European Review for Medical and Pharmacological Sciences 2020; 24: 2303-2312

MicroRNA-370 suppresses SOX12 transcription and acts as a tumor suppressor in bladder cancer

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Abstract. – OBJECTIVE: Dysregulation of microRNA-370 (miR-370) is involved in a variety of cancers, but its roles in bladder cancer (BC) remain largely unexplored. Therefore, we designed this study to explore the role of miR-370 in BC.

PATIENTS AND METHODS: We took advantage of biochemical assays, including RT-qPCR, Western blot, CCK-8, flow cytometry, transwell, xenograft tumor formation, and immunohistochemistry (IHC) for research.

RESULTS: The expression of miR-370 was found to be downregulated during the development of BC, highly correlating with the malignant transformation of tumors. The overexpression of miR-370 led to enhanced apoptosis in BC cells, while inhibiting cell proliferation, migration, and invasion, effectively blocking cancer metastasis. Additionally, we identified SOX12, a known human oncogene, as a direct target of miR-370, showing that upregulation of SOX12 attenuated miR-370-mediated tumor suppression, promoted tumor growth, and epithelial-mesenchymal transition (EMT) in BC.

CONCLUSIONS: Taken together, these findings help to elucidate the roles of miR-370 as a tumor suppressor in BC, providing a potential target for diagnosis and treatment of BC.

Key Words:
Bladder cancer, MiR-370, SOX12, Tumor suppressor.

Introduction

Bladder cancer (BC) is the most common form of malignant tumor found in the urinary tract. The incidence rate of BC is the highest among middle-aged and elderly people over the age of 50[1], and the prevalence in men is rising in China. Risk factors related to BC include diet, smoking, and drinking[2]. Fortunately, there are many treatments available for BC patients, the most common being surgical. In those patients with advanced BC, however, it is necessary to consider a variety of pathological factors when choosing a suitable therapeutic strategy[3], given that surgical trauma can lead to a slow postoperative recovery and poor prognosis to some extent. Also, a comparison of an over 60% five-year survival rate in patients with non-metastasized BC to a less than 50% three-year survival rate in patients with metastasized BC[4] highlights the importance of its early detection.

MicroRNAs (miRNAs) are small siRNA-like molecules encoded by the genomes of higher eukaryotes and assembled into RNA-induced silencing complexes. After recognition and base pairing of the target mRNA, miRNA can direct the silencing complex to degrade the target mRNA, or repress the translation of the target mRNA, depending on the degree of complementation[5]. Due to the ability of miRNAs to regulate cell growth and tissue differentiation, they are implicated in development and disease throughout life[6]. MiRNAs, therefore, have many biological functions, while only a small fraction of them have been elucidated until now. Among these miRNAs, the extensive role of miR-370 (all the miR-370 mentioned in the paper refers to miR-370-3p) in disease and cancer has drawn increasing attention. It has been shown that miR-370 inhibits the angiogenic activity of endothelial cells, vascular inflammation, and oxidative stress[7,8]. Additionally, altering miR-370 expression contributes to hepatic ischemia-reperfusion injuries by regulating the levels of nuclear kappa B (NF-kappaB) related factors[9]. The current research on this topic has presented contradictory effects of miR-370 in different malignant tumors. In breast cancer, upregulated miR-370 acts as an onco-miR[10], while overexpression of miR-370

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in esophageal squamous-cell carcinoma inhibits cancer progression[11]. These results suggest that miR-370 is a tissue-specific microRNA. To date, the role and mechanism of miR-370 in the development of BC remain elusive.

To identify the genes that miR-370 may act on, we performed a scan using the TargetScan database (http://www.targetscan.org/). This scan revealed a binding site of miR-370 in the 3' UTR (untranslated region) of SRY (sex determining region Y)-box 12 (SOX12), a SOX gene that functions as a tumor suppressor or oncogene in human cancer. SOX12 has been found to promote Treg differentiation in the periphery during colitis[12], and it has been proposed as a potential target for acute myeloid leukemia[13]. In human cancers, such as hepatocellular carcinoma, the upregulation of SOX12 has been found to promote metastasis[14]. The knockdown of SOX12 expression consistently inhibited the proliferation and migration of lung cancer cells[15]. In addition, SOX12 was predictive of prognosis in patients with clear cell renal cell carcinoma[16]. Moreover, as a target gene of miRNAs, it was reported that the targeting of SOX12 by miR-874 inhibited metastasis and EMT in hepatocellular carcinoma[17].

Our study seeks to understand miR-370's tumor-suppressive function in BC and how this may relate to SOX12. Our results indicate that miR-370 attenuates malignant formation of BC through the suppression of SOX12 expression. This information leads us to propose the miR-370/SOX12 axis as a potential target for the prognosis or treatment of BC.

