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ORIGINAL RESEARCH

# MiR-582-5p Inhibits Bladder Cancer-Genesis by Suppressing TTK Expression

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**Background:** Bladder cancer (BC) refers to the malignant growth found in the cells and tissues of the urinary bladder. While many studies have researched the progression of BC, scientists are yet to fully understand the mechanism of BC. This research aimed to explore the role of miR-582-5p and its target gene TTK in BC pathogenesis.

**Methods:** The evaluation of miR-582-5p and TTK mRNA expression in BC tissues or cells was performed using qRT-PCR. TargetScan was then used to predict the binding site of miR-582-5p on TTK mRNA. Subsequently, dual-luciferase reporter and RNA pull-down assays were employed to validate the binding relationship between miR-582-5p and TTK mRNA. CCK-8, BrdU, flow cytometry, and caspase-3 activity assays were later conducted to evaluate the viability, proliferation, cell cycle, and apoptosis of BC cells.

**Results:** Investigations revealed that miR-582-5p was downregulated in BC tissues and cells. Meanwhile, miR-582-5p inhibited the viability and proliferation of BC cells while stimulating the apoptosis and cycle arrest of the cells. TTK, the target gene of miR-582-5p, was later found to be over-expressed in BC tissues and cells. TTK, however, was observed to exhibit an opposite effect on miR-582-5p. Simply put, it stimulated BC cell malignant phenotypes, and this stimulation could be directly reversed by miR-582-5p.

**Conclusion:** This research confirmed that miR-582-5p could restrain bladder carcinogenesis by inhibiting TTK expression.

**Keywords:** bladder cancer, miR-582-5p, TTK, proliferation, apoptosis

## Introduction

Bladder cancer (BC) refers to a malignant tumor that is typically found in the urinary bladder mucosa.[1] The global incidence of BC is becoming alarming, especially for anyone concerned about improving quality healthcare in society. In 2018, BC was estimated to account for over 543, 000 new cancer cases worldwide, with a global mortality rate of 2.1% (201,600 deaths).[2] A 2015 report also estimated that in China, about 80,500 patients displayed BC symptoms, and over 32,900 deaths could be linked to BC cases.[3] Adjuvant therapies such as radiotherapy, chemotherapy, and immunotherapy have improved the survival rate of BC patients.[1,4,5] Emerging research on biomarkers has also offered new approaches to BC diagnosis and treatments.[6] However, due to the metastasis and recurrence of BC, the cure rate of patients is still low and unpalatable. By studying and understanding the mechanism underlying BC, the chances of reducing the death rate and incidence rate of BC are considerably high.

MiRNAs are single-stranded non-coding RNAs that are mainly involved in biological processes, such as mRNA degradation and the translational inhibition of target

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genes. Scientific efforts have been invested in unraveling how miR-582 influences cancer development during the past 20 years. Many studies found that miR-582 played an important role in the proliferation, migration, and invasion of cancer cells.[7,8] Several studies also reported its potentials as a biomarker for cancer treatment.[9–11] More specifically, the literature has noted the suppressive effect of miR-582-5p on several cancers, including gastric cancer,[12] non-small cell lung cancer,[10] human endometrial carcinoma,[13] and prostate cancer.[14] Two scientific articles in the last two decades have demonstrated the ability of miR-582-5p to restrain BC development.[15,16] However, scientists are yet to understand the restraining mechanism of miR-582-5p on BC cells.

TTK gene comprises 23 exons on the 6q14.1 chromosome. This gene encodes a dual-specific protein kinase that is involved in cell mitosis and proliferation. Besides, the literature is replete with information regarding the role of TTK in cancer. A number of studies have found TTK to be highly expressed in a variety of cancers with a poor prognosis outcome.[17,18] Some researchers described TTK as an oncogene that could facilitate the pathological process of cancer.[19–21] What's more, TTK has been identified as a target gene of miRNA.[22] Although TTK's role in cancer has attracted a plethora of attention, only a few studies have examined the relationship between BC and TTK. Among them, one research showed that TTK promoted BC cell proliferation and thus accelerated cancer deterioration.[23] However, it is not yet clear whether the regulation of TTK by miRNA can enhance or suppress BC development.

In this paper, we aimed not only to investigate the relationship between miR-582-5p and TTK but also to explore the effect of this microRNA and its target gene on BC.

# Materials and Methods

## Tissue Harvest and Cell Culture

All BC tissues and adjacent healthy tissues were collected from 47 patients with BC from The First Affiliated Hospital of Zhengzhou University. The study protocol was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University. The characteristic of the BC patients is shown in Table 1. BC cell lines (T24, RT4, J82, and 5637) and normal urothelial epithelial cell lines (SV-HUC-1) were purchased from the Shanghai Institute for Biological Center (China). SV-HUC -1 and J82 cells were cultured in an atmosphere containing 5% $CO_2$ at 37°C and in a MEM-EBSS medium (Sangon, China) containing 10% fetal bovine serum and 100 U/mL streptomycin. 5637 and T24 cells were cultured under 5% $CO_2$ at 37°C in an RPMI1640 medium (Sangon, China) containing 10% fetal bovine serum and 100 U/mL streptomycin. RT4 cells were cultured under 5% $CO_2$ at 37°C in a McCoy's 5A medium (Sigma, US) containing 10% fetal bovine serum, 2.2g/l $NaHCO_3$, and 100 U/mL streptomycin.

