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TATDN1 promotes the development and progression of breast cancer by targeting microRNA-140-3p

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Abstract. – OBJECTIVE: To explore whether long non-coding RNA (lncRNA) TATDN1 can promote the proliferation and cell cycle progression of breast cancer cells by adsorbing microRNA-140-3p, thus participating in the development of breast cancer (BCa).

PATIENTS AND METHODS: Expressions of TATDN1 and microRNA-140-3p in BCa tissues and paracancerous tissues were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Meanwhile, TATDN1 expression in BCa cell lines was detected as well. Regulatory effects of TATDN1 and microRNA-140-3p on proliferation and cell cycle progression of BCa cells were evaluated by Cell Counting Kit-8 (CCK-8) and flow cytometry, respectively. The binding relationship of microRNA-140-3p to NOVA1 and TATDN1 was examined by dual-luciferase reporter gene assay. Finally, rescue experiments were conducted to explore whether TATDN1 can regulate NOVA1 expression by adsorbing microRNA-140-3p to exert its biological function in BCa.

RESULTS: TATDN1 was highly expressed in BCa tissues and cell lines. Upregulation of TATDN1 promoted the proliferative potential and cell cycle progression of MCF-7 and MDA-MB-231 cells. Dual-luciferase reporter gene assay indicated that TATDN1 could bind to microRNA-140-3p, which was lowly expressed in BCa. Overexpression of microRNA-140-3p inhibited the proliferative potential and cell cycle progression of MCF-7 and MDA-MB-231 cells. Moreover, microRNA-140-3p partially inhibited the role of TATDN1 in regulating cellular behaviors of BCa cells. NOVA1 was predicted to be the target gene of microRNA-140-3p. Overexpression of NOVA1 partially abolished the inhibitory effects of microRNA-140-3p on proliferation and cell cycle progression of MCF-7 and MDA-MB-231 cells.

CONCLUSIONS: TATDN1 promotes the proliferative potential and cell cycle progression of BCa cells through adsorbing microRNA-140-3p to upregulate NOVA1 expression.

Key Words:
Breast cancer, TATDN1, MicroRNA-140-3p, NOVA1.

Introduction

Breast cancer (BCa) is a highly heterogeneous malignant tumor, and it is the most common malignant tumor in women. In the past decade, the mortality rate of BCa in China has been increasing year by year[1,2]. Responses of BCa patients to the same treatment option vary a lot, manifesting with different drug reactions, recurrences, and survivals. At present, clinical comprehensive treatment of BCa mainly depends on the specific molecular subtypes. The distinction between molecular subtypes of BCa only bases on protein-coding genes that are less than 2% of the human genomes[3]. Therefore, in-depth exploration on BCa pathogenesis is essential for improving individualized and precise treatment efficacies.

Recent studies have shown the crucial functions of miRNA and long non-coding RNA (lncRNA) in tumor development. Endogenous RNAs have miRNA target sites that indirectly regulate the target miRNAs *via* competitively binding to them. This complex regulatory network is known as ceRNA (competitive endogenous RNA) hypothesis[4]. Wang et al[5] have found that the pseudo-gene OCT4-p94 acts as a natural miRNA sponge to competitively bind to miR-145. As a result, the inhibitory effect of miRNA-145 on OCT4 was weakened, thus promoting the development of liver cancer. KRASPI (KRAS pseudogene 1) has certain homologous sequences with those of KRAS, and both of them contain miRNA binding sites. KRASP1 can regulate KRAS expression by competitively binding to

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these miRNAs, further affecting the biological process of prostate cancer[6]. CASC2 expression is significantly downregulated in colorectal cancer tissues and cancer cells. A series of functional experiments indicated that CASC2 can competitively bind to miR-18a and alleviate the inhibitory effect on PIAS3 expression[7]. Both FOXO1-3'UTR and E-cadherin-3'UTR regions contain miR-NA9 binding sites. FOXO1 can be used as a ceRNA to induce E-cadherin expression, further inhibiting the metastasis of BCa[8].