Patients and Methods

Clinical Tissues

Tumor tissues used in this experiment were obtained from 63 patients with BC at Beijing Rehabilitation Hospital, affiliated with Capital Medical University and Beijing Tsinghua Changgung Hospital. Patients did not receive radiotherapy or chemotherapy before surgery, and they provided written informed consent before participating in the research. The approval for the investigation was obtained from the Human Ethics Committees of the respective hospitals.

Cell Culture and Transfection

The immortalized bladder cell line SV-HUC-1 (ATCC® CRL-9520™) and the human BC cell lines 5637 (ATCC® HTB-9™) and J82 (ATCC® HTB-1™) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37°C in an atmosphere with 5% CO_2.

Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was applied to transfect 5637 cells at 70-80% confluency with SOX12 plasmid (Generay Biotech, Shanghai, China) and miR-370 mimics or inhibitors.

RNA Extraction and RT-qPCR

The total RNA was extracted from BC samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), followed by cDNA synthesis performed using PrimeScript RT reagent (TaKaRa, Dalian, China). RT-qPCR was conducted using SYBR Green Master Mix II (TaKaRa, Dalian, China) on a 7500 Fast Real-Time PCR system (ABI, Foster City, CA, USA). U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as controls for miR-370 and SOX12, respectively. The expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. The primers used were the same as those in previous reports[12,18]. Specifically, SOX12 forward, 5'-CCCGAGGTTACCGAGATGATC-3' and reverse, 5'-GCTGACGGTGGGCTCAGTAG-3'; GAPDH forward, 5'-AGACAGCCGCATCTTCTTGT-3' and reverse, 5'-TGATGGCAACAATGTCCACT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3', and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; miR-370 forward, 5'-TACTCAGGATCCTGTGCAAGGCGGGCTACT-3' and reverse, 5'-TACTCAAAGCTTCCCTCCCTCACCCAAATC-3'.

Western Blot Analysis

Protein samples were obtained by lysing cells with radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotech, Nantong, China). The protein lysate (25 μg) was separated on a 10% odium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel by electrophoresis, followed by transferring to polyvinylidene difluoride (PVDF) membranes. The blots were then blocked with 5% non-fat milk and incubated at 4°C overnight with primary antibodies for E-cadherin, N-cadherin, Bax, Bcl-2, and GAPDH (Abcam, Cambridge, MA, USA). Afterwards, horse-

radish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cambridge, MA, USA) were added and incubated for 1 h. An enhanced chemiluminescence (ECL) kit (Beyotime Biotech, Nantong, China) was used to assess the protein bands. Protein was quantified using Image Lab Software (Bio-Rad, Kidlington, UK).

CCK-8 Assay

The 5637 cells were seeded in a 96-well plate (4×10^3/well) and cultured in a 37°C incubator for 24 h. The cells were then transfected with miR-370 mimics and inhibitors for 24, 48, 72, and 96 h. After transfection, 10 ml of CCK-8 (Dojindo Laboratories, Kumamoto, Japan) solution was added and the cells were incubated for other 4 h. The absorbance was measured at 450 nm using a microplate reader (Molecular Devices LLC: Sunnyvale, CA, USA).

Flow Cytometry Analysis

Annexin-V/PI double-staining assay was used to evaluate cell apoptosis. First, the transfected 5637 cells (3×10^5 cell/well) were seeded in 6-well plates. After 48 h, the cells were collected and washed with cold phosphate-buffered saline (PBS), and then, the Annexin V- Fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Biovision, San Francisco, CA, USA) was used to stain the cells. Briefly, the cells were stained with 100 µL binding buffer (1×) containing 5 µL Annexin V-FITC for 15 min, followed by staining with 100 µg/mL propidium iodide (PI) in working solution (Sigma-Aldrich, Shanghai, China). A flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze the apoptotic 5637 cells.

Transwell Assay

Transwell chambers (8 µm pore size) were applied in 24-well plates to evaluate the migration and invasion of the 5637 cells. 4×10^3 cells were seeded in the upper chamber of each transwell and 10% FBS was added in the lower chamber to test cell migration. For the invasion assay, the upper chamber was pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). After an 8-h incubation, the cells were stained with 0.1% crystal violet, and the non-migrated or non-invaded cells on the upper surface of membrane were removed. The migrated and invaded cells were counted using a microscope (Olympus, Beijing, China).

Dual-Luciferase Reporter Assay

The pcDNA3.1 plasmid vector (Promega, Madison, USA) containing the 3'-UTR of wild type or mutant SOX12 was generated. The Luciferase vector and miR-370 mimics were co-transfected into 5637 cells. After 48 h, the Luciferase activities were measured using Dual-Luciferase assay system (Promega, Madison, USA).

Xenograft Tumor Formation Assay

The nude mice (6 weeks old) were purchased from the Shanghai SLAC Laboratory Animals (Shanghai, China). The cells (5×10^5) transfected with miR-370 plasmid or NC were injected into the right shoulder area of mice. The tumor volumes were measured every 3 days. After 2 weeks, the mice were sacrificed for analysis of tumors. All animal experiments were approved by the Ethics Committee of Beijing Rehabilitation Hospital Affiliated to Capital Medical University and Beijing Tsinghua Changgeng Hospital.

