MiR-188-5p and MiR-141-3p Promote Bladder Cancer Synergistically via Targeting PTEN to Activate AKT/c-MYC Signal Pathway

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Research Article

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Abstract

MicroRNAs play a key role in the progress of bladder cancer (BC), which may lead to poor prognosis. A single MicroRNA can be used as an independent factor to regulate the progress of BC, while two MicroRNAs can have a synergistic regulatory effect on BC progress. It has been confirmed in our previous studies that miR-188-5p and miR-141-3p demonstrated high expressions in BC tissues and cells, which can promote the progress of BC and affect patient's prognosis. As a follow-up research, this study has made further exploration in many aspects. We predicted and confirmed that miR-188-5p and miR-141-3p had a common target gene PTEN which had low expression in BC tissues and cells. Down regulation of PTEN can promote the progress of BC, and significantly reverse the inhibitory effect of down-regulated miR-188-5p and miR-141-3p on BC progress. PTEN is a cancer suppressor gene. Experiments further verified that pAKT and c-MYC were downstream effector proteins of PTEN, and their expressions increased with the decrease of PTEN expression. Experiments manifested that down-regulating miR-188-5p and miR-141-3p could significantly inhibit the volume and weight of subcutaneous tumors in mice, and half dose co-transfection of the two miRs made the tumor smaller and lighter. Therefore, it was concluded that miR-188-5p and miR-141-3p promoted the progress of BC synergistically by inhibiting PTEN to activate AKT/c-MYC pathway.

Introduction

Bladder cancer (BC) is the tenth most commonly diagnosed tumor around the world. There were nearly 570,000 new cases and 210,000 death cases in 2020, while the incidence rate and mortality of male were 4 times that of female [1]. The average age of diagnosis of BC is from 70 to 84 years old [2]. According to World Health Organization, BC is classified into high-grade and low-grade diseases based on its standardized histomorphology features and into non-muscle invasive and muscle invasive diseases based on the depth of tumor infiltration [3]. Nearly 75% of high-grade non-muscle invasive BCs will recur within 10 years among which 41% are simple recurrence and 33% will progress to deeper muscle invasive BCs. 40% of the deeper muscle invasive BCs lead to death [4]. By 2013, treatments including surgery, radiotherapy, chemotherapy and intravesical instillation have failed to significantly improve the 5-year survival rate of BC patients [5]. Checkpoint inhibitors developed in recent years are expected to improve the survival rate of BC patients. It has been reported that checkpoint inhibitors can significantly relive or even cure advanced BC. However, the effectiveness only reaches about 20% [6]. In view of the above-mentioned diagnosis and treatment background, it is of great importance whether new methods can be discovered to make breakthroughs in the treatment of BC, such as predicting and preventing the recurrence and progress of BC through molecular targets to improve its prognosis.

MicroRNA is an endogenous RNA composed of 21-24 nucleotides. It can not encode, but it has certain regulatory functions [7, 8]. MiRNAs play a significant regulatory role by inhibiting the translation of target genes or pyrolyzing the meRNA of target genes [9, 10]. MiRNAs regulate several important functions of cells, such as cell differentiation, proliferation, cycle, apoptosis, metabolism and angiogenesis [11-16]. Therefore, changes in miRNA expressions are closely related to the occurrence and development of

cancer [17]. Since miRNA is a key regulator in BC [18], researches on the relationship between BC and miRNAs have never ceased [19, 20]. Scholars are eager to analyze or intervene BC from the perspective of non-coding genes, thus to benefit BC patients.

This study found and verified through experiment in vitro that PTEN was the common target gene of miR-188-5p and miR-141-3p and presented low expression in BC tissues and cells. This study further manifested that miR-188-5p and miR-141-3p activated AKT/c-MYC pathway through the down-regulation of PTEN and then promoted BC synergistically. In vivo experiment found that down-regulation of miR-188-5p and miR-141-3p expressions could significantly inhibit the volume and weight of subcutaneous tumors in nude mice, while co-transfection of miR-188-5p and miR-141-3p with half-dose down-regulated expressions made the tumors much smaller and lighter.

Patients And Specimen

All experiments were approved by the Ethics Committee of the Fourth Affiliated Hospital of China Medical University and Informed Consents about the process and purpose of sampling were signed by patients. A total of 44 pairs of BC and adjacent normal urothelial tissues specimens were taken from invasive urothelial carcinoma patients who had received radical cystectomy in The Fourth Affiliated Hospital of China Medical University between March 2016 and September 2017 (Table 1). Patients who had received other non-surgical treatment before were not included in this experiment. In addition, patients with other non-urinary diseases, postoperative recurrence and incomplete information were also excluded.

Table 1 Clinical information of all patients

Characteristics	Data
Tissue pair	44
Age (years)	68（48-85）
Gender	
Male	40（91%）
Female	4（9%）
Relative expression of miR-188-5p	
Low	22
High	22
Relative expression of miR-141-3p	
Low	20
High	24
Follow-up （months）	65
Death	31

Cell culture and transfection

Human BC cell line 5637 was bought from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). 5637 cells were cultured in medium with 10% virus inactivated fetal bovine serum (FBS; Biological Industries, Cromwell, CT, USA) and placed at 37°C in an incubator with 5% CO_2. Follow-up cell experiments were carried out when cell fusion reached 60%. MiR-188-5p mimics, miR-188-5p inhibitor, miR-141-3p mimics, miR-141-3p inhibitor, inhibitor negative control (NC) and the Phosphatase and Tensin Homolog small interfering RNA (si-PTEN) were purchased from GenePharma (Shanghai, China). 5637 cells were then transfected transiently with the above reagents and Lipofectamine® 3000 (Invitrogen, New York, USA) according to manufacturer's instruction. Transfected cells were analyzed after 48h. Untransfected cells were set as the control group. The primer sequence is shown in Table 2.