It is reported[9] that lncRNA TATDN1 (*Homo sapiens* TatD DNase domain containing 1) is highly expressed in NSCLC, indicating the poor prognosis. Its role in BCa, however, is rarely reported. Our previous researches found that TATDN1 was highly expressed in BCa samples. This study mainly focused on its biological function in the progression of BCa.

Patients and Methods

General Data

BCa tissues and paracancerous tissues were harvested from 24 patients undergoing surgery in our hospital, and preserved in liquid nitrogen. Clinical data of enrolled BCa patients were recorded, including gender, age, tumor size, and tumor numbers. All patients were followed-up. They did not receive preoperative treatments and denied family history and pathologically diagnosed as BCa. Patients volunteered to participate in the study and signed the written informed consent. This investigation has been approved by the Ethics Committee of Health Science Center, Peking University, China.

Cell Culture

Breast cell line (Hs578Bst) and BCa cell lines (MCF-7, MDA-MB-231, and BCap-37) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum – FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Gibco, Rockville, MD, USA) at 37°C, 5% CO_2 incubator.

Cell Transfection

Transfection was performed when the cell density reached 70%-80%. Cells were transfected with microRNA-140-3p mimics, pcDNA-TATDN1, pcDNA-NOVA1 or negative controls using Lipofactamine 2000 (Invitrogen, Carlsbad, CA, USA). After standing at room temperature for 20 min, transfection reagent was slowly applied in each well. Complete medium was replaced at 5 h, and harvested at 24 h.

RNA Extraction

Tissues (50 mg) or cells (5×10^6 cells) were lysed in 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA). After maintenance for 5 min, 200 μL of chloroform was added, mixed and stand at room temperature for 5 min. The supernatant was transferred into a new RNase-free centrifuge tube after centrifugation at 4°C, 12000 rpm for 15 min. Isopropanol with the same volume of the supernatant was added for harvesting RNA precipitate by centrifugation. The extracted RNA was air dried, quantified and dissolved in diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNA was subjected to a reverse transcription system using a PrimeScript RT reagent kit to obtain a complementary deoxyribose nucleic acid (cDNA; TaKaRa, Otsu, Shiga, Japan). QRT-PCR was carried out in accordance with the instruction of SYBR Green PCR Kit (Applied Biosystems, Foster City, CA, USA). The total qRT-PCR system was 10 μL and performed as pre-denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. U6 was considered as the internal reference. Primer sequences were as follows: MicroRNA-140-3p, F: 5′-ACACTCCAGCTGGGTACCACAGGGTAGAA-3′, R: 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCGTGGTT-3′; U6, F: 5′-CTCGCTTCGGCAGCACATATA-3′, R: 5′-AAATATGGAACGCTTCACGA-3′; TATDN1, F: 5′-AGGAGAATGCGGACTTGATTTT-3′, R: 5′-CCCCTACACACCGATCTCTATTT-3′; GAPDH, F: 5′-GAAGAGAGAGACCCTCACGCTG-3′, R: 5′-ACTGTGAGGAGGGGAGATTCAGT-3′; NOVA1, F: 5′-TGCCATCTTCCCCAACTACC-3′, R: 5′-TCTCCACTCACAGTGACAACCCT-3′.

Dual-Luciferase Reporter Gene Assay

The transcript 3'UTR sequence of NOVA1/TATDN1 was cloned into the vector pGL3 containing the luciferase reporter gene, which was the NOVA1/TATDN1 WT group. NOVA1/

TATDN1 MUT group was constructed by mutating the core binding sequences using a site-directed mutagenesis kit. Cells were co-transfected with microRNA-140-3p mimics or negative control and NOVA1/TATDN1 WT or NOVA1/TATDN1 MUT, respectively. After transfection for 24 hours, cells were lysed and centrifuged at 10,000 g for 5 min. 100 μL of the supernatant was collected for determining the luciferase activity.

Cell Cycle Progression Assay

Transfected cells for 72 h were digested, resuspended in 70% alcohol and fixed for over 18 h. Cell density was adjusted to 1×10^6 cells/mL. 1 mL of cell suspension was washed with phosphate-buffered saline (PBS) for three times, incubated in 1 mL of 50 μg/mL Propidium Iodide (PI) at 37°C for 30 min. Finally, cells were subjected to flow cytometry analysis.