Immunohistochemistry (IHC) Staining

The tissue sections of xenograft tumors from nude mice were analyzed by IHC staining. All 4-µm thick, formalin-fixed and paraffin-embedded sections were dewaxed and rehydrated. The slides were heated in sodium citrate buffer (10 mM, pH 6.0) to retrieval antigen, followed by blocking with bovine serum albumin (Sigma-Aldrich, San Francisco, CA, USA). The slides were then incubated with anti-Ki67 (Abcam, Cambridge, MA, USA) antibody at 4°C overnight, and a goat anti-rabbit HRP conjugated secondary antibody (Abcam, Cambridge, MA, USA) at room temperature for 1 h. A DAB solution was used for brown color development. The strength of positivity was semi-quantified by considering both the intensity and proportion of positive cells.

TCGA Database Analysis

The Cancer Genome Atlas (TCGA) data for hsa-mir-370 expression in BC tissues and clinicopathological data from the patients were grabbed from https://gdc.cancer.gov/. Those patients who had not been operated on and who had been followed up for more than a month were selected. A total of 19 normal tissues and 418 BC tissues were obtained from the TCGA database of the corresponding patients. Hsa-mir-370 expression in BC and its correlations with clinical parameters of BC were analyzed. The statistical cutoff value, which is the highest Youden index (specificity+sensitivity-1) from the receiver operating

characteristic (ROC) curves, was used to define the high and low hsa-mir-370 expression. The mRNA expression of hsa-mir-370 >41 was used to define BC as high hsa-mir-370 expression, and the mRNA expression of RALY ≤41 was defined as low hsa-mir-370 expression.

Statistical Analysis

The data were calculated using Statistical Product and Service Solution (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism 6 and are represented as mean ± SD. The difference between the groups was calculated through the Chi-squared test or One-way ANOVA, followed by the Bonferroni post-hoc test. A significant difference was defined as $p<0.05$.

Results

MiR-370 Expression is Reduced in Bladder Cancer

To measure the status of miR-370 in the development of BC, we compared the transcription of miR-370 in 63 bladder cancers with their matched adjacent normal tissues. As shown in Figure 1A, compared with normal tissues, the expression of miR-370 was reduced in BC ($p<0.01$). Furthermore, based on the expression ratio of miR-370 in normal and BC tissues, the patients with BC can be divided into two groups: high or low miR-370 expression group (Cut-off = 1). We found that in BC patients, the aberrant expression of miR-370 was related to TNM stage ($p=0.03$) and lymph node metastasis ($p=0.04$; Figure 1B and Table I), but not related to age, gender, and tumor size (Table I). In addition, we observed that miR-370 expression was lower in 5637 and J82 cell lines than in SV-HUC-1 cells ($p<0.01$, Figure 1C). Our data suggest that the aberrant expression of miR-370 is associated with BC development. To obtain more samples for consideration of the expression pattern and clinical value of miR-370 in a larger cohort, a TCGA database was further used as validation set. TCGA database analysis also showed that miR-370 was highly associated with BC ($p<0.01$, Figure 1D), and patients with high hsa-mir-370 expression had a higher BC risk score (Figure 1E).

MiR-370 Restricts Bladder Cancer Cell Proliferation, Migration, and Invasion

To assess the effects of miR-370 on BC development, we transfected miR-370 mimics or inhibitor into the 5637 cell line that contains low level of miR-370. As shown in Figure 2A, the effects of mimics or inhibitor on miR-370 expression were confirmed by qRT-PCR assays ($p<0.01$). Subsequently, flow cytometry analysis revealed that the overexpression of miR-370 suppressed 5637 cell proliferation, while the silence of miR-370 elicited the opposite effect on cell proliferation ($p<0.05$ or 0.01, respectively, Figure 2B). We also noticed that compared with the control cells, the overexpression of miR-370 promoted the apoptosis of 5637 cells ($p<0.01$, Figure 2C). To determine whether the overexpression of miR-370 affects cancer cell migration and invasion, we utilized the transwell assay, and found that compared with cells treated with vehicle or inhibitor, miR-370 overexpression significantly suppressed both migration and invasion of BC cells ($p<0.01$, Figure 2D and 2E). Taken together, our data demonstrate that miR-370 could suppress BC cell proliferation, migration, and invasion.