**Table 1** Clinical Characteristics of 47 Patients with Bladder Cancer

| Characteristics | Total = 47 | Percentage (%) |
|---|---|---|
| Age(years) | | |
| >69 | 20 | 42.6% |
| ≤69 | 27 | 57.4% |
| Gender | | |
| Male | 31 | 66.0% |
| Female | 16 | 34.0% |
| Tumor diameter(cm) | | |
| >3 | 21 | 44.7% |
| ≤3 | 26 | 55.3% |
| Histological grade | | |
| Low | 10 | 21.3% |
| High | 37 | 78.8% |
| TNM stage | | |
| I | 10 | 21.3% |
| II | 14 | 29.8% |
| III | 20 | 42.6% |
| IV | 3 | 6.3% |
| Lymph nodes metastasis | | |
| Positive | 14 | 29.8% |
| Negative | 33 | 70.2% |

## Cell Transfection

MiR-582-5p negative control (NC), miR-582-5p inhibitor or mimic (RiboBio, China), and TTK over-expression (TTK-OE) plasmids (GeneCopoeia, China) were transfected into BC cells using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, US) at room temperature. After that, the qRT-PCR was conducted to verify the transfection efficiency.

## qRT-PCR

The Cell Total RNA Isolation Kit (Foregene, China) was used to extract the RNAs. The reverse-transcription of miRNA was performed using mirVana qRT-PCR miRNA Detection Kit (Invitrogen, US). The reverse-transcription

of mRNA was performed using the RT EasyTM II system (Foregene, China). The MonAmp ChemoHS qPCR Mix (Monad, US) was then used to perform qRT-PCR analysis. U6 acted as the reference for miR-582-5p, while GAPDH acted as the reference for TTK mRNA. The sequences of primers are shown in Table 2.

## Western Blot Assay

The proteins were obtained with RIPA Lysis (Sangon, China) and separated using 12% SDS-PAGE gel electrophoresis at 100 V for 1 h. After equilibrating the protein-containing gel with the transfer buffer, the protein was transferred to the PVDF membrane (Sangon, China). The hybridization membrane was then incubated with anti-TTK (1:1000, Sangon, China) and β-actin (1:1000, Sangon, China) for 2 h and blocked at 4°C overnight. After 24-hincubation, the membrane was treated with a secondary antibody (Rabbit-Mouse; Sangon, China) for 2 hours. ImageJ software was eventually used to quantify the intensity of bands.

## CCK-8 Assay

A total of 100 μL 5637 and T24 cells, which were suspended (2000 cells/well) in a logarithmic growth phase, were cultured in a 96-well plate for the following periods: 0 h, 24 h, 48 h, and 72 h. Each well was treated for 2 h with 10 μL of CCK-8 solution reagents (Sangon, China). After the treatment, the optical absorbance was measured with a microplate at 450nm.

**Table 2** The Primer Sequences for RT-qPCR

| GENE | Primer Sequences (5′-3′) |
|---|---|
| miR-212-3p | Forward: GGTAACAGTCTCCAGTCA<br>Reverse: GCAATTGCACTGGATACG |
| miR-582-5p | Forward: GCGGTTACAGTTGTTCAACC<br>Reverse: CTCAACTGGTGTCGTGGA |
| U6 | Forward: GCTTCGGCAGCACATATACTAAAAT<br>Reverse: CGCTTCACGAATTTGCGTGTCAT |
| CCNB2 | Forward: CACAGGATACACAGAGAATG<br>Reverse: CTTGATGGCGATGAATTTAG |
| TTK | Forward: TCCCCAGCGCAGCTTTCTGTAGA<br>Reverse: CCAGTCCTCTGGGTTGTTTGCCAT |
| GAPDH | Forward: GAAGGTGAAGGTCGGAGTC<br>Reverse: GAAGATGGTGATGGGATTTC |

## BrdU Assay

The BrdU Cell Proliferation Assay Kit (Cell signaling, US) was employed to detect the cells. The transfected cells were cultured for 48 h before 10 μM BrdU solution was added to 5637 and T24 cells. The cells were later immobilized with 100 μL Fixing/Denaturing Solution for half an hour. The prepared 1× test antibody solution was added to the cells for 1-h incubation. The cells were subsequently washed thrice with 1× Wash Buffer. The next procedure involved the addition of the prepared 1× HRP labeled secondary antibody solution to the cells. After that, the mixture was incubated incubation. The samples were then treated with 100 μL STOP Solution. Finally, the stained cells were observed in a microscope at 450 nm.

## Caspase-3 Activity Assay

The Caspase-3 Activity Assay Kit (Elabscience, China) was utilized to carry out cell apoptosis detection. Then, $5\times10^4$ cells/well were seeded in a 96-well plate. After that, 30 μL (each well) cell lysis buffer was added to the samples. The cells were subsequently placed on ice for 5 min. The next procedure involved the addition of 5 μL Ac-DEVD-pNA to the samples, followed by 4-h incubation. The activation of Caspase-3 was eventually determined using a spectrophotometer at 400 nm.

## Flow Cytometry

The cell cycle of 5637 and T24 cells was detected with flow cytometry assay. More specifically, the Cell Cycle Assay Kit- PI/RNase Staining (Dojindo, Japan) was used to perform cell-cycle detection. After resuspending the cells with pre-chilled 1 × PBS, pre-chilled 75% anhydrous ethanol was used to fix the cells overnight. Twenty microliter RNase was then added to the samples for 30 min at 37°C to digestion. The cells were stained with 20 μL PI for half an hour in the absence of light. The red fluorescence was detected with flow cytometry at 488 nm.

## Dual-Luciferase Reporter Assay

TTK 3′UTR mutant type (MUT) was obtained by mutating the binding sequence "AACUGUAA". TTK 3′UTR wild type (WT) remained the same. Both sequences were subsequently subcloned into the pmiR-GLO vector. The cells co-transfected with miR-582-5p mimic and WT or MUT; miR-582-5p mimic NC and WT or MUT were seeded in a 96-well plate. The Dual-Luciferase Reporter Gene Assay Kit (YEASEN, China) was utilized to measure the

luciferase activity of cells. After the cell medium was discarded, the cells were added to 100 μL firefly-luciferase solution to determine luciferase activities. Next, 100 μL renilla luciferase reaction solution was added to ascertain renilla luciferase activity. The measurement was performed with a microplate reader (Biotek, US).