Cell Proliferation Assay

Cells were cultured in 96-well plates with 1×10^4 cells per well. Each sample set 5 replicate wells. After incubation for 0, 12 h, 24 h, 48 h, and 96 h, 10 μL of cell counting kit-8 (CCK-8) was added each well. Absorbance was recorded at 450 nm with a microplate reader for plotting the growth curve.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were represented as mean ± SD. The *t*-test was used for analyzing differences between two groups. $p<0.05$ indicated the significant difference.

Results

High Expression of TATDN1 in BCa

We examined TATDN1 expression in BCa tissues and paracancerous tissues by qRT-PCR. Results showed that TATDN1 was highly expressed in tumor tissues compared with those of controls (Figure 1A). Moreover, TATDN1 expression was higher in BCa tissues with stage III-IV relative to those with stage I-II (Figure 1B). Further analysis

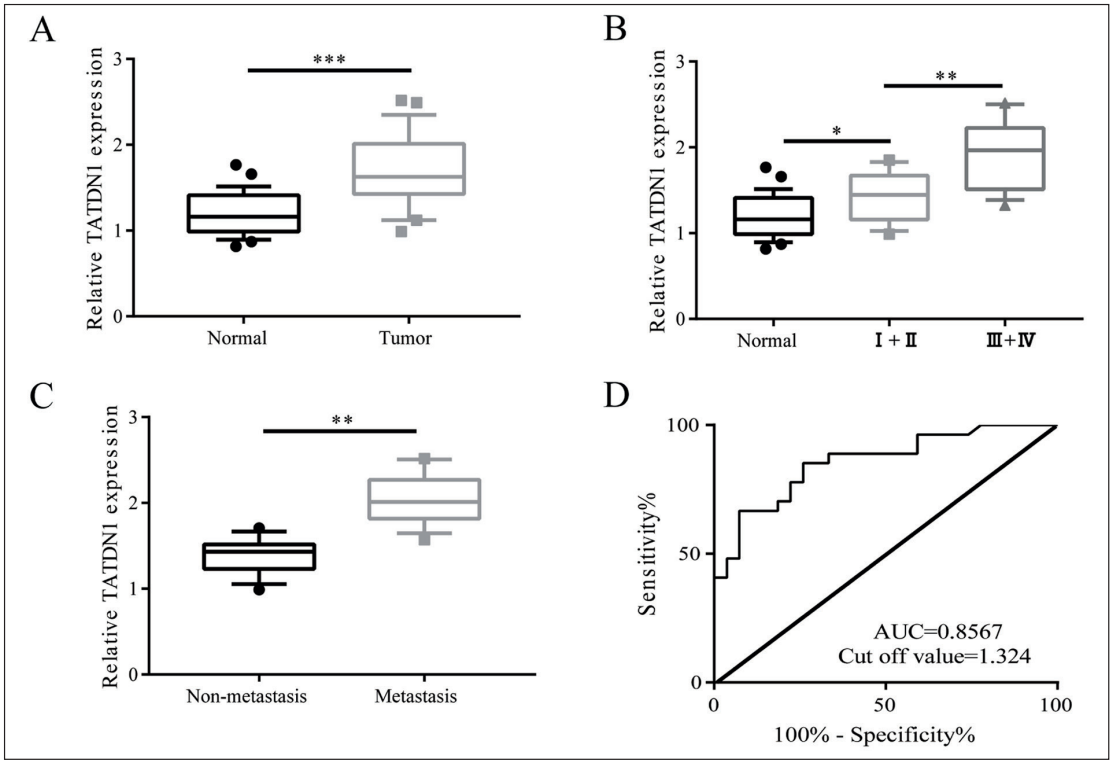

Figure 1. High expression of TATDN1 in BCa. ***A,*** QRT-PCR results showed that TATDN1 was highly expressed in BCa tissues than that of paracancerous tissues. ***B,*** TATDN1 expression was higher in BCa tissues with stage III-IV relative to those with stage I-II. ***C,*** TATDN1 expression in metastatic BCa tissues was higher than those of non-metastatic ones. ***D,*** Survival curve plotted based on follow-up data of enrolled BCa patients indicated the diagnostic potential of TATDN1 in BCa (AUC=0.8567, cut-off value=1.324).

showed that TATDN1 expression in metastatic BCa tissues was higher than those of non-metastatic ones (Figure 1C). Survival curve plotted based on follow-up data of enrolled BCa patients indicated the diagnostic potential of TATDN1 in BCa (AUC=0.8567, cut-off value=1.324, Figure 1D).