Table 2 Sequences of all Primers

Items	Sequence
PTEN F	GACCATAACCCACCACAGC
PTEN R	ACCAGTTCGTCCCTTTCC
U6 F	GCTTCGGCAGCACATATACT
U6 R	GTGCAGGGTCCGAGGTATTC
hsa-miR-188-5p F	GCG CAT CCC TTG CAT GGT
hsa-miR-188-5p R	AGT GCA GGG TCC GAG GTA TT
hsa-miR-188-5p RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCTCC
hsa-miR-141-3p F	CGTCGCTAACACTGTCTGGTAA
hsa-miR-141-3p R	GTGCAGGGTCCGAGGTATTC
hsa-miR-141-3p RT Primer	GTTGGCTCTGGTGCAGGGTCCGAG GTATTCGCACCAGAGCCAACCCATCT
miR-188-5p mimics	CAUCCCUUGCAUGGUGGAGGG CUCCACCAUGCAAGGGAUGUU
miR-188-5p inhibitor	CCCUCCACCAUGCAAGGGAUG
miR-141-3p mimics	UAACACUGUCUGGUAAAGAUGG AUCUUUACCAGACAGUGUUAUU
miR-141-3p inhibitor	CCAUCUUUACCAGACAGUGUUA
inhibitor NC	UUCUCCGAGUGUC ACGUTT

5637 cells were incubated for 48 hours after transfected with lentivirus. After collection, the transfected cells were added to RIPM-1640 complete medium (Gibco, Carlsbad, CA, USA) mixed with 1 mg-/ml puromycin. The same medium was replaced every two days, and then cell lines with stable expression were screened out for further experiment.

Timed quantitative reverse transcription qRT-PCR

According to the manufacturer's instruction, total RNA was extracted from BC tissues and cells using Trizol reagent (Invitrogen, NY, USA). Stem loop primers from TaqMan™ MicroRNA Reverse Transcription Kit were used to reversely-transcribe total RNA into cDNA. A total of 20μl PCR reaction mixture containing SYBR Premix EX Taq II (TaKaRa, Dalian, China) was prepared on ice. PCR amplification reaction was carried out on ABI7500 Real-Time PCR Amplifier to perform quantitative analysis and make standard curve. All samples were added to 3 wells to reduce error. Data were standardized with U6 as the internal

reference gene and 2-ΔΔCT value was introduced to evaluate the expression difference of mRNAs of each group.

CCK8 assay

CCK8 assay was performed to evaluate cell proliferation ability. Evenly mixed 5637 cell suspension was seeded into 96 well plate with a density of 3×10^3 cells per well. 10μl of 10% CCK8 solution (CCK-8; Dojindo, Kumamoto, Japan) was added to each well. The 96 well plate had been incubated for 1h at 37℃ before the absorbance at 450nm wavelength was measured on microplate reader (Multiskan; Devices, Menlo Park, CA, USA).

Wound healing assay

Wound healing assay was conducted to compare the difference of cell migration ability. Transfected 5637 cells were seeded into 6 well plate and cultured to monolayer fused cells. Cells were scratched by 200μl pipette tip and washed three times with PBS before pure culture medium was added. After 24h incubation, cells in each group were photographed respectively by phase contrast microscope.

TransWell assay

TransWell assay was performed to investigate cell invasiveness. TransWell chambers (BD Biosciences, Franklin Lakes, NJ, USA) were coated with Matrigel (BD Biosciences). Transfected 5637 cells were transferred to the upper chamber with a density of 1.5×10^4 per well. Chambers were placed in 24 well plate after 200μl pure medium had been added. A total of 500μl culture solution with 20% FBS was added to lower chambers. The medium was removed after 24h of incubation and chambers were washed twice with PBS. Cells were stained with 0.4% crystal violet (MedChem Express, Shanghai, China) and then washed with distilled water. Cells which had invaded to lower chambers were counted and photographed by 200-fold inverted microscope.

Western Blot assay

Buffer solution (Beyotime, China) with protease inhibitor was prepared according to manufacturer's instruction. Protein was extracted and the protein concentration was measured by BCA Protein Assay Kit (Beyotime, China). Polyacrylamide gel with 10% SDS was used as carrier and protein molecules were separated by electrophoretic separation. The protein was transferred to PVDF membrane (Millipore, Billerica, MA, USA). The membrane was incubated with primary antibodies and secondary antibodies, and fluoresced by ECL (echochemiluminescence) detection system. Thereafter, protein bands were analyzed by gel image processing system (Gel-Pro-Aanlyzer software). The following antibodies were used in the protein-related studies of this assay: primary antibodies, including PTEN antibodies (ab32199), AKT antibodies (ab179463), anti-pAKT antibodies (ab38449) and anti-c-MYC antibodies (ab32072), were diluted 1:500; secondary antibody was goat anti-rabbit IgG-HRP (ab6721, 1:5000); internal reference antibody was GAPDH (ab181602, 1:5000). All antibodies were purchased from Abcam.

Statistical analysis

All experiments were repeated three times and continuous variables in experiments were represented by mean value(±SD). The difference between two groups was represented by independent sample Student T obtained from GraphPad. P value was calculated by one-way analysis of variance (ANOVA). A p value less than 0.05 was considered to be statistically significant. Spearman rank test was performed to evaluate the relationship between miR-188-5p and PTEN expressions as well as the relationship between miR-141-3p and PTEN expressions.