MiR-370 Ameliorates Malignant Transformation of Bladder Cancer In Vivo

To further assess the tumor-suppressive effects of miR-370 *in vivo*, we stably transfected miR-370 plasmid or miR-NC into 5637 cell line, and subcutaneously injected into nude mice. As shown in Figure 3A and 3B, the presence of miR-370 significantly restricted tumor growth when compared to control ($p<0.01$). In addition, the IHC staining showed that compared with control, the tumors with miR-370 overexpression had lower levels of Ki-67, a cell proliferative marker (Figure 3C). Additionally, we measured the expression of genes associated with EMT and cell apoptosis in 5637 cells with or without miR-370 mimics. As shown in Figure 3D, the expression of E-cadherin was downregulated by miR-370 in 5637 cells compared to cells treated with vehicle or miR-370 inhibitor. Conversely, the overexpression of miR-370 inhibited the expression of N-cadherin. Correspondingly, miR-370 inhibitor showed opposite effect of the mimics on both mRNA and protein levels of E-cadherin and N-cadherin ($p<0.01$, Figure 3D). We also assessed the Bcl-2/Bax expression, which is related to apoptosis. As shown in Figure 3D, the overexpression of miR-370 suppressed Bcl-2 expression and promoted Bax expression compared with NC or inhibitor. Silencing of miR-370 elicited opposite effects on

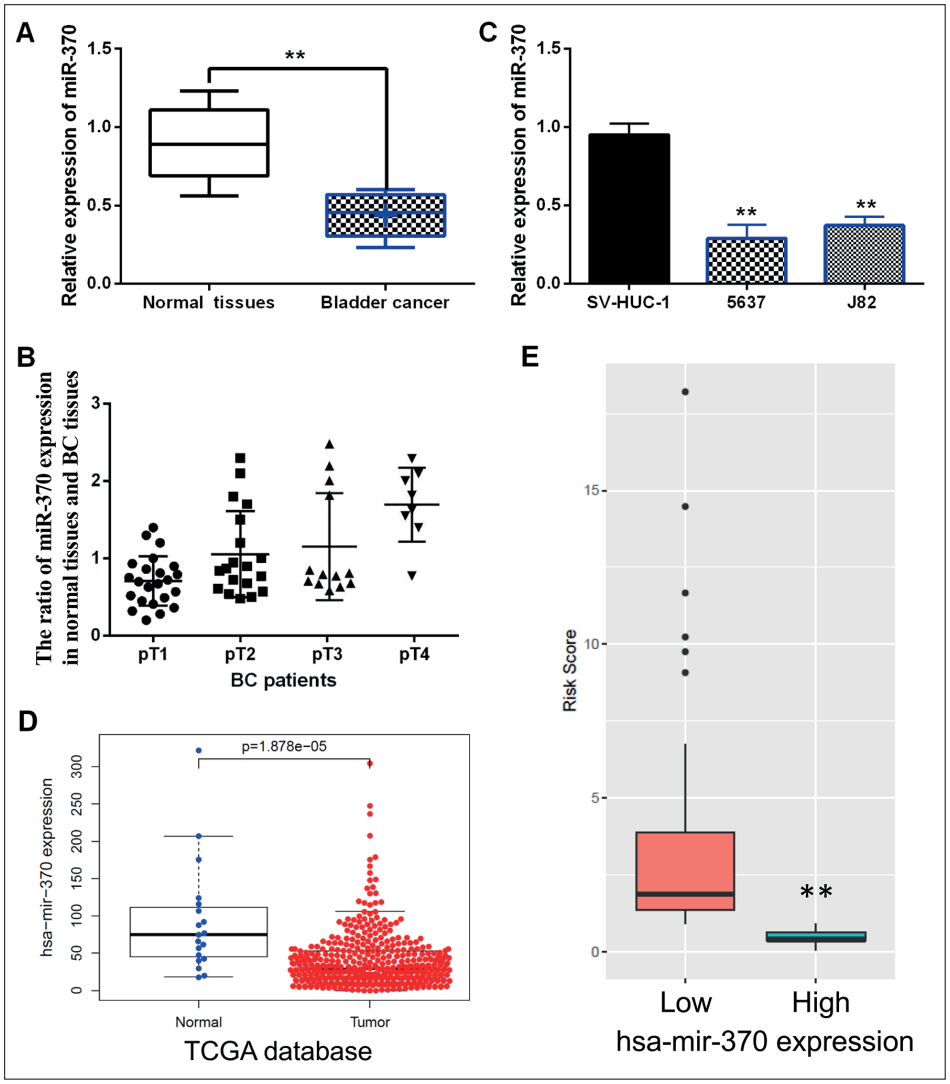

Figure 1. Clinical analysis of miR-370 expression in BC. **A,** Expression of miR-370 mRNA in BC tissues. Data were obtained from 63 BC patients. **B,** Graphic analysis of the relationship between miR-370 expressions during tumor stages. **C,** Expression of miR-370 in the 5637, J82, and SV-HUC-1 cell lines. **D,** mRNA expression of miR-370 was significantly lower in BC tissues than in normal bladder tissues according to an analysis of TCGA database. **E,** In the TCGA set, the mean risk score increased with decreasing miR-370 expression. **$p< 0.01$.