TATDN1 Promoted Proliferative Potential and Cell Cycle Progression of BCa

Since TATDN1 was highly expressed in BCa, we next explored its underlying mechanism. Firstly, TATDN1 expression in BCa cell lines was determined by qRT-PCR. Identically, TATDN1 was highly expressed in BCa cells than that of breast cells (Figure 2A). The transfection of constructed pcDNA-TATDN1 in MCF-7 and MDA-MB-231 cells remarkably upregulated the TATDN1 expression (Figure 2B). BCa cells overexpressing TATDN1 showed higher viability than controls as CCK-8 assay indicated (Figure 2C, 2D). Besides, TATDN1 overexpression markedly accelerated cell cycle progression in MCF-7 and MDA-MB-231 cells (Figure 2E, 2F).

TATDN1 Exerted its Functions in BCa Through Sponging MicroRNA-140-3p

Potential binding sites between TATDN1 and microRNA-140-3p were found through online prediction (Figure 3A). Further studies revealed that microRNA-140-3p was lowly expressed in BCa tissues (Figure 3B). Transfection of microRNA-140-3p mimics greatly upregulated microRNA-140-3p expression in MCF-7 and MDA-MB-231 cells, indicating a sufficient transfection efficacy (Figure 3C). Through dual-luciferase reporter gene assay, we confirmed the binding of microRNA-140-3p to TATDN1 (Figure 3D, 3E). To verify whether TATDN1 exerted its functions by sponging microRNA-140-3p, BCa cells were co-overexpressed with microRNA-140-3p and TATDN1. MicroRNA-140-3p overexpression partially reversed the promotive effect of TATDN1 on viability of MCF-7 and MDA-MB-2319 cells (Figure 3F, 3G). Similarly, microRNA-140-3p overexpression also partially reversed the promoted cell cycle progression due to overexpressed TATDN1 (Figure 3H, 3I). Taken together, we confirmed that TATDN1 regulated cellular behaviors of BCa cells by sponging microRNA-140-3p.

MicroRNA-140-3p Exerted its Functions in BCa Through Degrading NOVA1

MicroRNAs degrade their target genes to exert biological functions. We subsequently predicted that NOVA1 was the potential target for microRNA-140-3p (Figure 4A). The NOVA1 expression in BCa tissues was examined, and we found that was highly expressed in BCa than controls (Figure 4B). The transfection of pcDNA-NOVA1 sufficiently upregulated the NOVA1 expression in BCa cells (Figure 4C). Moreover, the dual-luciferase reporter gene assay verified the binding of NOVA1 to microRNA-140-3p (Figure 4D, 4E). Rescue experiments were carried out by co-transfection of pcDNA-NOVA1 and microRNA-140-3p mimics in BCa cells. In co-overexpressed MCF-7 and MDA-MB-231 cells, the upregulated NOVA1 partially reversed the inhibitory effect of microRNA-140-3p on cell proliferation (Figure 4F, 4G). In addition, the upregulated NOVA1 reversed the inhibited cell cycle progression in BCa cells overexpressing microRNA-140-3p (Figure 4H, 4I). It is demonstrated that TATDN1 may exert its function by adsorbing microRNA-140-3p to degrade the downstream NOVA1.

