Pathology - Research and Practice 215 (2019) 152598

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

# MiR-188-5p and MiR-141-3p influence prognosis of bladder cancer and promote bladder cancer synergistically

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ARTICLE INFO

*Keywords:*
Bladder cancer
miR-188-5p
miR-141-3p
Prognosis

ABSTRACT

MicroRNA (miRNA) plays a significant role in suppressing the occurrence and development of tumor by inhibiting the translation of target proteins. Although previous researches have verified many miRNAs' functions in bladder cancer (BC), the function of miR-188-5p and miR-141-3p in BC still remains unknown. Our experiment manifested that miR-188-5p and miR-141-3p were highly expressed in BC tissues and cells, which indicated a poor prognosis. In vitro functional assays suggested that down-regulated miR-188-5p and miR-141-3p inhibited the proliferation, migration and invasion of BC cells, while a combination of half dose down-regulated miR-188-5p and half dose down-regulated miR-141-3p demonstrated a more obvious inhibition effect. All results indicated that miR-188-5p and miR-141-3p promoted BC respectively and synergistically. Therefore, miR-188-5p and miR-141-3p will not only assist the diagnosis of BC, but also serve as more effective joint markers to predict the progression of BC.

## 1. Introduction

Bladder cancer (BC) is the most frequently seen urinary tumor. Worldwide, BC ranks 7$^{th}$ among the most frequently diagnosed malignancies of male and 17$^{th}$ among that of female [1]. In Europe, age-standardized incidence rates of BC of male and female are 19.1/100,000 and 4.0/100,000 respectively, while morbidity and mortality rate of BC vary according to changing risk factors [2]. BC is classified into non-muscle invasive BC and muscle invasive BC. 30% of BC is muscle invasive and 70% is non-muscle invasive which may further develop into muscle invasive and eventually cause death [3]. Although doctors use radical surgery and chemoradiotherapy combined with TURBT (transurethral resection of bladder tumor) as major treatments for muscle invasive BC patients, the five-year mortality rate of such patients still reaches up to 60% [4]. Therefore, a "new weapon" which can reverse BC oncogene is in urgent need to fight against this malignancy.

MicroRNA (miRNA) is an endogenous RNA of non-coding protein. It is a single stranded RNA that consists of 19–23 nucleotides. MiRNAs, with a variety of regulatory functions in cells, can inhabit the post-transcriptional expression of proteins [5]. MiRNAs play a significant role in tumor cell proliferation, differentiation, apoptosis and other physiological processes [6]. Many researchers suggested that miRNAs promoted or inhibited the development of BC [7,8]. Current studies have proved that miR-188-5p presents low expression in gastric cancer, liver cancer and oral squamous cell carcinoma and functions as tumor inhibitor [9–11]. MiR-141-3p demonstrates low expression and functions as tumor inhibitor in malignant gliomas and papillary thyroid carcinoma [12,13], while presenting high expression and functioning as tumor promoter in cervical cancer and prostate cancer [14,15]. In view of previous studies, little work has been performed on the mechanism of single or combined action of miR-188-5p and miR-141-3p. Therefore, this research will further investigate whether miR-188-5p and miR-141-3p play a role in the development and progression of BC as well as their way of function.

Firstly, it was detected that both miR-188-5p and miR-141-3p demonstrated high expressions in BC tissues and cells which indicated a poor prognosis of patients. Secondly, this study verified that miR-188-5p and miR-141-3p were cancer promoters which promoted the proliferation, migration and invasion of BC. They worked both respectively and synergistically to promote BC progression.

*Abbreviations:* miRNA, microRNA; BC, bladder cancer; TURBT, transurethral resection of bladder tumor; FBS, fetal bovine serum; qRT-PCR, timed quantitative reserve transcription; PBS, phosphate-buffered saline; ANOVA, one-way analysis of variance; NC, negative control

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https://doi.org/10.1016/j.prp.2019.152598
Received 8 June 2019; Received in revised form 28 July 2019; Accepted 16 August 2019

**Table 1**
MiRNA expressions and overall survival rates of 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 |

**Table 2**
Sequences of all Primers.

| Primers Sequence (5'-3') | |
|---|---|
| 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 | GTCGTATCCAGTGCAGGGTCCGAGGT |
| RT Primer | ATTCGCACTGGATACGACCCCTCC |
| hsa-miR-141-3p F | CGTCGCTAACACTGTCTGGTAA |
| hsa-miR-141-3p R | GTGCAGGGTCCGAGGTATTC |
| hsa-miR-141-3p | GTTGGCTCTGGTGCAGGGTCCGAG |
| RT Primer | GTATTCGCACCAGAGCCAACCCATCT |
| miR-188-5p inhibitor | CCCUCCACCAUGCAAGGGAUG |
| miR-141-3p inhibitor | CCAUCUUUACCAGACAGUGUUA |
| inhibitor NC | UUCUCCGAGUGUC ACGUTT |

Abbreviation: NC-negative control.