Table I. Relationship between miR-370 expression and their clinic-pathological characteristics of BC patients.

Characteristics	Cases	miR-370		*p*-value
		High	Low	
Age (years)				0.06
≥ 60	33	8	25	
< 60	30	13	17	
Gender				0.21
Male	38	7	31	
Female	25	14	11	
Tumor size				0.33
< 3 cm	36	8	28	
≥ 3 cm	27	13	14	
TNM stage				0.03*
I-II	42	32	10	
III-IV	21	10	11	
Lymph node metastasis				0.04*
No	44	27	17	
Yes	19	4	15	

Statistical analyses were performed by the χ^2-test. *$p<0.05$ was considered significant.

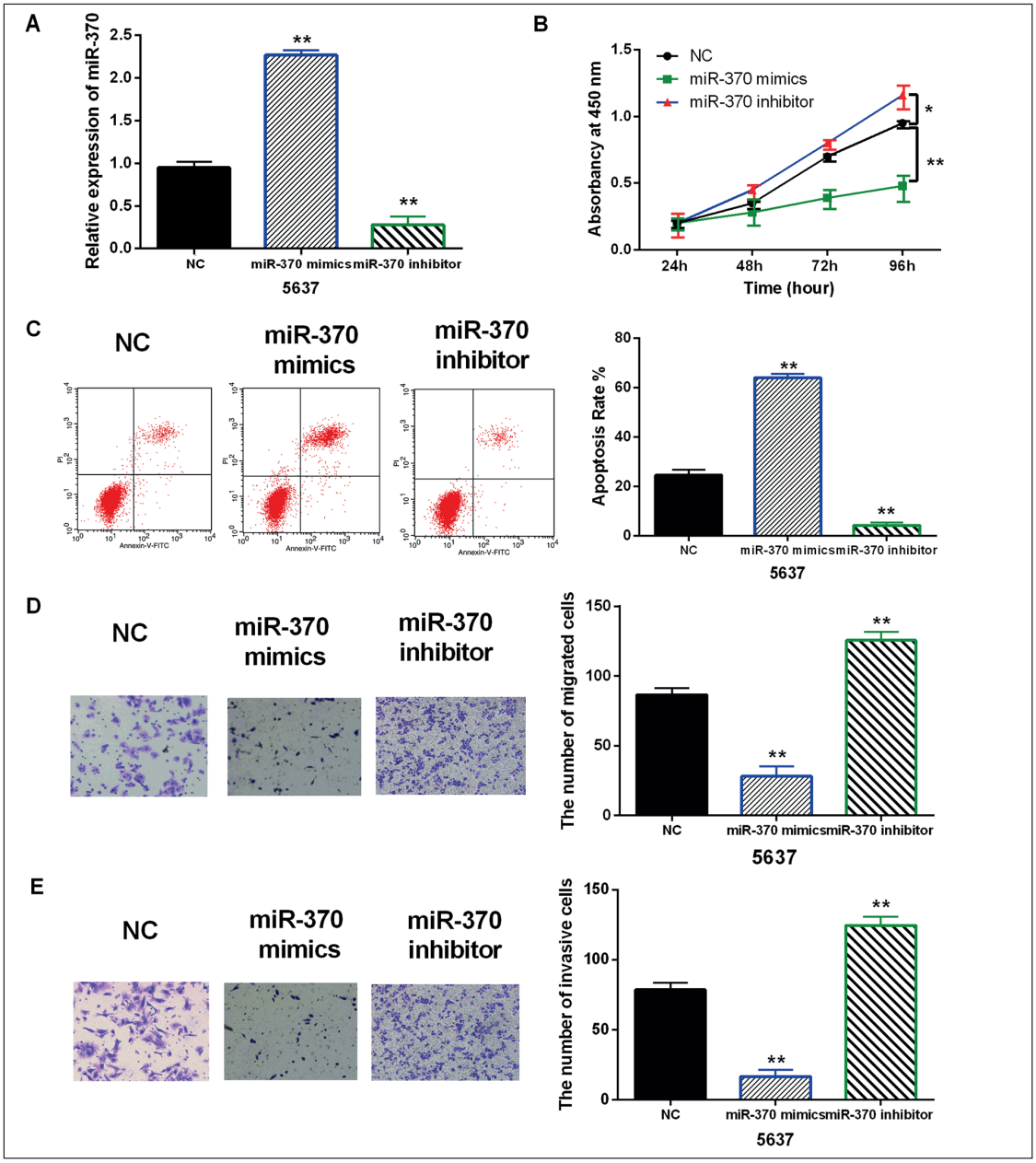

Figure 2. MiR-370 inhibited viability, migration, and invasion and induced apoptosis in BC cells. **A,** Expression of miR-370 regulated by its mimics or inhibitor in 5637 cells. **B,** MiR-370 mimics or inhibitor regulated cell proliferation. **C,** Apoptosis rate of 5637 cells with miR-370 mimics or inhibitor as analyzed by flow cytometry. **D, E,** MiR-370 mimics or inhibitor regulated cell migration and invasion, (20× magnifications). *p<0.05, **p<0.01.