Discussion

LncRNA is a non-coding RNA containing more than 200 nucleotides[10]. It participates in cellular behaviors, and is closely related to the occurrence and development of many human diseases. LncRNA has been identified to be involved in the occurrence, development, invasion, and metastasis of tumors, which has been widely explored[11]. Recent studies[12-14] have revealed multiple changes in gene expressions and transcriptional regulations in the malignant progression of BCa, and among which, abnormally expressed lncRNAs are involved in. LncRNA PANDAR participates in the progression of BCa by targeting p16 to regulate the cell cycle progression[15]. In this research, TATDN1 was highly expressed in BCa. Further analyses showed that TATDN1 expression was correlated to the stage and metastasis of BCa. Through *in vitro* experiments, we found that TATDN1 markedly promoted the proliferative potential and cell cycle progression of BCa cells, suggesting its potential role in the development of BCa.

CeRNA hypothesis proposes a new regulatory network involving mRNAs and non-coding RNAs in the pathological progression of diseas-

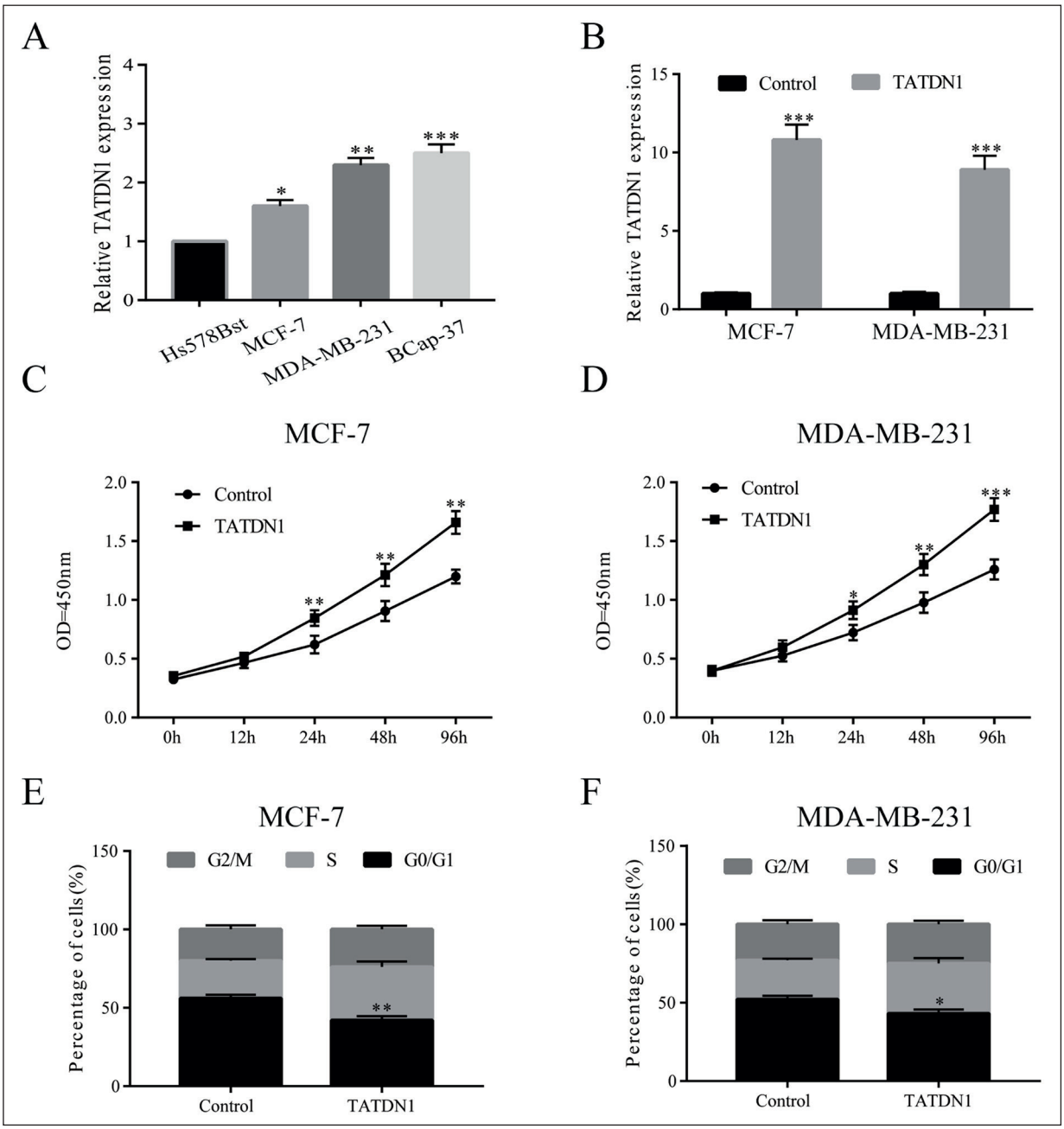

Figure 2. TATDN1 promoted proliferative potential and cell cycle progression of BCa. ***A,*** TATDN1 was highly expressed in BCa cells than breast cells. ***B,*** Transfection of constructed pcDNA-TATDN1 in MCF-7 and MDA-MB-231 cells remarkably upregulated TATDN1 expression. ***C, D,*** CCK-8 assay indicated that MCF-7 and MDA-MB-231 cells overexpressing TATDN1 showed higher viability than controls. ***E, F,*** TATDN1 overexpression markedly accelerated cell cycle progression in MCF-7 and MDA-MB-231 cells.

es[16]. Through bioinformatics analysis, we found that microRNA-140-3p was a potential target gene for TATDN1. MicroRNA-140-3p inhibits the proliferative and invasive abilities of NSCLC cells, serving as a tumor-suppressor gene[17,18]. This study revealed a low expression of microRNA-140-3p in BCa, and further results from the dual-luciferase reporter gene assay confirmed the binding of microRNA-140-3p to TATDN1. More importantly, microRNA-140-3p could partially inhibit the regulatory effects of TATDN1 on proliferative potential and cell cycle progression.