## RNA Pull-Down

MiR-582-5p mimic-biotin (Bio-miR-582-5p, RiboBio, China) and its negative control (Bio-NC, RiboBio, China) were transfected into 5637 and T24 cells. The RNA pull-down Kit (gzscbio, China) was then used to analyze the samples. A cell lysis buffer was added to the cells. After that, the sample was centrifuged to obtain supernatant. The supernatant was then treated with streptavidin magnetic beads (Invitrogen, US) to capture biotin-coupled RNA complex. After magnetic grates (Life, US) were used to remove streptavidin magnetic beads, TTK mRNA expression level was measured by qRT-PCR.

## Statistical Analysis

All experiments were repeated three times, and the data collected were presented in the format of mean ± SD (standard deviation). Student's *t*-test and ANOVA were used for statistical analysis. $P< 0.05$ was assumed to be statistically significant, and $P< 0.001$ was considered extremely significant.

# Results

## TTK and miR-582-5p Were Identified as Probable Biomarkers in BC

We first identified 32 differentially expressed mRNAs in the GSE37815 data series and GEPIA2 database with adjusted P value <0.05 and logFC>1.5 (http://gepia2.cancer-pku.cn/) (Figure 1A). The 32 mRNAs were uploaded into the STRING algorithm to visualize the potential interaction network between these genes and their potential signaling pathways. It was found that the cell cycle pathway was the most significant signaling pathway that involved four of these genes: SFN, TTK, CCNB2, and CDC20 (Figure 1B). For this reason, we scrutinized the GSE37815 data matrix and explored the relative expression of the four genes (Figure 1C). After conducting a KEGG enrichment analysis of the 35 common differentially expressed mRNAs in the GSE37815 data series and GSE76211 data series (Figure 1D), we found that CCB2 and TTK were the two genes that participated in cell cycle signaling (Figure 1E). We then detected the expression of TTK and CCNB2 mRNA in adjacent healthy and BC tissues using qRT-PCR. Compared with adjacent healthy tissues, TTK mRNA and CCNB2 mRNA in BC tissues were upregulated about 2.5 times and 1.5 times, respectively (Figure 1F). After using GEPIA algorithm, TTK was also found to be significantly upregulated in BC samples (Figure 1G). Therefore, we chose TTK for our follow-up study. To identify an upstream miRNA of TTK mRNA, we investigated two prediction tools, miRDB and TarBase. The two miRNAs identified were miR-212-3p and miR-582-5p (Figure 1H). We noticed that miR-582-5p restrained the growth of BC.[15,16,24] We later analyzed how miR-212-3p and miR-582-5p were expressed between adjacent healthy and BC tissues. Our findings indicated that both of them were downregulated in BC tissues. However, the downregulation of miR-582-5p expression was far more significant than miR-212-3p (Figure 1I). Hence, we selected miR-582-5p as the gene of interest in this study.

## MiR-582-5p Suppressed the Malignancy Phenotypes of BC Cells

Using qRT-PCR, we analyzed miR-582-5p expression in different BC cell lines, including T24, RT4, J82 and 5637 and the normal urothelial epithelial cell line (SV-HUC-1). We found that miR-582-5p expression was lower in BC cell lines than in the normal urothelial epithelial cell line. This result revealed that miR-582-5p downregulation occurred during the pathological process of BC (Figure 2A). In addition, the expression level of miR-582-5p was lower in 5637 and T24 cell lines than in other BC cell lines. Therefore, we transfected miR-582-5p negative control, mimic, or inhibitor into 5637 and T24 cells. Compared with the blank control group, qRT-PCR data indicated that miR-582-5p mimic dramatically enriched miR-582-5p by almost 2-fold, while miR-582-5p inhibitor reduced it by nearly 65% in 5637 cells. We also noticed that miR-582-5p mimic significantly enriched miR-582-5p by 2-fold, whereas its inhibitor decreased it by almost 70% in T24 cells. These outcomes showed that cell transfection was successful (Figure 2B). Based on the CCK-8 data, the upregulation of miR-582-5p weakened the viability of 5637 and T24 cells, while the downregulation of miR-582-5p enhanced it significantly (Figure 2C).

Furthermore, the BrdU assay result showed that miR-582-5p could restrict the proliferation capacity of 5637 and