Bcl-2/Bax expression (p<0.01, Figure 3D). Our results demonstrate that miR-370 could suppress BC tumor growth *in vivo*.

MiR-370 Regulates the Development of BC by Targeting SOX12

To investigate the mechanism by which miR-370 suppresses BC development, we used the TargetScan database (http://www.targetscan.org/) to find targets of miR-370. As shown in Figure 4A, we identified a binding site for miR-370 localized in the 3'-UTR of SOX12, confirmed by Luciferase reporter assay. Treatment with the miR-370 mimic resulted in a decline in the Luciferase activity of Wt-SOX12 but had no effect on Mut-SOX12 Luciferase activity (p<0.01, Figure 4B). We also assessed the expression of SOX12 in BC tissues, and found that

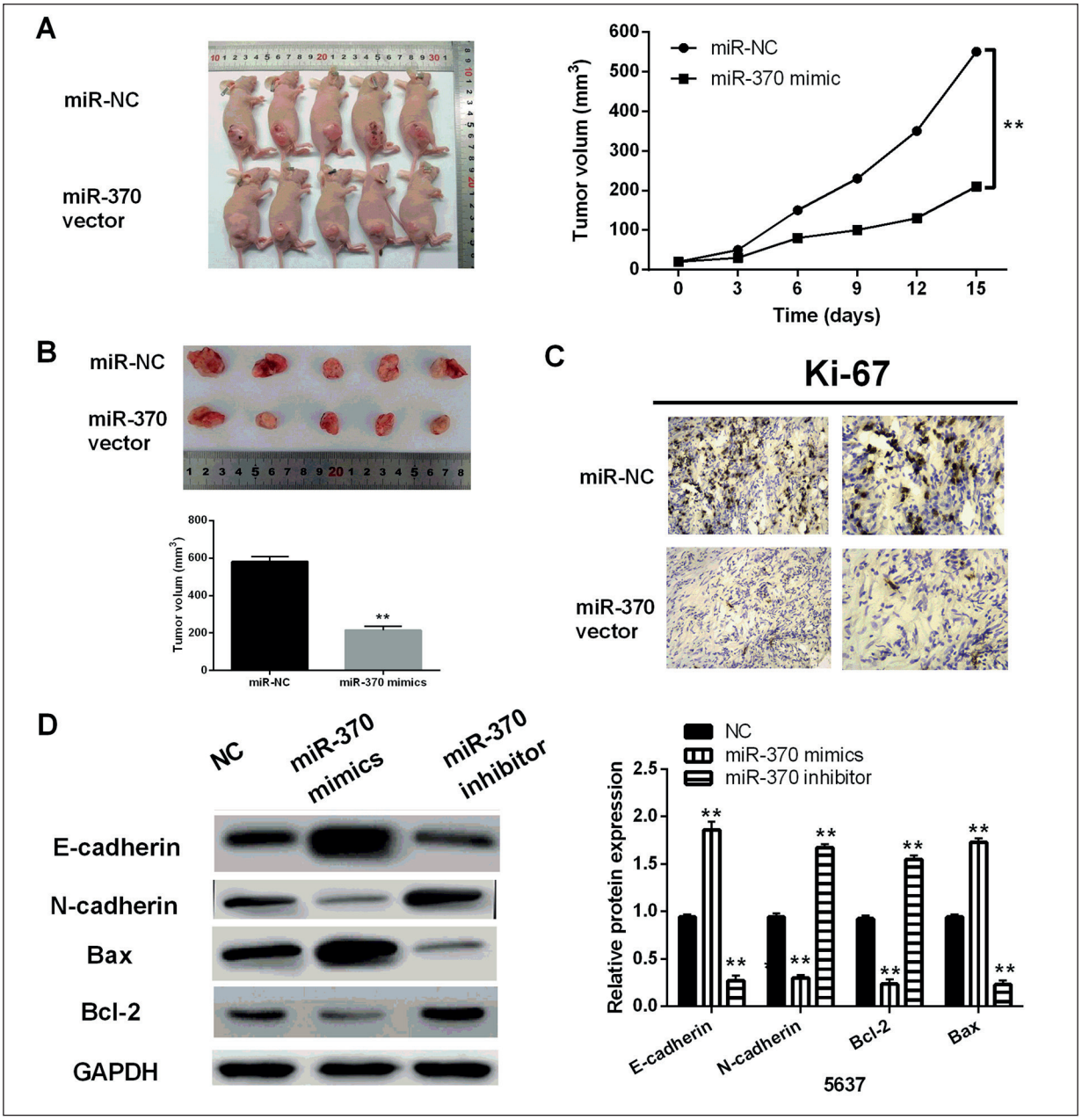

Figure 3. MiR-370 inhibited BC tumor growth. **A, B,** Tumor volumes and the growth curves in the miR-370 group and the miR-NC group. **C,** Lower Ki-67 expression was detected in tumors with miR-370 vector, (200× magnifications). **D,** MiR-370 regulated protein expressions of E-cadherin, N-cadherin, Bax, and Bcl-2 in 5637 cells. $^{**}p < 0.01$.