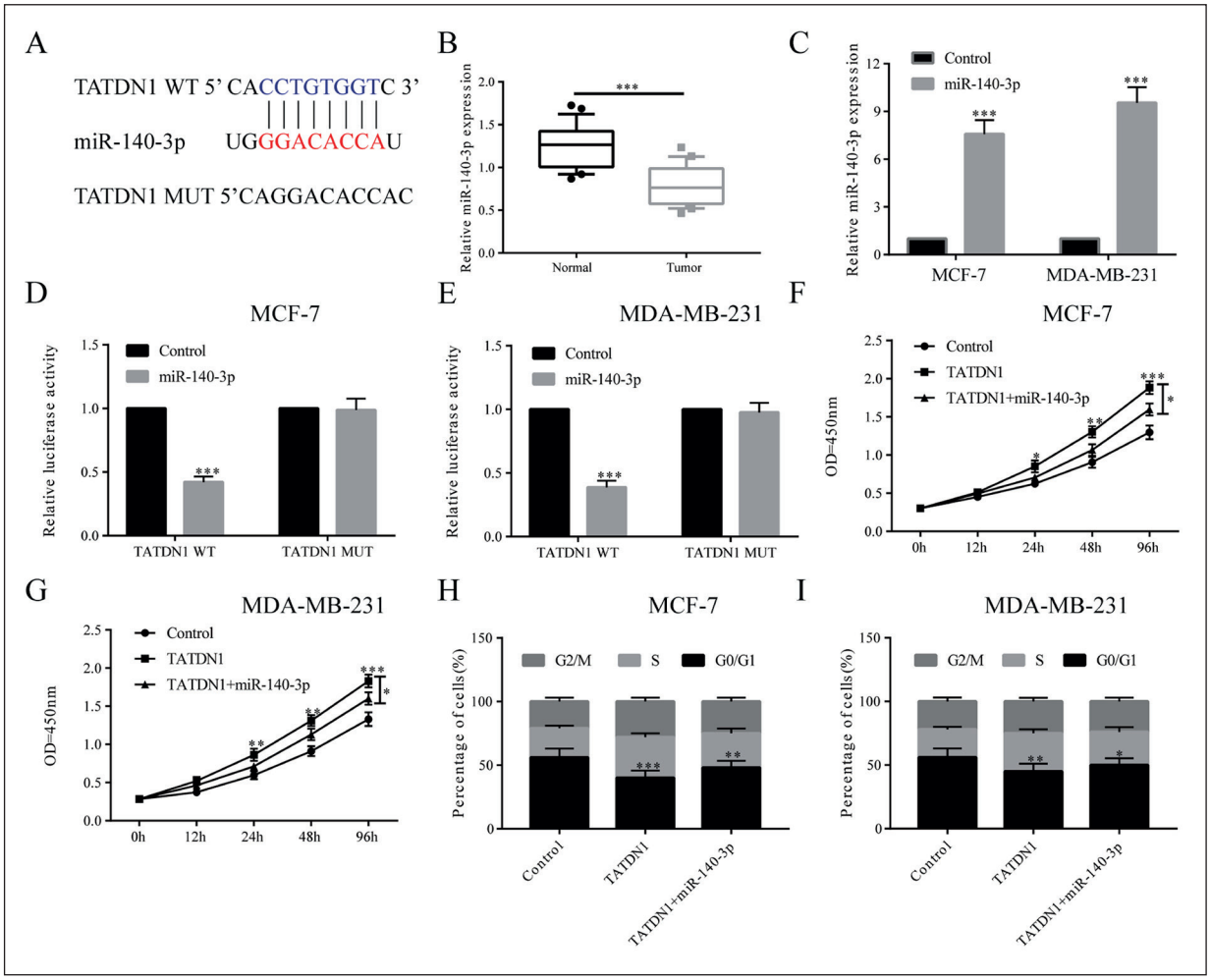

Figure 3. TATDN1 exerted its functions in BCa through sponging miR-140-3p. ***A,*** Potential binding sites between TATDN1 and miR-140-3p. ***B,*** BiR-140-3p lowly expressed in BCa tissues than that of paracancerous tissues. ***C,*** Transfection of miR-140-3p mimics greatly upregulated miR-140-3p expression in MCF-7 and MDA-MB-231 cells. ***D, E,*** Dual-luciferase reporter gene assay confirmed the binding of miR-140-3p to TATDN1 in MCF-7 and MDA-MB-231 cells. ***F, G,*** CCK-8 assay indicated that MCF-7 and MDA-MB-231 cells co-overexpressed with miR-140-3p and TATDN1 partially reversed the promotive effect of TATDN1 on viability. ***H, I,*** MCF-7 and MDA-MB-231 cells co-overexpressed with miR-140-3p and TATDN1 partially reversed the promoted cell cycle progression due to overexpressed TATDN1.

Tutar[19] have confirmed that miRNAs exert their functions by directly degrading target genes. Through online prediction and analyses, NOVA1 was found to be the potential target gene of microRNA-140-3p. In osteosarcoma, NOVA1 promotes the cancer progression as an oncogene[20]. Here, we verified that microRNA-140-3p bound to NOVA1, and NOVA1 was highly expressed in BCa. Co-overexpression of microRNA-140-3p and NOVA1 partially abolished the inhibitory effects of microRNA-140-3p on proliferative ability and cell cycle progression of BCa cells. It is indicated that lncRNA TATDN1 regulated the progression of BCa by adsorbing microRNA-140-3p to mediate NOVA1 expression.

Conclusions

We found that TATDN1 promotes the proliferative potential and cell cycle progression of BCa cells through adsorbing microRNA-140-3p