## 2. Materials and methods

### 2.1. Tissue specimens

This experiment was approved by the Ethics Committee of The Fourth Affiliated Hospital of China Medical University. Informed Consents had been signed with patients before specimens were taken. A total of 44 pairs of BC and adjacent normal urothelial tissues specimens were obtained 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 received preoperative or postoperative radiotherapy, chemotherapy and other non-surgical treatment were excluded from this experiment. Besides, patients with incomplete information, tumor recurrence or underlying diseases of other systems were also excluded. All specimens were frozen in liquid nitrogen immediately after taken.

### 2.2. Cell culture and transfection

Human BC cell lines 5637, T24, BIU87 and SV-HUC-1 were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). 5637, T24 and BIU87 cell lines were cultured in RMMI 1640 medium (Gibco, Carlsbad, CA, USA) mixed with 10% virus inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 100 U/ml streptomycin. SV-HUC-1 cell line was cultured in 20%F-12K medium (Sigma, Munich, Germany). All medium was placed at 37 °C in incubator with 5% $CO_2$. Follow-up experiments were performed when cell fusion reached 60%. MiR-188-5p inhibitor, miR-141-3p inhibitor, inhibitor negative control (NC) were synthesized by GenePharma (GenePharma, Shanghai, China). According to the manufacturer's instruction, the above primers were transfected transiently against 5637 cell line using Lipo3000 (Invitrogen, New York, USA) at a final concentration of 50 nM. Cell experiment was performed after 48 h. Untransfected cells were considered as a control group.

The primer sequence is shown in Table 2.

### 2.3. 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 and TaqMan MiRNA Reverse Transcription kit were used to reversely-transcribe 2 μg total RNA into cDNA (Applied Biosystems, Foster City, CA, USA). Samples were standardized with U6 as the internal reference. A 20 μl reaction system was built which included SYBR Premix EX Taq II (TaKaRa, Dalian, China) in each group. Thermal cycling and quantitative gene expression analysis were performed in the ABI PRISM 7500 timed quantitative fluorescence PCR System. 2-ΔΔCT value was introduced to evaluate expressions of miR-188-5p and miR-141-3p.

### 2.4. CCK8 assay

CCK8 assay was conducted to evaluate cell proliferation. 5637 Cells were seeded into 96 well plate with a density of $3 \times 10^3$ cells in each well. 100 μl of 10% CCK8 solution, which was made away from light, was added to each well. The 96 well plate was incubated for one hour at 37 °C before placed into microplate reader. Then the absorbance of 450 nm wavelength was measured on microplate reader respectively after 0, 24, 48 and 72 h of incubation.

### 2.5. Wound healing assay and TransWell assay

Wound healing assay was performed to investigate cell migration ability. Transfected 5637 cells were seeded in 6 well plate and cultured to a monolayer fusion state. Then serum-free medium was replaced. Cells in each group were scratched by 200 μl pipette tip, and the surfaces of cells were washed three times with phosphate-buffered saline (PBS). Then cells were observed and photographed by inverted microscope. After cultured for 24 h, cells in each group were photographed and recorded again. TransWell assay was conducted to investigate cell migration and invasion ability. TransWell chamber membrane in 24 well plate was coated with Matrigel. Transfected 5637 cells were transferred to the upper chamber with serum-free medium with a density of $1.5 \times 10^4$ per well. Culture solution with 20% FBS was added to lower chambers as chemotactic factor. The medium was removed after 24 h of incubation and chambers were washed twice with PBS. Cells were fixed with 4% paraformaldehyde at room temperature, stained with 0.4% crystal violet and washed with distilled water. Cells which had invaded to lower chambers were counted and photographed by 200-fold inverted microscope. Five fields of microscope were randomly captured for cell counting and the mean value was taken as the number of invasive cells in each well.