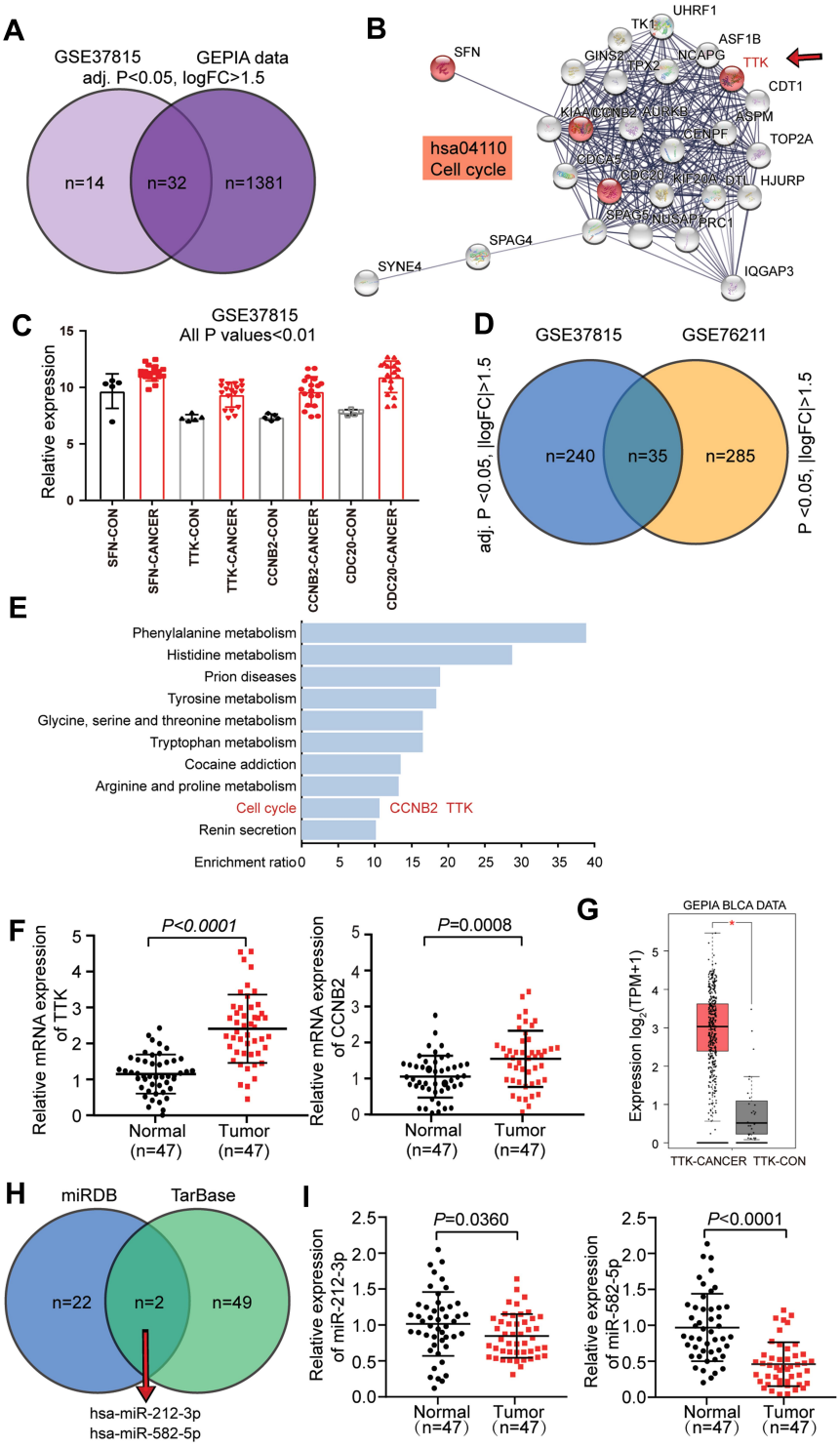


**Figure 1** The identification of TTK and miR-582-5p in BC. (**A**) A total of 32 significant genes were identified to play important roles in BC. The DEGs of GSE37815 data series were scrutinized using GEO2R algorithm. (**B**) STRING analysis results of the 32 DEGs from A. The red nodes represented genes that participated in the cell cycle signaling pathway. (**C**) A general view on the expression of the four nodes from B in the GSE37815 data series. (**D**) We introduced another GEO data series, GSE76211, to identify the significant DEGs. Together with GSE37815 data series, 35 DEGs were identified. (**E**) The 35 DEGs were subjected to enrichment analysis at http://www.webgestalt.org/enrichment analysis. CCNB2 and TTK were considered the enriched term: cell cycle. (**F**) The expression of TTK and CCNB2 mRNA in BC tissues and adjacent healthy tissues was measured using qRT-PCR. Normal: adjacent healthy tissue. Tumor: BC tissue. (**G**) TTK overexpression in BC tissues was validated with the GEPIA algorithm. The number of cancer tissues is 404, and the number of healthy tissues is 28. *P< 0.05 compared with control group. (**H**) The potential upstream miRNAs of TTK mRNA were identified using miRDB (http://mirdb.org/cgi-bin/search.cgi) and TarBase (from DIANA toolbox). (**I**) The expression levels of miR-212-3p and miR-582-5p were analyzed using qRT-PCR in BC tissues and adjacent healthy tissues. Normal: adjacent healthy tissue, Tumor: BC tissue.

**Abbreviations:** adj. P, adjusted P; FC, fold change; CON, control healthy tissues.

T24 cells (Figure 2D). Compared with the blank control group, miR-582-5p upregulation restrained cell proliferation by about 40%, whereas miR-582-5p downregulation facilitated cell proliferation by about 50% in 5637 cells. In short, both 5637 cells and T24 cells showed a similar trend (Figure 2D). Our caspase-3 activity assay results showed that the apoptosis ability of 5637 and T24 cells with miR-582-5p mimic was about 3-fold compared with the blank control group, while the apoptosis ability of cells with miR-582-5p inhibitor was significantly reduced by around 60% compared to the blank control group (Figure 2E). As depicted in Figure 2F and G, miR-582-5p upregulation reduced the cell population in S (Synthesis) phase by 50% and increased the cell population in G2 (Gap 2) phase by 80% compared to the blank control group in 5637 cells.

In addition, miR-582-5p downregulation enhanced cell population in S phase by 25% and reduced cell population in G2 phase by 30% compared to the blank control group in 5637 cells (Figure 2F and G). As for T24 cells, we observed a similar trend (Figure 2F and G). The cell cycle of 5637 and T24 cells could be restrained at G2 phase by miR-582-5p mimic. Overall, miR-582-5p repressed the viability and proliferation abilities of BC cells but facilitated the cycle-arrest and apoptosis abilities of BC cells.