SOX12 was upregulated ($p<0.01$, Figure 4C). Moreover, we identified a negative correlation between miR-370 expression and SOX12 expression in BC tissues ($p<0.0001$, $R^2=0.8654$; Figure 4D), showing that miR-370 overexpression reduced the expression of SOX12, while the miR-370 inhibitor promoted SOX12 expression ($p<0.01$, Figure 4E). To illuminate the interaction between miR-370 and SOX12, we transfected miR-370 mimics along with the SOX12 vector into 5637 cells. The inhibition of SOX12 expression that was induced by the miR-370 mimics was restored by upregulation of SOX12 (Figure 4E). Functionally, the upregulation of SOX12 impaired the suppressive effect of miR-370 on the proliferation of 5637 cells (Figure 4F). Consistently, the overexpression of SOX12 attenuated miR-370-mediated cell death (Figure

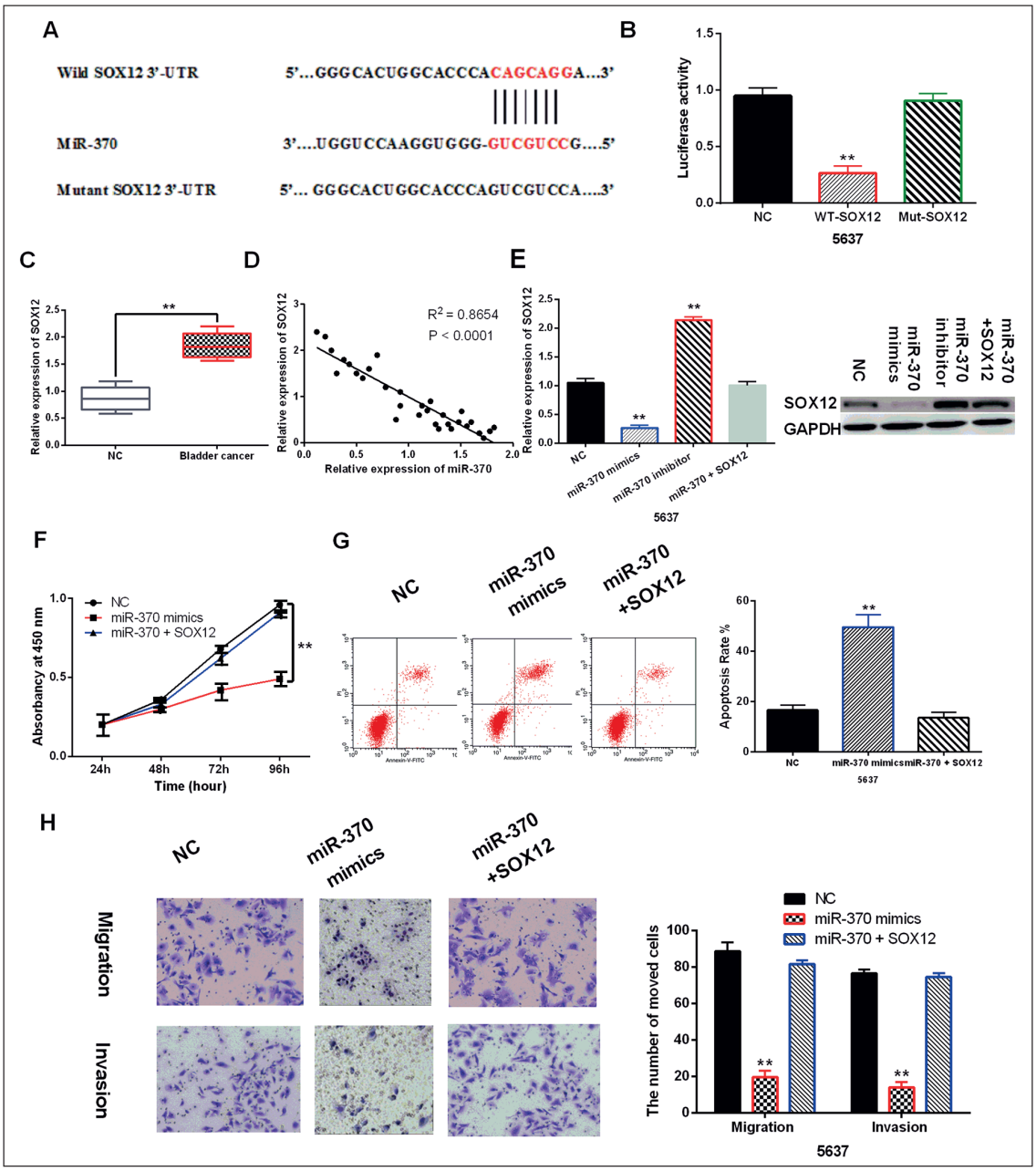

Figure 4. MiR-370 regulated the development of BC by targeting SOX12. **A,** Binding sites between SOX12 and miR-370. **B,** Luciferase reporter assay. **C,** Expression of SOX12 mRNA in BC tissues. Data were obtained from 63 BC patients. **D,** Negative correlation between miR-370 and SOX12 in BC tissues. 32 samples were randomly selected from 63 samples for correlation analysis. **E,** mRNA abundance and protein expression of SOX12 regulated by miR-370 mimics, miR-370 inhibitor, or SOX12 vector + miR-370 mimics in 5637 cells**. F, G, H,** SOX12 vector and miR-370 mimics regulated cell proliferation, apoptosis, migration, and invasion (20× magnifications) in 5637 cells. **$p<0.01$.

4G). In addition, miR-370-mediated inhibition of cell migration and invasion was reduced by SOX12 overexpression in 5637 cells (Figure 4H). Collectively, these findings indicate that SOX12 is required for the suppressive function of miR-370 in modulation of BC development.

Discussion

In the past few years, accumulative research has shown the important roles of miRNAs in the development of BC. For example, miR-218 was found to suppress cell migration, invasion,

and proliferation in BC by targeting BMI-1[19]. Inversely, the oncogene miR-187-5p is associated with cellular proliferation, migration, invasion, and apoptosis, as well as the risk in recurrence of BC[16]. As a miRNA associated with tumorigenesis, miR-370 exhibited contradictory effects in different cancers (e.g., upregulation of miR-370 contributes to progression of gastric carcinoma *via* suppression of FOXO1[20]). In most cancers, miR-370 exerts an inhibitory effect (e.g., the downregulation of miR-370 has been detected in glioblastoma and laryngeal squamous cell carcinoma[21,22]). Functionally, the upregulation of miR-370 promotes cell apoptosis and inhibits proliferation in human gastric cancer[23]. Furthermore, miR-370 directly targets FOXM1 to inhibit osteosarcoma cell proliferation and migration[24].