Luciferase reporter assay

Luciferase reporter assay was carried out to investigate whether miR-188-5p and miR-141-3p combined with PTEN directly. Bioinformatics software Targetscan and miRDB were introduced to predict the binding sites of miR-188-5p, miR-141-3p and PTEN. The predicted miR-188-5p binding site (pmiR-3'UTR WT1) and miR-141-3p binding site (pmiR-3'UTR WT2, pmiR-3'UTR WT3) were mutated to generate mutant miR-188-5p binding site (MT1) and mutant miR-141-3p binding site (MT2, MT3). All mRNA 3' UTR of WT and MT human PTEN were inserted into pmirGLO dual luciferase reporter vector (Promega, Madison, WI, U.S.A.). 5636 cells (2×10^5) were seeded into 12 well plate and co-transfected with 1.5μg pmirGLO-PTEN UTR(WT, MT) and 75pmol/L miR-188-5p mimics or 75pmol/L miR-188-3p mimics or NC by Lipo3000 for 48h. 100μl Renilla luciferase detection reagent (AmyJet Scientific，Wuhan, China) was added for correction. Thereafter, microplate reader was used to detect fluorescence intensity.

Tumor formation in nude mice

All nude mice experiment had been approved by the Animal Research Committee of The Fourth Affiliated Hospital of China Medical University. Subcutaneous tumor formation experiment in nude mice was performed according to Animal Care and Use Committee's requirements. 5-week-old healthy female BALB/c nude mice were selected and divided into 4 groups: A: control group; B: miR-188-5p silent group; C: miR-141-3p silent group; D: miR-188-5p and miR-141-3p silent group. 1×10^7/ml cell suspension was injected into the armpit of mice in each group. Tumor volume was measured every 5 days after the tumor had reached $100mm^3$. This experiment lasted for 30 days after which the mice were euthanized. Tumor tissue was cut out and weighted, and a growth curve was drawn.

Results

Down regulation of miR-188-5p or miR-141-3p inhibits the volume and weight of subcutaneous tumor

In order to compare to the results of previous in vitro cell experiment, this study carried out an in vivo experiment which was the subcutaneous tumor formation in nude mice. Compared with the control group, down-regulating the expression of miR-188-5p or miR-141-3p significantly reduced the size of tumor, and ultimately reduced the weight of tumor. A combination of half dose down-regulated miR-188-5p and half dose down-regulated miR-141-3p had greater inhibiting effect on the volume and weight of

tumor than full dose down-regulation of each respective miR (Fig. 1A and B). In vivo, down-regulating miR-188-5p or miR-141-3p also inhibited BC progress, and a combination of half dose down-regulation of both miRs demonstrated a more obvious inhibiting effect than full dose down-regulation of each respective miR. This indicated that both miR-188-5p and miR-141-3p promoted BC progress and the two miRs worked together to demonstrate a synergistic effect.

PTEN is a common target protein of miR-188-5p and miR-141-3p

Bioinformatics software Targetscan and miRDB were introduced to predict the target genes of miR-188-5p and miR-141-3p. Venn diagram was made to obtain the target gene intersection of the two miRNAs predicted by the two software. Results suggested that miR-188-5p and miR-141-3p had 13 potential common target genes (Fig. 2) among which PTEN was selected in our experiment. Expression level of PTEN mRNA in tissues was verified by qRT-PCR. Results manifested that the expression level of PTEN mRNA in BC tissues was lower than that in adjacent tissues (Fig. 3A). Correlation study showed that the expression levels of miR-188-5p and miR-141-3p were negatively correlated with the expression level of PTEN mRNA (Fig. 3B and 3C). Western Blot assay and luciferase reporter assay were performed to investigate whether miR-188-5p and miR-141-3p were combined with the 3'-UTR of PTEN.

Western Blot assay: 5637 cells were transfected with miR-188-5p inhibitor, or miR-141-3p inhibitor, or the combination of half dose miR-188-5p inhibitor and half dose miR-141-3p inhibitor. Compared with control group, expression levels of PTEN protein in transfected cells increased significantly, among which the PTEN expression level of cells transfected with a combination of half dose miR-188-5p inhibitor and half dose miR-141-3p inhibitor marked the highest increase (Fig. 3D). Result showed that both miR-188-5p and miR-141-3p inhibited the expression of PTEN protein, and the two miRs worked together to inhibit the expression of PTEN protein synergistically.

Luciferase reporter assay: binding sites of miR-188-5p and PTEN or miR-141-3p and PTEN were predicted by Targetscan (Fig. 3E). Normal 3'-UTR of PTEN and mutant 3'-UTR of PTEN were inserted into the downstream of pmirGLO dual-luciferase miRNA target expression vector respectively to generate luciferase reporters consisting of wild-type (WT) or mutant (MU) 3'-UTR. Luciferase activity was significantly inhibited in 5637 cells co-transfected with miR-188-5p mimic and WT vector, or miR-141-3p mimic and WT vector (Fig. 3F). However, in 5637 cells co-transfected with miR-188-5p mimic and MU vector or miR-141-3p mimic and MU vector, luciferase activity was not affected (Fig. 3G). Above results showed that down-regulating expressions of miR-188-5p and miR-141-3p increased PTEN expression. Half dose co-transfection of miR-188-5p and miR-141-3p had a synergistic effect in up-regulating PTEN expression. MiR-188-5p and miR-141-3p can inhibit the expression of PTEN synergistically. 3'-UTR of PTEN had 1 binding site with miR-188-5p and 2 binding sites with miR-141-3p. PTEN can be combined with miR-188-5p and miR-141-3p.

1. (A) qRT-PCR manifested that the expression level of PTEN mRNA in BC tissues was lower than that in adjacent tissues(****P < 0.00001).
2. (B) and (C) correlation study showed that the expression levels of miR-188-5p and miR-141-3p were negatively correlated with the expression level of PTEN mRNA.
3. (D) In Western Blot assay, expression levels of PTEN protein in transfected cells increased significantly, among which the PTEN expression level of cells transfected with a combination of half dose miR-188-5p inhibitor and half dose miR-141-3p inhibitor marked the highest increase.
4. (E) Binding sites of miR-188-5p and PTEN or miR-141-3p and PTEN were predicted by Targetscan.
5. (F) Luciferase activity was significantly inhibited in 5637 cells co-transfected with miR-188-5p mimic and WT vector, or miR-141-3p mimic and WT vector(****P < 0.00001).
6. (G) In 5637 cells co-transfected with miR-188-5p mimic and MU vector or miR-141-3p mimic and MU vector, luciferase activity was not affected.