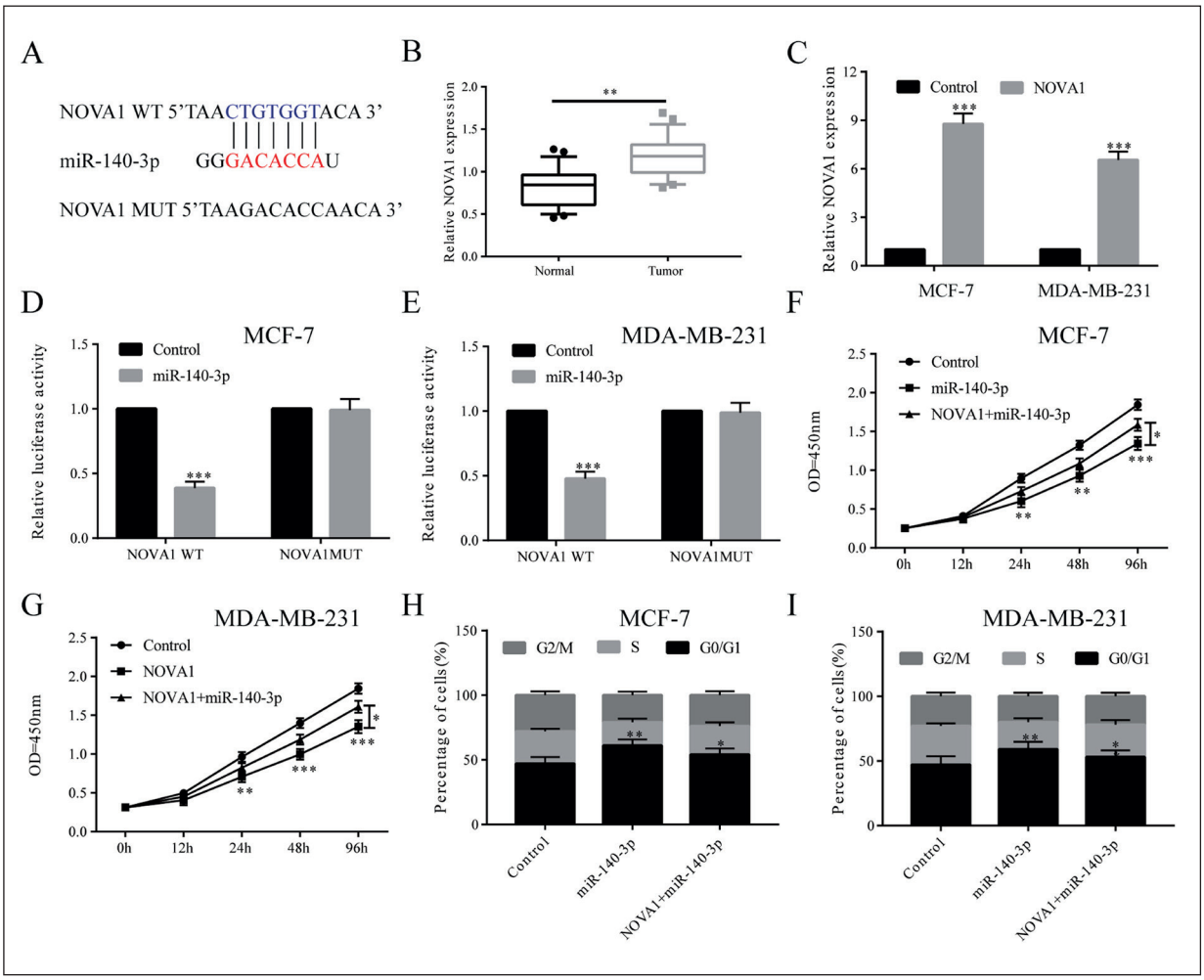

Figure 4. MiR-140-3p exerted its functions in BCa through degrading NOVA1. ***A,*** Potential binding sites between NOVA1 and miR-140-3p. ***B,*** NOVA1 was highly expressed in BCa tissues than that of paracancerous tissues. ***C,*** Transfection of pcDNA-NOVA1 sufficiently upregulated NOVA1 expression in MCF-7 and MDA-MB-231 cells. ***D, E,*** Dual-luciferase reporter gene assay confirmed the binding of miR-140-3p to NOVA1 in MCF-7 and MDA-MB-231 cells. ***F, G,*** CCK-8 assay indicated that MCF-7 and MDA-MB-231 cells co-overexpressed with miR-140-3p and NOVA1 partially reversed the inhibitory effect of miR-140-3p on cell proliferation. ***H, I,*** MCF-7 and MDA-MB-231 cells co-overexpressed with miR-140-3p and NOVA1 partially reversed the inhibitory effect of miR-140-3p on cell cycle progression.

to upregulate NOVA1 expression. TATDN1 could be used as a novel therapeutic target for BCa.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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