs 578T cells co-transfected miR-513a-5p and ZFP36-wt plasmid was strikingly weakened (Figure 4C). In addition, the expression of ZFP36 was degraded in miR-513a-5p mimics treated MDA-MB-231 and Hs 578T cells when compared with miR-Ctrl group (Figure 4D).

miR-513a-5p Regulates ZFP36 to Affect Breast Carcinoma Cell Proliferation and Invasion

MDA-MB-231 and Hs 578T cells were transfected with miR-513a-5p mimics alone or co-transfected with pcDNA-ZFP36 and miR-513a-5p mimics. The protein expression of ZFP36 was lower in miR-513a-5p transfected cells and restored in pcDNA-ZFP36 group (Figure 5A). The colony formation of MDA-MB-231 and Hs 578T cells in vitro was weaker in miR-513a-5p mimics alone transfection and notably rescued by pcDNA-ZFP36 (Figure 5B). Consistently, transwell assay displayed that MDA-MB-231 and Hs 578T cells invasion capacity markedly decreased in miR-513a-5p mimics group and pcDNA-ZFP36 reversed this trend (Figure 5C). Next, we observed that patients with lower ZFP36 expression had worse OS than those with higher expression, using Kaplan Meier plotter analysis (Figure 5D).

ADAMTS9-AS1 Affects Breast Carcinoma Cells Growth in vivo

To study the function of ADAMTS9-AS1 in the growth of breast carcinoma cells in vivo, the subcutaneously transplanted tumor model of MDA-MB-231 cells was constructed in nude mice. Vector or pcDNA-ADAMTS9-AS1 transfected MDA-MB-231 cells were inoculated into nude mice. The growth of subcutaneous xenograft tumors was observed every week. Tumors were collected 35 days after transplantation. The macroscopic appearance of dissected tumor tissues is shown in Figure 6A. Furthermore, the tumor volume and tumor weight in the pcDNA-ADAMTS9-AS1 group were smaller than that in the