MiR-188-5p and miR-141-3p promote the progress of BC synergistically by targeting PTEN

MiR-188-5p silent lentivirus and miR-141-3p silent lentivirus were prepared to transfect BC cells respectively to get miR-188-5p silent cell line and miR-141-3p silent cell line. A combination of half dose miR-188-5p silent lentivirus and half dose miR-141-3p silent lentivirus were used to co-transfect BC cells to obtain miR-188-5p and miR-141-3p combined silent cell line. PTEN siRNA-1/2/3 fragment and NC were designed and synthesized based on PTEN gene sequence information. PTEN siRNA-2 with the best interference effect was screened out through qRT-PCR (Fig. 4A) to transfect the control silent cell line, miR-188-5p silent cell line, miR-141-3p silent cell line as well as miR-188-5p and miR-141-3p combined half dose silent cell line.

Western Blot assay indicated that compared with control group, PTEN protein expression level of miR-188-5p silent cell line and miR-141-3p silent cell line which had not been transfected by siPTEN rose significantly, and the PTEN protein expression level of miR-188-5p and miR-141-3p combined half dose silent cell line also rose. The later demonstrated a more obvious rising trend. PTEN protein levels of siPTEN-transfected miR-188-5p silent cell line, miR-141-3p silent cell line and the miR-188-5p and miR-141-3p combined half dose silent cell line decreased significantly compared with the group with untransfected cell lines (Fig. 4B).

CCK8 assay manifested that siPTEN transfection can decrease the expression of PTEN, thus increasing BC cell proliferation and reversing low expression miR's inhibition on BC cell proliferation (Fig. 4C). Wound healing assay indicated that siPTEN transfection can decrease PTEN expression, thus increasing BC cell migration and recovering low expression miR's inhibition on BC cell migration (Fig. 4D). Transwell assay showed that siPTEN transfection can reduce PTEN expression, thus increasing BC cell invasion and antagonizing low expression miR's inhibition on BC cell invasion (Fig. 4E). Therefore, we concluded that down-regulated PTEN expression can reverse the two tumor-promoting miRs' inhibition on BC progress, and low expression PTEN promoted BC progress. We also found that PTEN's functioning mechanism was negatively related to that of miR-188-5p and miR-141-3p, which indicated that PTEN

inhibited BC progress. MiR-188-5p or miR-141-3p promoted BC progress by directly targeting PTEN. The two miRs also have synergistic effect in promoting BC progress by directly targeting PTEN.

1. PTEN siRNA-2 with the best interference effect was screened out through qRT-PCR (***P < 0.0001).
2. PTEN protein levels of siPTEN-transfected miR-188-5p silent cell line, miR-141-3p silent cell line and the miR-188-5p and miR-141-3p combined half dose silent cell line decreased significantly compared with the group with untransfected cell lines.
3. SiPTEN transfection can decrease the expression of PTEN, thus increasing BC cell proliferation and reversing low expression miR's inhibition on BC cell proliferation.
4. SiPTEN transfection can decrease PTEN expression, thus increasing BC cell migration and recovering low expression miR's inhibition on BC cell migration.
5. Transwell assay showed that siPTEN transfection can reduce PTEN expression, thus increasing BC cell invasion and antagonizing low expression miR's inhibition on BC cell invasion.

MiR-188-5p and miR-141-3p promote BC progress by targeting PTEN to activate AKT/c-MYC pathway

AKT and c-MYC are common effect proteins of PTEN. While detecting the expression of PTEN, Western Blot also detected the expression of AKT, p-AKT and c-MYC.

MiR-188-5p silent cell line, miR-141-3p silent cell line and the miR-188-5p and miR-141-3p combined half dose silent cell line were transfected with siPTEN. Western Blot manifested that the expression levels of PTEN protein in all groups were lower than that of the control group (Fig. 4B), but the expression levels of AKT protein in all experiment groups showed no obvious change compared with that of the control group (Fig. 5A). The p-AKT expression levels and c-MYC expression levels of experiment groups were higher than that of the control group (Fig. 5B and 5C) and p-AKT accounted for an increasing proportion. Results suggested that down-regulated PTEN promoted the phosphorylation of AKT protein and increased the expression of c-MYC protein. This indicated that miR-188-5p and miR-141-3p reduced PTEN to activate AKT/c-MYC pathway, thus to promote BC progress.

Discussion

BC is one of the most commonly diagnosed urinary tumors, which has always been a serious threat to people's health. BC is classified into non-muscle invasive BC and muscle invasive BC. Although muscle invasive BC can be treated by surgeries and chemotherapy, the 5-year survival rate of muscle invasive BC has not been significantly improved[21–23]. Targeted therapy and chemotherapy are still the main clinical treatment strategies for advanced BC. Therefore, the screening of new and more effective therapeutic targets becomes a major focus of current studies[24]. While searching for new therapeutic targets, it has been found that miRNAs can regulate coding protein genes and play a regulatory role in the occurrence, development and metastasis of malignant tumors. At present, a variety of miRNAs in the tissue, blood or urine samples of BC patients have been identified to have diagnostic and prognostic functions [25, 26]. Similar results have been found in our previous experiment that miR-188-5p and miR-

141-3p demonstrate high expressions in BC tissues and cells, and they can promote BC progress and promote the prognosis of patients synergistically.