## MiR-582-5p Directly Targeted TTK

TargetScan was employed to predict the binding sequences of miR-582-5p on TTK (the binding scheme illustrated in Figure 3A). The binding sites were then mutated to construct TTK mutant luciferase reporter plasmids and, most importantly, to ascertain the relationship between miR-582-5p and TTK. MiR-582-5p mimic and TTK wild-type or TTK mutant luciferase reporter plasmids were co-transfected into 5637 and T24 cells. Compared to the negative control, the co-transfection of miR-582-5p mimic and TTK wild-type undermined the fluorescence intensity of BC cells. On the other hand, miR-582-5p mimic and TTK mutant co-transfection did not decrease fluorescence intensity (Figure 3B). RNA pull-down assay was later performed to verify the target relationship between miR-582-5p and TTK. As shown in Figure 3C, TTK expression in the Bio-miR-582-5p group was 6-fold more than that of the negative control group. The correlation detection exhibited a negative mRNA expression level between miR-582-5p and TTK (Figure 3D). We measured how TTK was expressed in BC cells and SV-HUC-1. Our findings revealed that TTK expression was 70% higher in 5637 cells than in SV-HUC-1 and that it was 1.2 times higher in T24 cells than in SV-HUC-1. This outcome confirmed that TTK was involved in the regulation of BC development (Figure 3E). After transfecting miR-582-5p mimic or inhibitor, we determined mRNA expression of TTK in 5637 and T24 cells. The results showed that miR-582-5p mimic decreased TTK mRNA expression by 50%, while miR-582-5p inhibitor increased TTK expression by 50% compared to the blank control group in 5637 cells and T24 cells (Figure 3F). Overall, TTK was found to be a reliable target of miR-582-5p.

## TTK Downregulated by miR-582-5p Advanced BC Development

To further explore whether the expression level of TTK inhibited by miR-582-5p affects the progression of BC, we transfected the negative control, TTK-OE, miR-582-5p mimic, and TTK-OE plus miR-582-5p mimic into 5637 and T24 cells. We observed that whereas miR-582-5p mimic transfection suppressed the TTK protein level by 50% compared to the blank control group, TTK-OE increased it by 40% (Figure 4A and B). In short, the TTK expression level was rescued by OE in miR-582-5p mimic transfecting the cells (Figure 4A and B). Thus, 5637 and T24 cells were successfully transfected. We later performed cell characterization experiments on the successfully transfected 5637 and T24 cells. As shown in Figure 4C, the upregulation of TTK significantly enhanced cell viability, whereas miR-582-5p mimic restored the enhancement. BrdU assay also showed that TTK-OE improved cell proliferation by 70% compared to the blank control group. This facilitation, however, was reversed by miR-582-5p mimic in 5637 and T24 cells (Figure 4D). Our caspase-3 activity assay indicated an approximately 50% decrease in cell apoptosis, and this decrease was due to the upregulation of TTK by miR-582-5p mimic (Figure 4E). In Figure 4F and G, the cell population of 5637 cells in G2 phase of the TTK upregulation group was 50% that of the blank control group, while the cell population in S phase increased by 3-fold. The T24 cells displayed a similar result (Figure 4F and G). Meanwhile, TTK-OE could relieve the cell cycle arrest of 5637 and T24 cells caused by miR-582-5p mimic. Taken together, TTK facilitated cell viability and proliferation, but decreased cell apoptosis, which all could be rescued by miR-582-5p mimic.