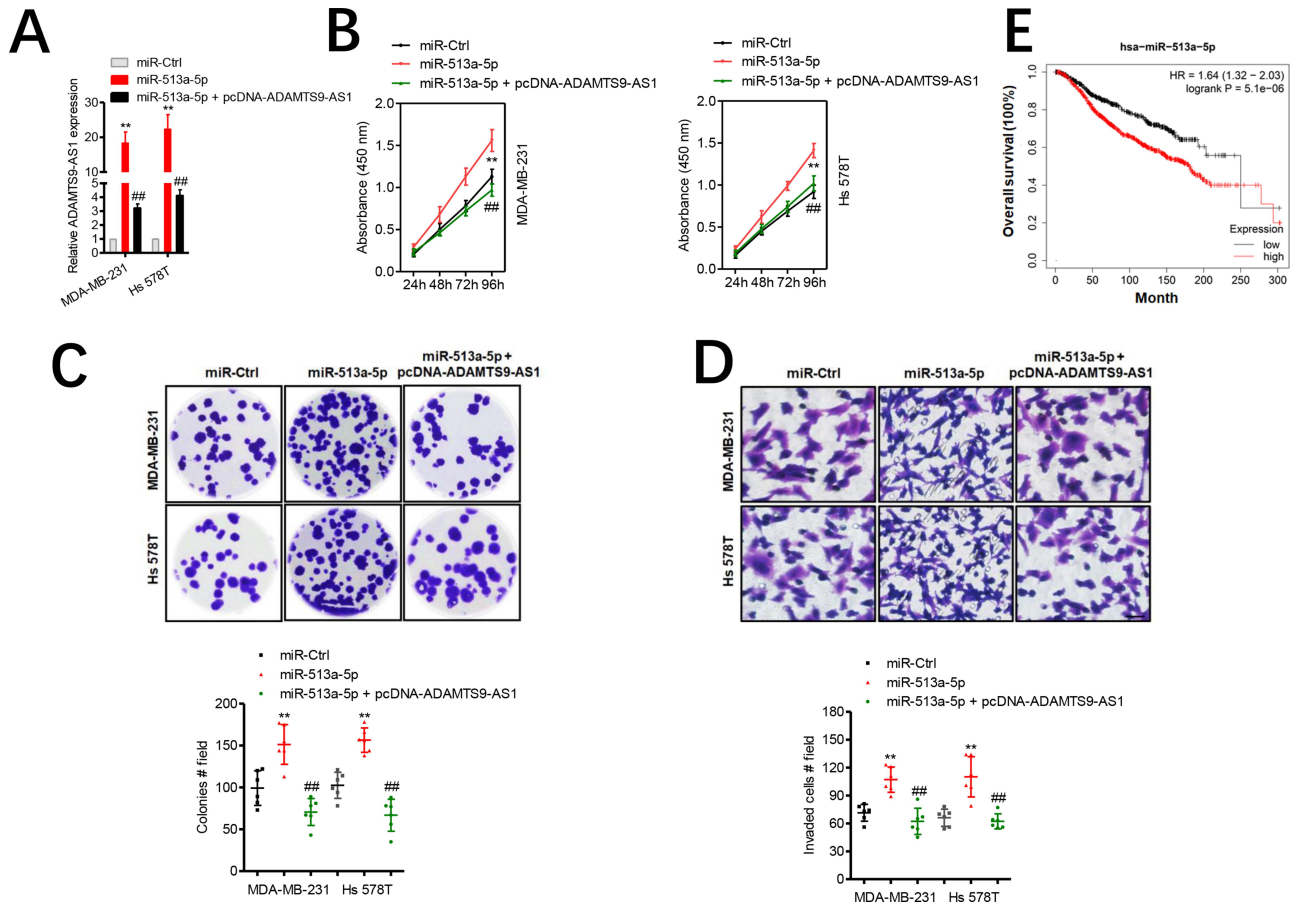

Figure 3 Effects of ADAMTS9-AS1 on breast carcinoma cell proliferation and invasion via regulating miR-513a-5p. (**A**) The expression of miR-513a-5p was significantly higher in mimics transfected group compared with miR-Ctrl group, whereas the expression of miR-513a-5p was significantly lower in miR-513a-5p and pcDNA-ADAMTS9-AS1 co-transfected group. (**B**) The results of CCK-8 assay showed that the proliferation of breast carcinoma cells was heightened whereas pcDNA-ADAMTS9-AS1 reversed the trend. (**C**) Colony formation analysis using MDA-MB-231 and Hs 578T cells. (**D**) The results of transwell assay showed that invasion cells number was markedly increased in miR-513a-5p mimics transfected group compared with miR-Ctrl group, whereas the invasion cells number was significantly decreased by pcDNA-ADAMTS9-AS1. $^{**}P<0.01$ compared with miR-Ctrl, $^{\#\#}P<0.01$ compared with miR-513a-5p. (**E**) Kaplan-Meier curves for overall survival rate of patients with breast cancer by miR-513a-5p expression in tumors.

vector group (Figure 6B and C). It was also observed that expression of miR-513a-5p was reduced, while ZFP36 expression was noticeably raised in the pcDNA-ADAMTS9-AS1 group (Figure 6D and E). All these observations indicated that lncRNA ADAMTS9-AS1 restrains the aggressive traits of breast carcinoma cells via sponging miR-513a-5p (Figure 6F).

Discussion

Over the past decade, significant improvements have been made in the treatment of breast carcinoma, but the outcomes of patients still need to be improved owing to recurrence and metastasis.[22,23] Hence, identifying new regulators that facilitate breast cancer malignancy and elucidating the molecular mechanisms are urgently required. Substantial investigations have shown that the dysregulated lncRNAs in various carcinomas exert critical roles in the development of carcinoma cells.[24–26] For example, silencing lncRNA HOX Transcript Antisense RNA (HOTAIR) exerts suppressive impacts on acute myeloid leukemia (ALL) by demethylation of Homeobox A5 (HOXA5).[27] The LOC285758 promotes the invasion of acute myeloid leukemia cells by downregulating miR-204-5p.[28] LncRNA PCED1B Antisense RNA 1 (PCED1B-AS1) promotes the Warburg effect and tumorigenesis by upregulating Hypoxia Inducible Factor 1 Subunit Alpha (HIF-1alpha) in glioblastoma.[29]