In this study, further in vivo experiments were carried out to perform subcutaneous tumor formation in nude mice after stable lentiviral transfection. Results showed that down-regulated miR-188-5p or miR-141-3p significantly reduced the size and weight of tumor. A combination of half dose down-regulated miR-188-5p and half dose down-regulated miR-141-3p inhibited the growth of tumor more observably. The experiments further confirmed that in BC, miR-188-5p and miR-141-3p were tumor-promoting genes which can reversely regulate tumor-suppressing genes, and the two miRs had the biological function of synergistically promoting BC progress.

Although a few people believe that miRNAs can stimulate the translation of target gene through combining mRNAs' 5' untranslated regions (UTRs) and open reading frames (ORFs)[27, 28], more researchers have proved that miRNAs' 5' UTR is partially or completely complementary to mRNAs' 3' UTR, thus to inhibit the translation of target genes or induce the degradation of target genes [29]. MiRNAs perform their biological functions through the negative regulation of target genes[30–32]. Based on this theory, we further predicted and studied the target genes. Firstly, potential target genes of miR-188-5p and miR-141-3p were predicted by TargetScan and miRDB. Secondly, intersections of the four target gene sets were taken and 13 common target genes were obtained, including PTEN.

PTEN is a traditional tumor suppressor gene which mutates frequently in a variety of cancers. PTEN mutations are closely related to high-level BC [33, 34]. Therefore, we selected PTEN as the target gene for further study. First, qRT-PCR verified that in BC tissues PTEN showed a low expression negatively correlated with expressions of miR-188-5p and miR-141-3p, suggesting that there is a negative regulation relationship between PTEN and miR-188-5p as well as PTEN and miR-141-3p. Second, WB assay manifested that PTEN expression rose significantly when miR-188-5p expression or miR-141-3p expression was down-regulated respectively through transfection in 5637 cells, while half dose down regulation of the combination of the two miRs showed a much greater inhibition on PTEN expression. Therefore, we conclude that miR-188-5p or miR-141-3p inhibits the expression of PTEN, and the two miRs also work synergistically to inhibit PTEN expression. Third, dual luciferase reporter assay verified that miR-188-5p or miR-141-3p can combine with 3'-UTR of PTEN. Accordingly, we conclude that PTEN is the common target gene of miR-188-5p and miR-141-3p, and the two miRs inhibit PTEN expression synergistically.

Following researches indicated that down regulation of PTEN can improve the proliferation, migration and invasion of BC cells, which means that down-regulated PTEN is a tumor suppressing gene in BC. Further experiments showed when PTEN was down-regulated together with a full dose down regulation of each miR or half dose down regulation of the combination of the two miRs, the two miRs' inhabitation on BC cell's proliferation, migration and invasion ability was reversed. The low expression of PTEN had a biological effect which was similar to the high expression of miR. As a target gene, PTEN played a

regulatory role between the two miRs and BC. To sum up, it is concluded that miR-188-5p and miR-141-3p promote BC progress synergistically by inhibiting PTEN expression.

Further pathway analysis indicated that down-regulated PTEN expression had no significant effect on the expression of AKT protein, but increased the pAKT protein expression, c-MYC protein expression and the ration of pAkt/Akt, suggesting that down-regulated PTEN expression promoted AKT phosphorylation, activated AKT and increased pAKT expression and c-MYC expression.

AKT regulates cell growth, proliferation, activity and many other processes[35]. AKT activity is strictly restricted under normal physiological conditions. However, AKT is often abnormally activated in many cancers[36, 37]. AKT mutation characterized by AKT overactivation can promote the occurrence and development of BC[38].

C-MYC is one of the three MYC genes which participates in various physiological processes, such as embryonic development, cell cycle, cell proliferation, cell apoptosis and protein synthesis[39]. C-MYC amplifies abnormally in BC and many other cancers, which makes it one of the oncogenes[40].

Therefore, miR-188-5p and miR-141-3p promote BC synergistically via inhibiting PTEN. AKT/c-MYC signal pathway is activated to promote AKT phosphorylation and BC cell proliferation, thus inhibiting the apoptosis of BC cells.

In conclusion, PTEN is the common target gene of miR-188-5p and miR-141-3p. Through in vivo and in vitro experiments, it has been found for the first time that miR-188-5p and miR-141-3p promote BC progress synergistically by inhibiting PTEN to activate AKT/c-MYC pathway.

This study manifested that miR-188-5p and miR-141-3p can act on a common target before acting on the same pathway, thus having a synergistic effect on promoting the development of BC. This is a further exploration about the complex molecular mechanism of miRs' regulation on BC. Although multiple miRs increase the workload and difficulty of research, the results are gratifying. It will be more effective to predict the occurrence and progress of BC by monitoring expressions of the two miRs. New medicines with combined effects of the two miRs would become a better choice.

Abbreviations

BC: bladder cancer

NC: negative control

Declarations

Ethics approval and consent to participate

The experimental protocol was established. All experiments were approved by the Ethics Committee of the Fourth Affiliated Hospital of China Medical University and Informed Consents about the process and purpose of sampling were signed by patients.

Consent for publication

The authors confirm that this work has not been published anywhere before. It is not under consideration for publication elsewhere and its publication in your journal has been approved by all authors.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no financial or non-financial competing interests.

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Authors' contributions

Dr. Xianxu YANG was responsible for experimental design and data collection. He prepared all figures and wrote the main manuscript. Mr. Zongze LI collected data in experiment and edited the manuscript. Both Dr. Xianxu YANG and Mr. Zongze LI reviewed the manuscript.