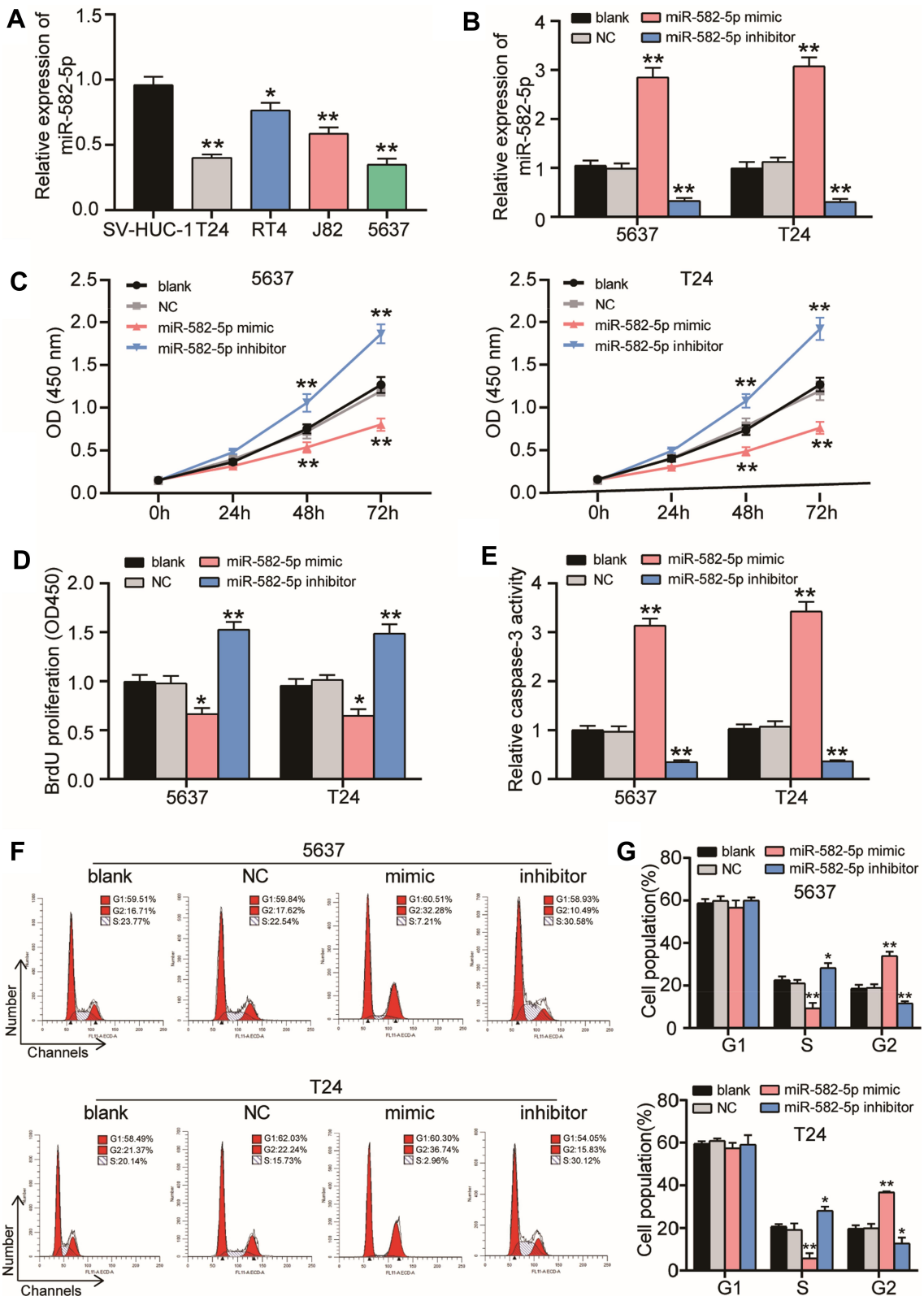


**Figure 2** MiR-582-5p suppressed the malignancy phenotypes of BC cells. (**A**) Using qRT-PCR, the expression of miR-582-5p was analyzed in BC cell lines, including T24, RT4, J82 and 5637 and the normal urothelial epithelial cell line (SV-HUC-1). (**B**) The transfection efficiency of miR-582-5p mimic or inhibitor in 5637 and T24 cells was determined using qRT-PCR. U6 was used as the internal control. (**C**) The viability of the transfected 5637 and T24 cells was measured with CCK-8 assay. (**D**) The proliferation of the transfected 5637 and T24 cells was determined with BrdU assay. (**E**) The apoptosis rate of the transfected 5637 and T24 cells was verified using caspase-3 activity assay. (**F, G**) The cell cycle of the transfected 5637 and T24 cells was obtained by performing flow cytometry analysis. (**A**–**G**) 5637 and T24 cells were transfected with miR-582-5p negative control, miR-582-5p mimic or miR-582-5p inhibitor. Three independent experiments were performed, and all the data were presented in the form of mean ±SD. *P< 0.05, **P< 0.001, compared with blank control group.
**Abbreviation:** NC, negative control.