Recently, ADAMTS9-AS1 has been found to be downregulated in prostate cancer and plays a tumor suppressive role in prostate cancer cell growth.[15] The higher expression of ADAMTS9-AS1 is also associated with the overall prognosis in patients with breast cancer.[17] However, the functions of ADAMTS9-AS1 in the growth and invasion of breast carcinoma remain unclear. Herein, we revealed

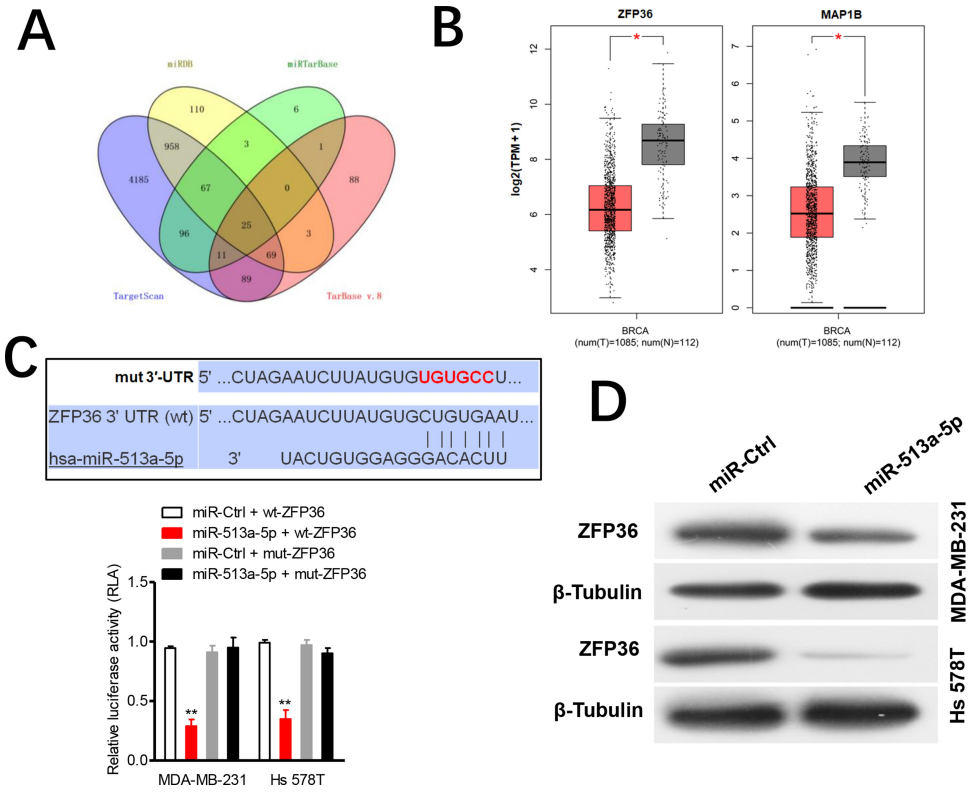

Figure 4 The negative correlation between ZFP36 and miR-513a-5p. (**A**) Venn diagram of numbers of target genes of miR-513a-5p detected in TargetScan, miRDB, miRTarBase and TarBase v.8 bioinformatics tools. (**B**) The expressions of ZFP36 and MAP1B in TCGA breast cancer samples were analyzed through GEPIA (http://gepia.cancer-pku.cn/index.html). *P<0.05 compared with normal (N). (**C**) The predicated miR-513a-5p binding sites in the 3′ UTR of ZFP36 (ZFP36-3′ UTR-wt) and the designed mutant sequence (ZFP36-3′ UTR-mut) were indicated. miR-513a-5p mimics in combination with ZFP36-AS1-wt or ZFP36-AS1-mut were co-transfected into breast cancer cells. The luciferase activity was detected. **P<0.01 compared with miR-Ctrl. (**D**) The expressions of ZFP36 in miR-513a-5p mimics transfected MDA-MB-231 and Hs 578T cells were detected using Western blot.

that ADAMTS9-AS1 was significantly down-expressed in breast carcinoma tissues and cells. Moreover, ectopic overexpression of ADAMTS9-AS1 not only suppressed cell growth and colony formation, but also decreased the invasive ability in breast carcinoma cells. Altogether, our observations imply that lncRNA ADAMTS9-AS1 may be a suppressor in breast carcinoma.