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Figures

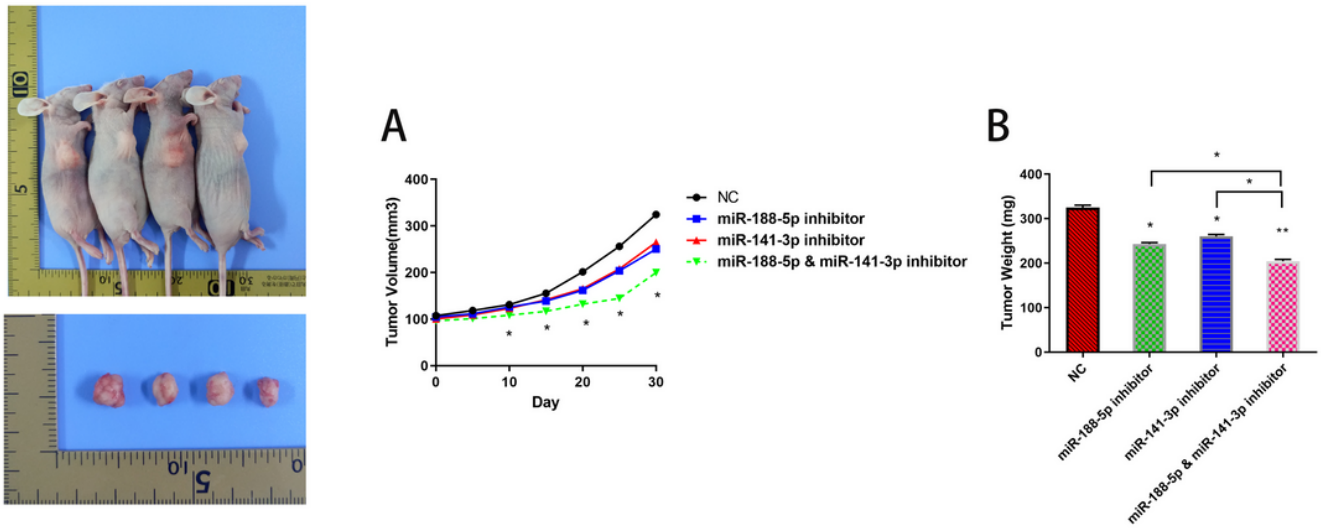

Figure 1

Down-regulating the expression of miR-188-5p or miR-141-3p significantly reduced the size and weight of tumor. (A) The size of tumor was reduced with the down regulation of miR-188-5p and miR-141-3p expressions (*P < 0.05). (B) The weight of tumor was reduced with the down regulation of miR-188-5p and miR-141-3p expressions (*P < 0.05, **P < 0.01).

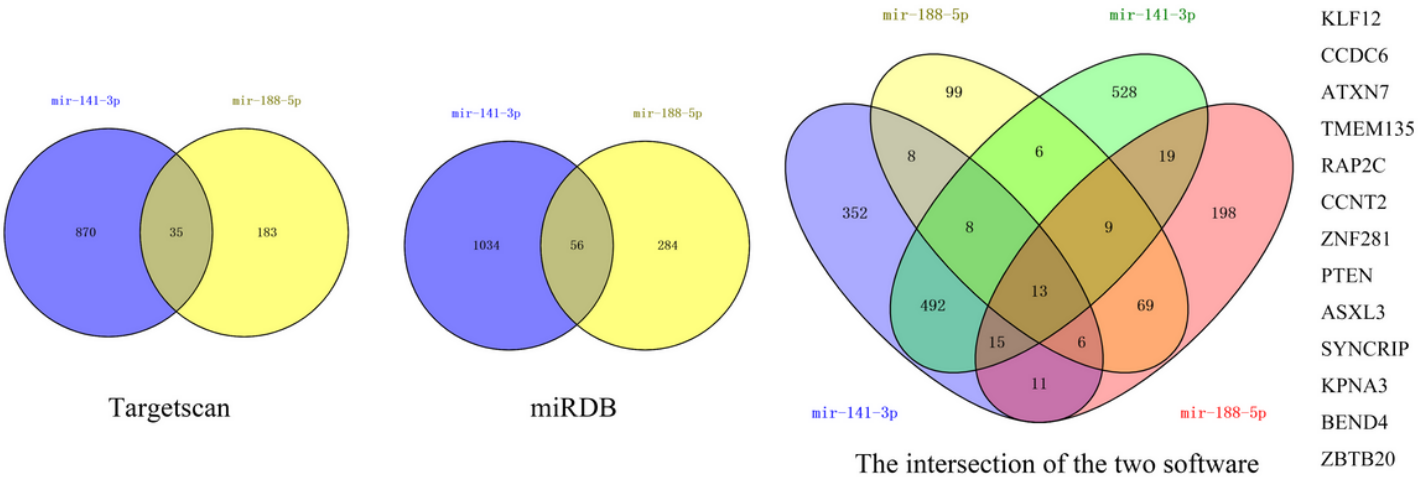

Figure 2

Bioinformatics software Targetscan and miRDB were introduced to predict the target genes of miR-188-5p and miR-141-3p. Results suggested that miR-188-5p and miR-141-3p had 13 potential common target genes including PTEN.