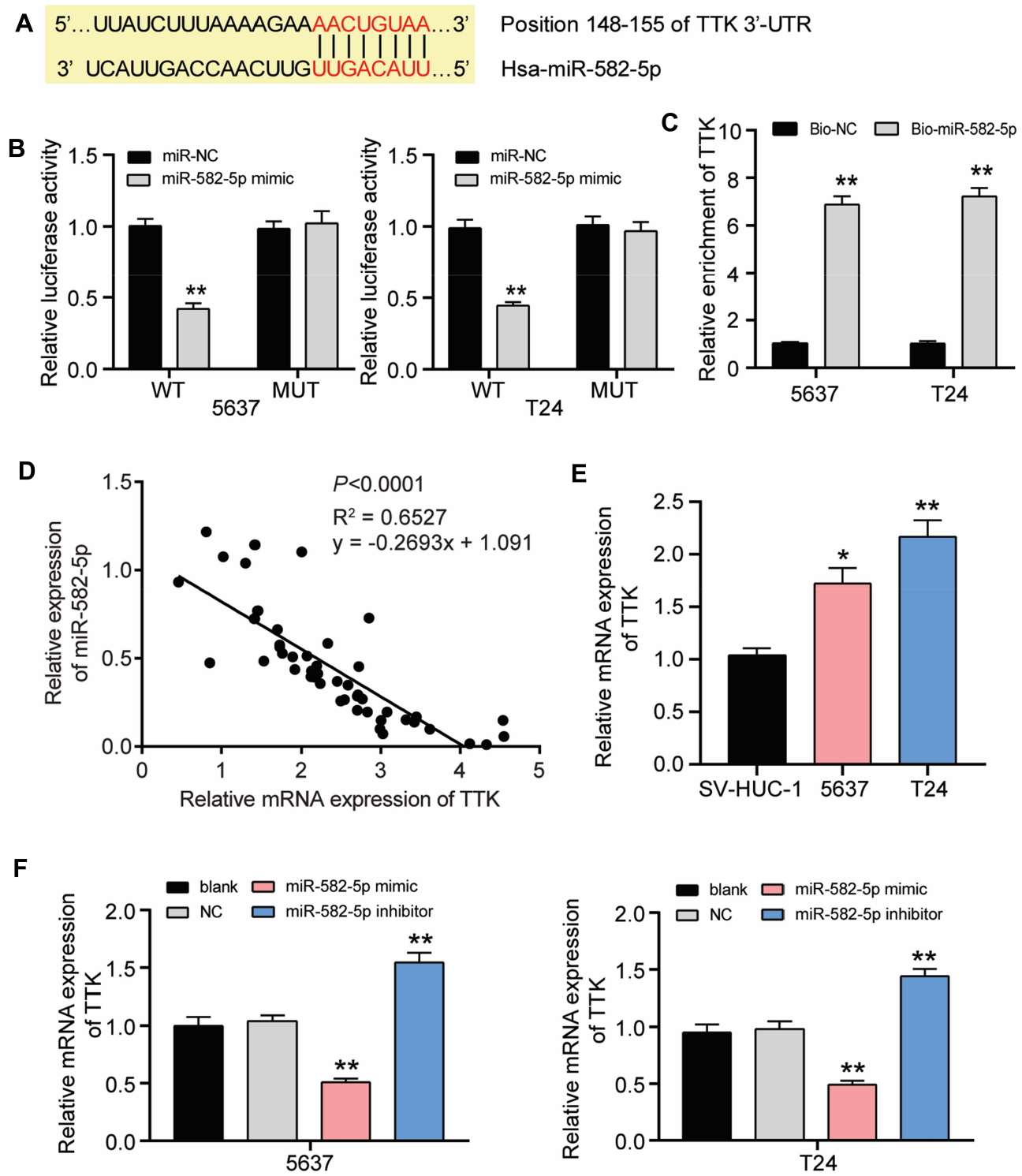


**Figure 3** MiR-582-5p directly targeted TTK. (**A**) The binding sequences of miR-582-5p and TTK mRNA were predicted by TargetScan Human 7.2. (**B**) The potential binding site between miR-582-5p and 3′UTR of TTK mRNA was reflected by the fluorescence intensity of the dual fluorescent plasmid. **P< 0.001, compared with WT plus the miR-NC group. (**C**) RNA pull-down assay was performed to verify the target relationship between miR-582-5p and TTK. **P< 0.001, compared with the Bio-NC group. (**D**) The correlation between miR-582-5p expression and TTK mRNA expression. (**E**) The expression of TTK mRNA in BC cells (5637 and T24) and normal urothelial epithelial cell line (SV-HUC-1) was measured using qRT-PCR. GAPDH was used as the internal control. *P< 0.05, **P< 0.001, compared with SV-HUC-1 cell. (**F**) The expression of TTK mRNA in the transfected 5637 and T24 cells was identified using qRT-PCR. GAPDH was used as the internal control. 5637 and T24 cells were transfected with miR-582-5p negative control, miR-582-5p mimic or miR-582-5p inhibitor. **P< 0.001, compared with the blank control group. (**B**–**F**) Three independent experiments were performed, and all the data were depicted in the form of mean ±SD.

**Abbreviations:** WT, TTK 3′UTR wild type; MUT, TTK 3′UTR mutant type; Bio-miR-582-5p, miR-582-5p mimic-biotin; Bio-NC, negative control-biotin.

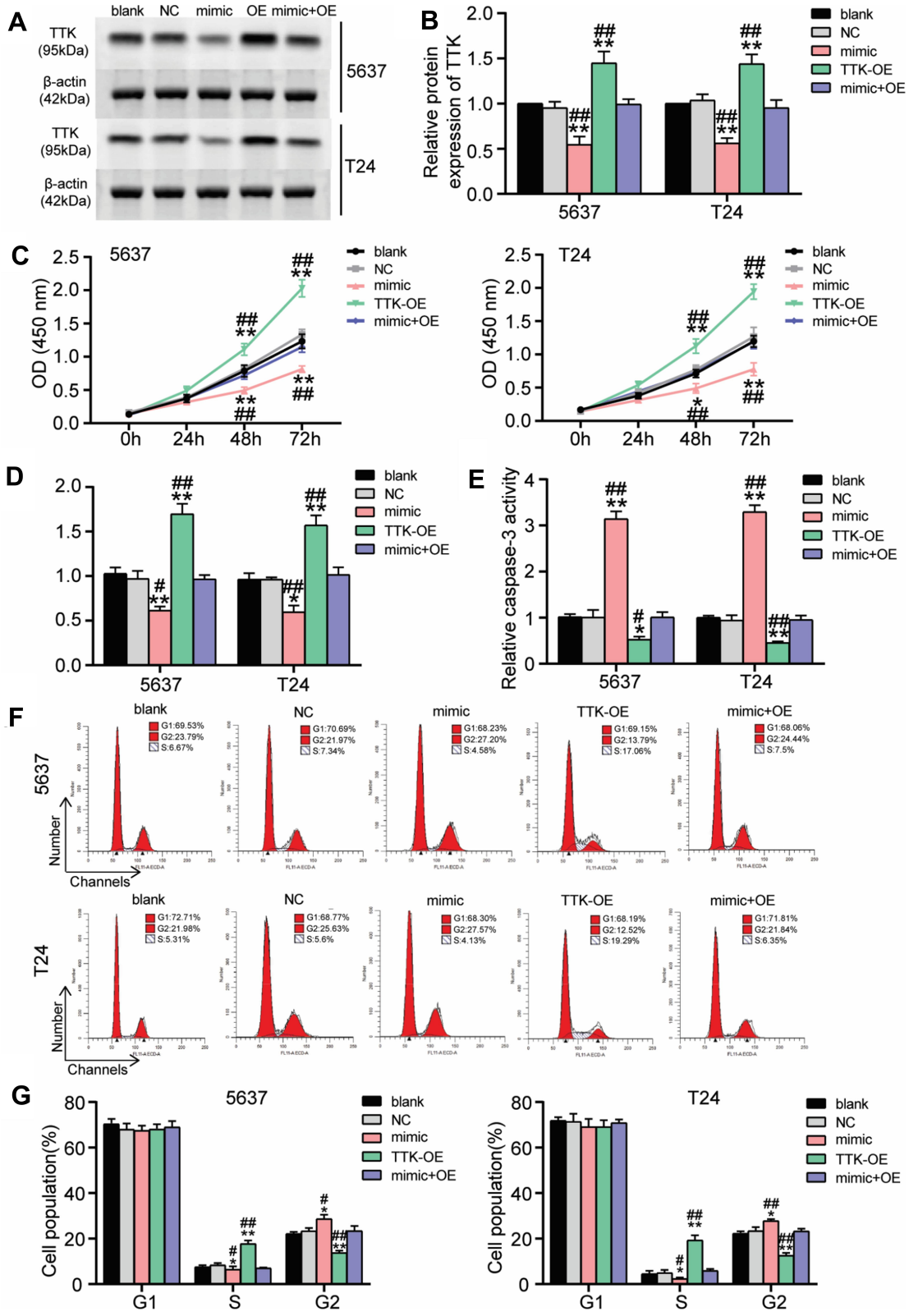


**Figure 4** TTK downregulated by miR-582-5p advanced BC development. (**A, B**) The transfection efficiency of 5637 and T24 cells was determined using Western blot analysis, with GAPDH as the loading control. (**C**) The viability of the transfected 5637 and T24 cells was measured with CCK-8 assay. (**D**) The proliferation of the transfected 5637 and T24 cells was determined using BrdU assay. (**E**) The apoptosis rate of the transfected 5637 and T24 cells was verified by caspase-3 activity assay. (**F, G**) The cell cycle of the transfected 5637 and T24 cells was obtained using flow cytometry analysis. (**A**–**G**) 5637 and T24 cells were transfected with negative control, miR-582-5p mimic, TTK-OE, or miR-582-5p mimic plus TTK-OE. Three independent experiments were performed, and all the data were presented in the form of mean ±SD. *P< 0.05, **P< 0.001, compared with the blank group. #P< 0.05, ##P< 0.001, compared with the co-transfection group of miR-582-5p mimic plus TTK-OE.