Systematic analysis focused on lncRNAs associated ceRNA networks has been performed in diverse cancers.[30,31] Using bioinformatics tools and luciferase reporter assay, we confirmed that ADAMTS9-AS1 and miR-513a-5p could fully bind to each other. In our experiments, miR-513a-5p was detected to be overexpressed in breast carcinoma tissue. MiRNAs exert crucial roles in various biological processes of carcinoma cells through regulating downstream genes.[32,33] In our experiments, ZFP36 was proved to be the downstream target of miR-513a-5p in breast carcinoma cells. Importantly, the higher level of ZFP36 was associated with better overall survival in breast cancer patients.

The lncRNA-miRNA axis in the progression of cancers has been extensively verified.[34] In our study, a new ADAMTS9-AS1/miR-513a-5p regulatory axis in breast carcinoma was revealed. LncRNA ADAMTS9-AS1 inhibited breast carcinoma growth and invasion traits through upregulating the expression of miR-513a-5p. And the expression of ZFP36, the direct target of miR-513a-5p, was reinforced by ADAMTS9-AS1. Although, our current study revealed the suppressive role of ADAMTS9-AS1 and its influence mechanism in breast carcinoma, some issues need to be explored. The findings derived from 56 breast carcinoma tissue specimens are limited and needed to be corroborated using a larger case-cohort. Additionally, it might be better to use an in vivo model to further illuminate the lncRNA ADAMTS9-AS1-miR-513a-5p regulatory axis.

Altogether, we demonstrated that lncRNA ADAMTS9-AS1 inhibited the proliferation, colony formation and invasion of breast cancer cells by sponging miR-513a-5p. Our results provide the experimental basis for exploring