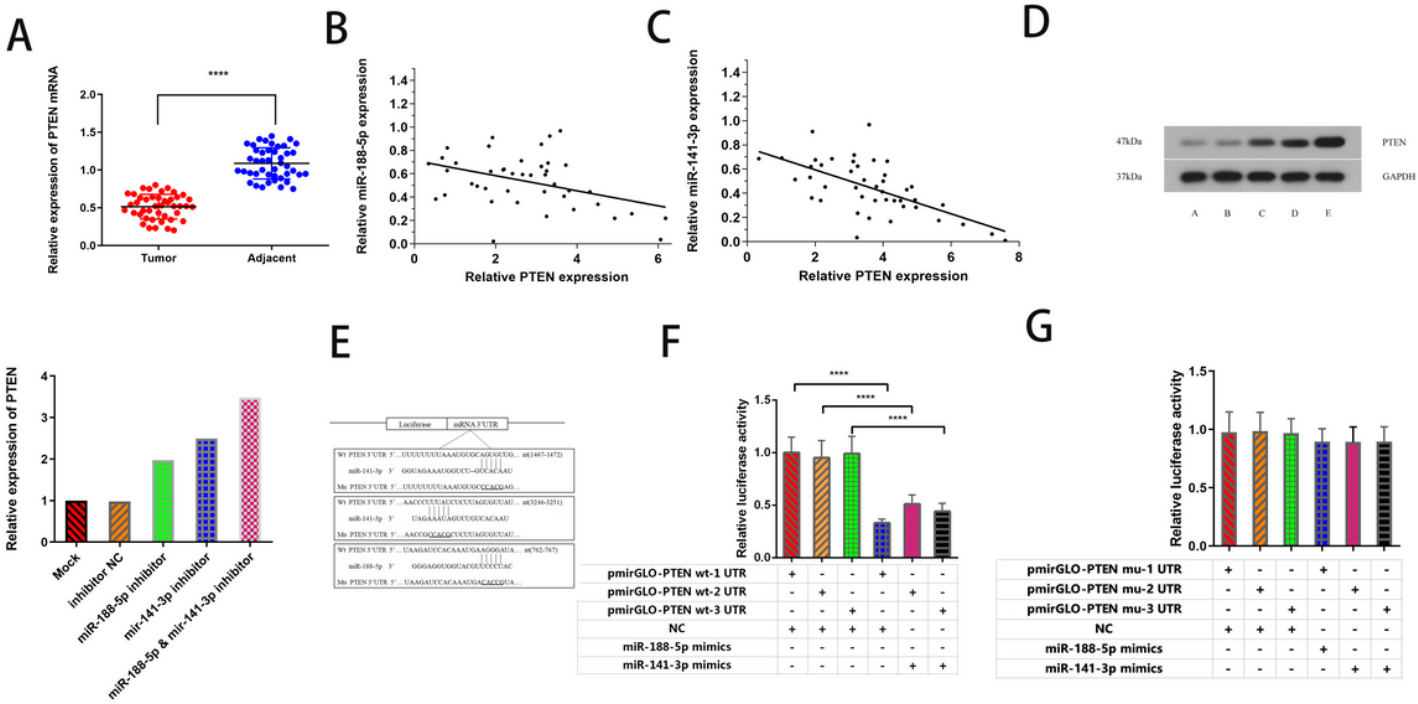

Figure 3

qRT-PCR, correlation study, Western Blot assay and luciferase reporter assay were conducted to verify PTEN as a common target gene of miR-188-5p and miR-141-3p. (A) qRT-PCR manifested that the expression level of PTEN mRNA in BC tissues was lower than that in adjacent tissues（****P < 0.00001）. (B) and (C) correlation study showed that the expression levels of miR-188-5p and miR-141-3p were negatively correlated with the expression level of PTEN mRNA. (D) In Western Blot assay, expression levels of PTEN protein in transfected cells increased significantly, among which the PTEN expression level of cells transfected with a combination of half dose miR-188-5p inhibitor and half dose miR-141-3p inhibitor marked the highest increase. (E) Binding sites of miR-188-5p and PTEN or miR-141-3p and PTEN were predicted by Targetscan.