**Abbreviations:** NC, negative control; mimic, miR-582-5p mimic; TTK-OE, TTK overexpression vector; mimic+OE, miR-582-5p mimic plus TTK overexpression vector.

## Discussion

In this research, we explored the key role of miR-582-5p and its target gene TTK in BC progression. More specifically, we showed that miR-582-5p downregulation and TTK upregulation occurred in BC tissues and cells. Furthermore, we demonstrated the suppressive effect of miR-582-5p on the viability and proliferation ability of BC cells and indicated the promotive effect of this microRNA on cell cycle arrest and apoptosis. Meanwhile, miR-582-5p was found to directly inhibit TTK expression by pairing with its 3′UTR sequence. In the end, TTK was observed to promote the malignant phenotype of BC cells, which was rescued by miR-582-5p.

Several studies have reported that miR-582-5p was expressed differentially during different cancer progressions, meaning its function was dependent on the types of tissues and cells. miR-582-5p was upregulated in oral and colorectal cancer tissues,[25,26] but it exhibited low expression in non-small cell lung cancer and gastric cancer tissues.[12,27] This differential expression was also related to the different roles (oncogenes or cancer resistance genes) of miR-582-5p during different cancer development. Besides, research in the literature has confirmed that miRNAs can target and manipulate different genes in different pathways, thereby exerting different functions. For instance, in the progression of colorectal cancer, miR-582-5p was upregulated and was found not only to target the APC gene but also to accelerate cell proliferation.[26] However, in gastric cancer, miR-582-5p had lower expression and restrained cell proliferation by targeting AKT3.[12]

In this research, we demonstrated that miR-582-5p had lower expression in BC tissues and cells by targeting TTK. Our result was consistent with the outcome of a previous study in which the low expression of miR-582-5p occurred during the process of high-grade BC.[15] As for the functional effect of miR-582-5p, we found that this microRNA could repress cell viability and proliferation and induce cell apoptosis and cell cycle arrest, thus inhibiting the pathogenesis of BC. Its inhibitory effect was also illustrated in another study,[16] which demonstrated that miR-582-5p facilitated the growth of 5637 and T24 cells. Besides, we demonstrated that miR-582-5p blocked the cell cycle of 5637 and T24 cells at G2 phase. In addition to the fact that our study was the first scientific research to explore the impacts of miR-582-5p on the cell cycle of BC cells, it was also the first to discover that miR-582-5p could target and slow down TTK in BC progression.

Furthermore, several research found that TTK was upregulated during the development of various cancers, including non-small cell lung cancer,[28] gastric cancer,[29] and pancreatic cancer.[30] We indicated that TTK expression was high in BC tissues and cells. Besides, higher TTK was found to result in a worse prognosis for breast cancer patients.[18,31,32] By silencing TTK, prostate cancer cell proliferation was impeded, thereby hindering cancer progression.[20] These pieces of researches clarified the promotional role of TTK in cancer progression, and their results were consistent with our research findings. We also observed similar cancer-promoting effects of TTK that occurred in BC carcinogenesis, that facilitated cell viability and proliferation, and that inhibited the apoptosis of BC cells. Our observation was in line with the result of a previous study, which showed that TTK exacerbated BC development.[23] Taken together, our data provided another detailed evidence that TTK aggravated the process of BC.

This study was the first to explore the impacts of TTK on the cell cycle of BC cells. In our study, we found that the upregulation of miR-582-5p resulted in the apoptosis of 5637 and T24 cells and that it arrested the cells at G2 phase and reduced the proportion of S phase. However, the interference with miR-582-5p or the over-expression of TTK produced an opposite result. In their study, Li et al[33] studied the effect of splicing factor ASF/SF2 on the programmed cell death of DT40-ASF cells. They found that ASF/SF2 inactivation leads to cell cycle arrest at G2 phase and induced cell death. Sun et al[34] also used the G2 phase proportional increase to assess the radiosensitivity of cells. The researchers found that the cell cycle was arrested at G2 phase when cells were dying. Therefore, we conjectured that the miR-582-5p/TTK axis could regulate cell proliferation and apoptosis by inhibiting some proteins, thereby preventing the cells from M phase. For instance, Zhang et al[35] found that knocking down TTK induced the cell cycle arrest at G2 phase in human endometrioid endometrial adenocarcinoma. This result might support our hypothesis. Hence, further exploration of this field is required.

This research comes with several limitations. Our study did not focus on how TTK exerted a kinase's role to activate its downstream signaling involved in promoting the BC progression. Besides, the xenograft experiment was not performed due to the limitation of experimental conditions. In the next study, we will explore the effects of

miR-582-5p and TKK on the kinase pathway and further explain the mechanism of miR-582-5p and TKK on BC, such as migration, invasion, and xenograft tumor. Moreover, clinical explorations are needed to examine whether miR-582-5p has metastatic effects on BC.

## Conclusion

Our findings revealed that miR-582-5p could inhibit BC by suppressing TTK expression. Put simply, we found that this microRNA directly targeted TTK in the regulation of BC progression. After evaluating the roles of miR-582-5p and TTK in the BC cell cycle using flow cytometry assay, our data further confirmed the suppressive effect of miR-582-5p and the promotive effect of TTK on BC development.

## Data Sharing Statement

The data used and analyzed during the current study are available from the corresponding author on reasonable request.

## Ethics Approval and Consent to Participate

The study protocols were approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University. All procedures used in this research met the ethical standards set out in the Helsinki Declaration. All patients signed written informed consent.

## Disclosure

The authors declare that no conflicts of interest exist in this research.

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