Consistent with the above studies, we found that miR-370 expression was decreased in BC and also associated with TNM stage and lymph node metastasis. We also showed that miR-370 inhibited cell viability and promoted apoptosis in BC cells, while repressing cell migration and invasion. These findings are consistent with previous studies and prove that miR-370 acts as a suppressive miRNA in BC. Moreover, for the first time, we observed that miR-370 inhibited tumor growth and blocked EMT in BC. In this study, SOX12 was found to be a direct target of miR-370. Also, miR-370 was shown to modulate the expression of SOX12, which is upregulated in BC tissues. In particular, SOX12 impaired the inhibitory effect of miR-370 in the development of BC. Finally, it is worth noting that miR-370 has several targets including MDM4, PAQR4, GUCD1, and WNK2 in other diseases[25-28], the expression levels of which have been detected in the samples of BC patients we collected. The expression levels of these genes are too low to be modulated by miR-370, which further indicates that the effects of miR-370 are dependent on the cell types.

Abnormal expression and dysregulation of SOX12 have been reported in several diseases and cancers. SOX12 has been verified to act as a potential novel target for acute myeloid leukemia[13], and during colitis, SOX12 was found to promote the differentiation of Tregs in the periphery[12]. For cancer, SOX12 upregulation has been implicated in the metastasis of hepatocellular carcinoma and ovarian cancer[29,30]. The results we obtained are consistent with those of previous studies, all of which revealed that the SOX12 gene functions as a carcinogen in cancer. In addition, it was reported that the silencing of SOX12 by shRNA suppressed migration, invasion, and proliferation of breast cancer cells[31]. In BC, we found that miR-370 overexpression suppressed SOX12 expression, and the inhibitory effect of miR-370 was weakened by SOX12. Based on the above results, we postulated that in BC, miR-370 might suppress cell viability, migration, and invasion, and induce apoptosis by inhibiting SOX12 expression. Similar to miR-370, miR-29b was found to suppress cell proliferation and mobility by targeting SOX12[32] in pancreatic cancer. Hence, we conclude that miR-370 acts as an inhibitor of BC through the targeting of SOX12.

Conclusions

Collectively, our data demonstrate that miR-370 is downregulated in the development of BC, relating to poor prognosis. Furthermore, by targeting of SOX12, miR-370 restricts cancer cell proliferation, migration, and invasion, eventually blocking the development of BC. We believe that the miR-370/SOX12 axis could shed new light on the diagnosis of BC, resulting in better treatment options and outcomes for patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

This work was supported by the Fund of Capital Medical Development Research (2009-3176).

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