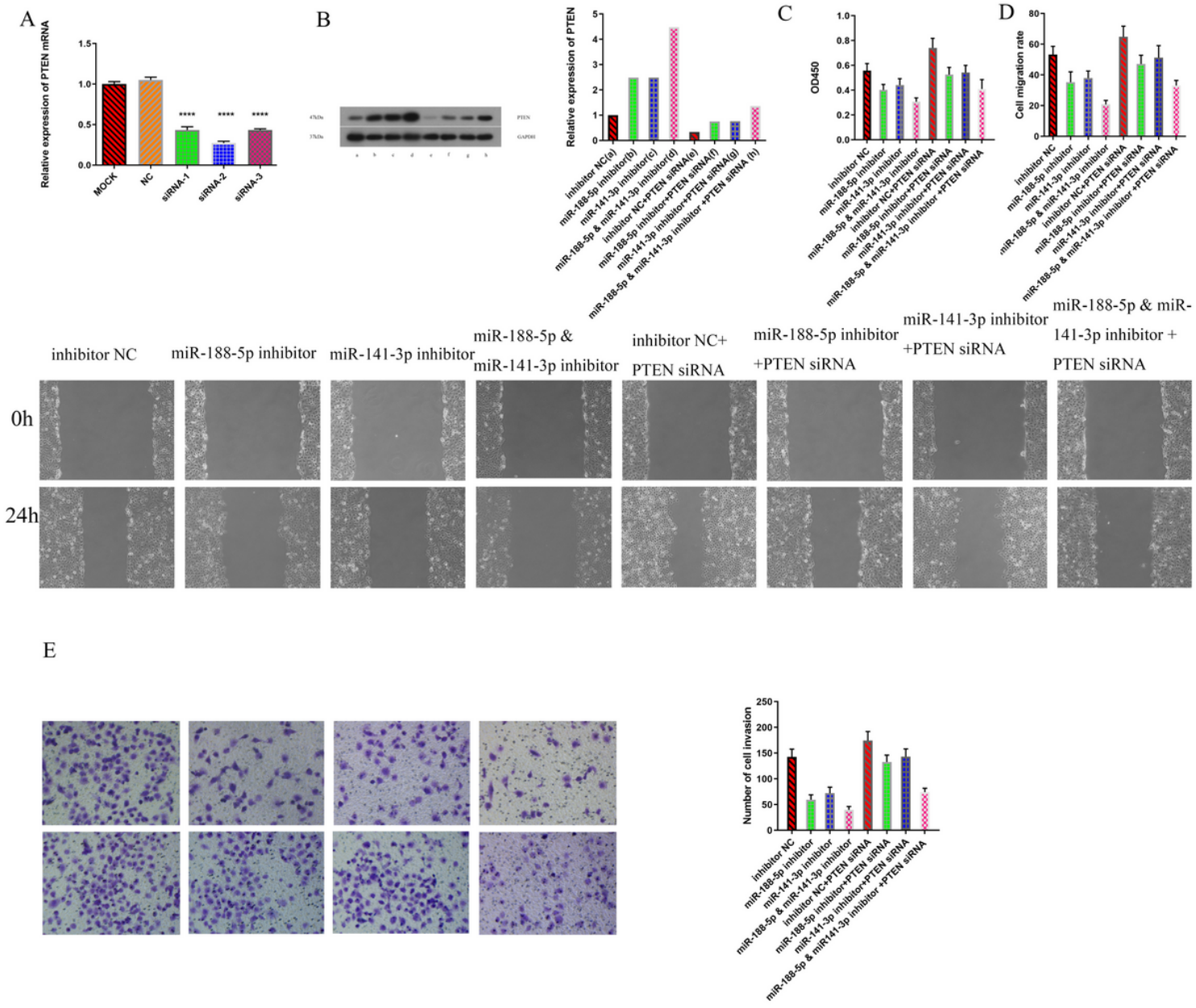

Figure 4

qRT-PCR, Western Blot assay、CCK8 assay、Wound Healing assay and Transwell assay were conducted to clarify the function of the common target gene PTEN. (A) PTEN siRNA-2 with the best interference effect

was screened out through qRT-PCR (***P < 0.0001). (B) PTEN protein levels of siPTEN-transfected miR-188-5p silent cell line, miR-141-3p silent cell line and the miR-188-5p and miR-141-3p combined half dose silent cell line decreased significantly compared with the group with untransfected cell lines. (C) SiPTEN transfection can decrease the expression of PTEN, thus increasing BC cell proliferation and reversing low expression miR's inhibition on BC cell proliferation. (D) SiPTEN transfection can decrease PTEN expression, thus increasing BC cell migration and recovering low expression miR's inhibition on BC cell migration. (E) Transwell assay showed that siPTEN transfection can reduce PTEN expression, thus increasing BC cell invasion and antagonizing low expression miR's inhibition on BC cell invasion.

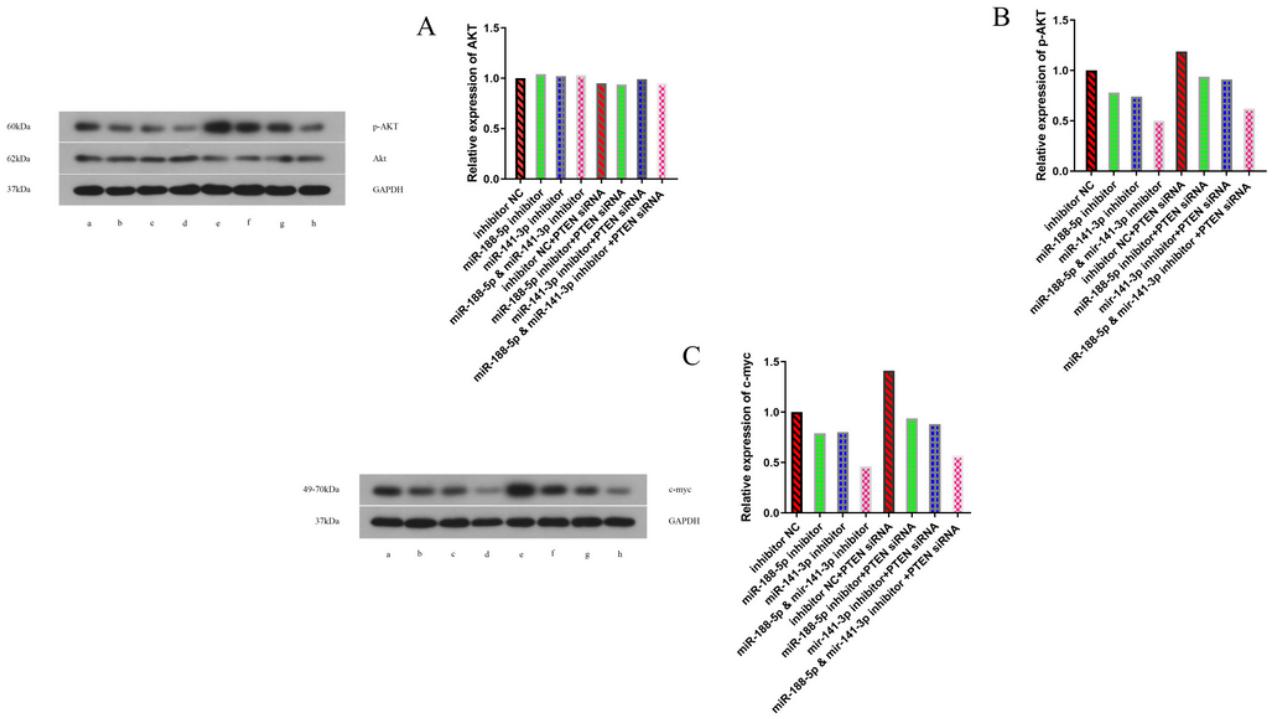

Figure 5

Verification of downstream pathway genes AKT and c-MYC. (A) SiPTEN transfection decreased PTEN expression, but the expression levels of AKT protein in all experiment groups showed no obvious change compared with that of the control group. (B) SiPTEN transfection decreased PTEN expression, the p-AKT expression levels of experiment groups were higher than that of the control group. (C) SiPTEN transfection decreased PTEN expression, the c-MYC expression levels of experiment groups were higher than that of the control group.