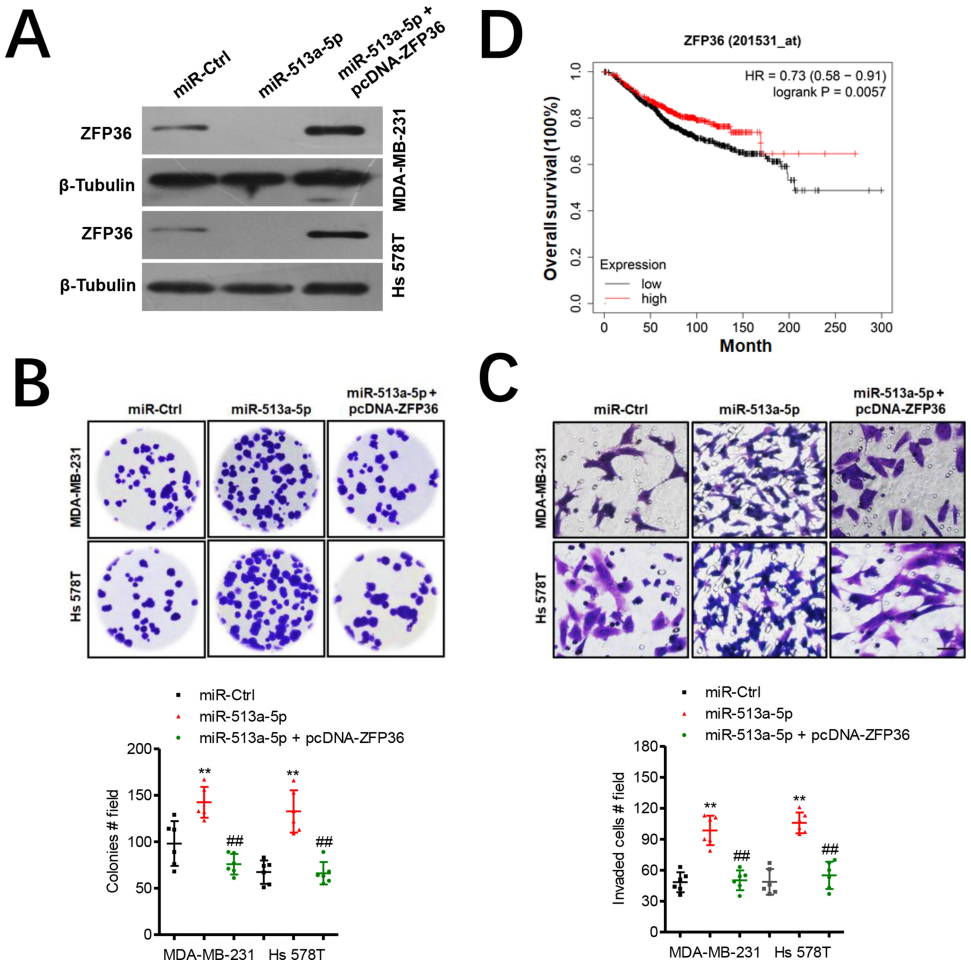

Figure 5 Effects of miR-513a-5p on breast carcinoma cell growth and invasion via regulating ZFP36. (**A**) MDA-MB-231 and Hs 578T cells were transfected with miR-513a-5p alone or co-transfected with miR-513a-5p mimics and pcDNA-ZFP36. The expression of ZFP36 was determined by Western blot. (**B**) The results of colony formation assay showed that the number of colonies was higher in miR-513a-5p mimics group compared with miR-Ctrl group, whereas the colonies number was lower in pcDNA-ZFP36 group. (**C**) The results of transwell assay showed that invaded cells number was higher in miR-513a-5p mimics group compared with miR-Ctrl group, whereas the invaded cell number was lower in pcDNA-ZFP36 group. $^{**}P<0.01$ compared with miR-Ctrl, $^{\#\#}P<0.01$ compared with miR-513a-5p. (**D**) Kaplan-Meier curves for overall survival rate of patients with breast cancer by ZFP36 expression in tumors.

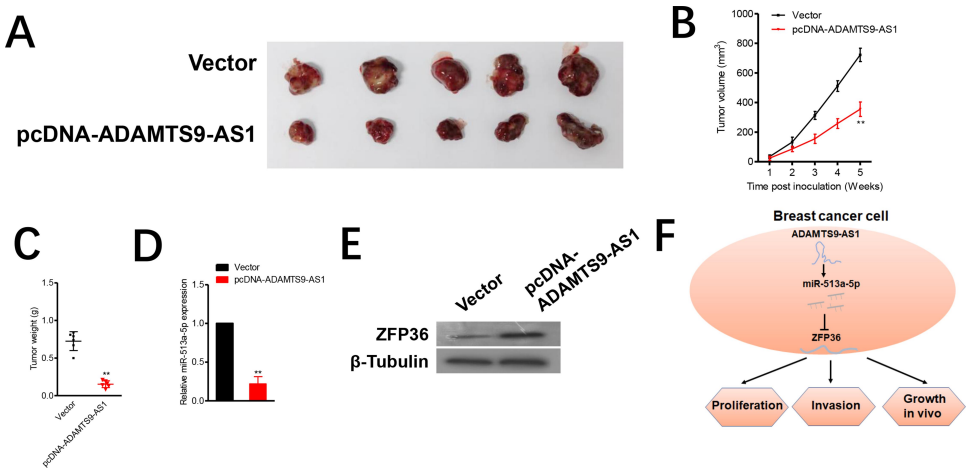

Figure 6 ADAMTS9-AS1 inhibits the breast carcinoma tumor growth in mice in vivo. (**A**) Negative vector or pcDNA-ADAMTS9-AS1 transfected MDA-MB-231 cells were inoculated into nude mice. Tumor xenografts excised from nude mice after five-weeks were shown. (**B**) The volume of tumor in pcDNA-ADAMTS9-AS1 group grew slower that in vector group. (**C**) The weight of tumor in pcDNA-ADAMTS9-AS1 group was smaller than in vector group. (**D**) The expression level of miR-513a-5p in pcDNA-ADAMTS9-AS1 group was significantly lower than that in vector group. (**E**) The results of Western blot assay showed that the expression of ZFP36 inpcDNA-ADAMTS9-AS1 group was higher than that in vector group. $^{**}P<0.01$ compared with vector. (**F**) Model depicting the implication of ADAMTS9-AS1 in the growth and invasion of breast cancer cells.

the pathogenesis of breast cancer and developing potential treatment targets for breast carcinoma.

Disclosure

The authors report no conflicts of interest for this work.

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