### 2.6. Statistical analysis

Each experiment procedure was repeated three times. All data were represented by mean value ( ± SD). Student *T* test for independent samples was performed with GraphPad. P-value was calculated with one-way analysis of variance (ANOVA). Patients' prognosis was analyzed using Kaplan–Meier. P values of less than 0.05 were statistically significant.

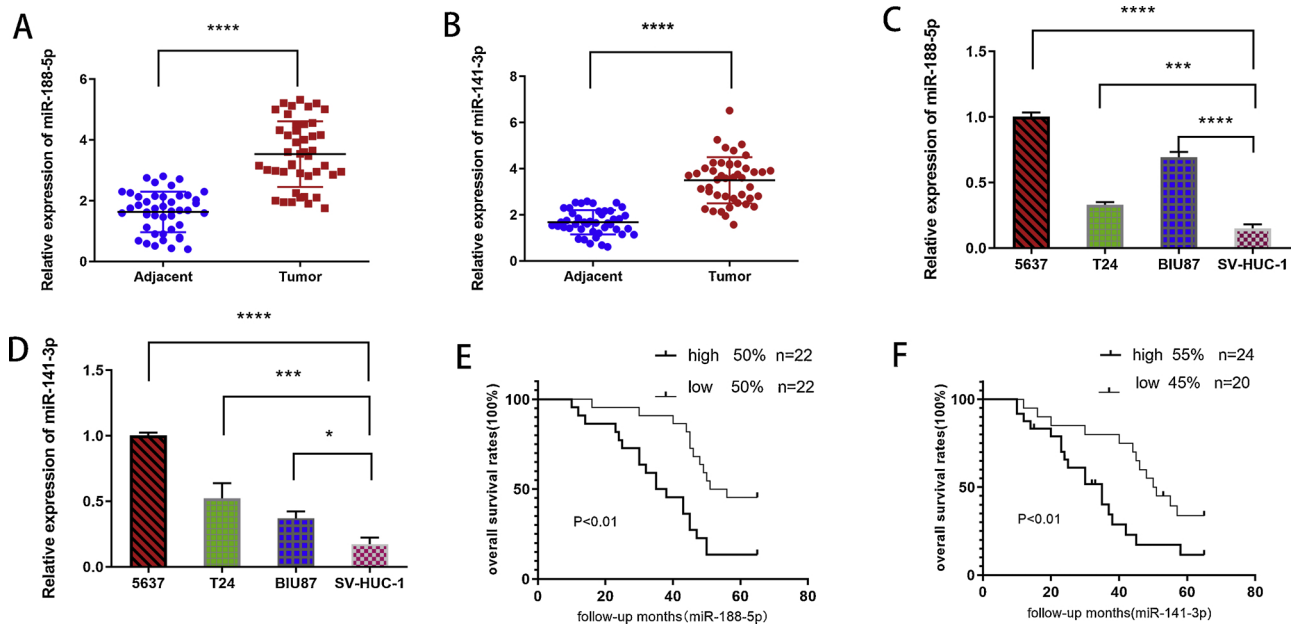


**Fig. 1.** MiR-188-5p and miR-141-3p are over-expressed in BC tissues and cells, which indicates a poor prognosis. Compared with normal adjacent tissues, (A) miR-188-5p presents higher expression in 44 pairs of BC tissues (****P < 0.00001), (B) miR-141-3p presents higher expression in 44 pairs of BC tissues (****P < 0.00001). Compared with normal cell line SV-HUC-1, (C) miR-188-5p is over-expressed in 5367, T24 and BIU87 cell lines (****P < 0.00001, ***P < 0.0001), (D) miR-141-3p is over-expressed in 5367, T24 and BIU87 cell lines (****P < 0.00001, ***P < 0.0001, *P < 0.05). Kaplan–Meier curve suggests that (E) patients with over-expressed miR-188-5p suffer worse prognosis (P < 0.01), and (F) patients with over-expressed miR-141-3p suffer worse prognosis (P < 0.01).

## 3. Results

### *3.1. MiR-188-5p and miR-141-3p presented high expression in BC cells, indicating a poor prognosis*

Expression levels of miR-188-5p and miR-141-3p in tissues and cells were detected by qRT-PCR. Results showed that compared with normal adjacent tissues, miR-188-5p and miR-141-3p expressions were significantly higher in BC tissues (Fig. 1A and B); compared with normal urothelial cells SV-HUC-1, miR-188-5p and miR-141-3p expressions were much higher in BC tissues (expression level of 5637 cells are highest) (Fig. 1C and D). According to the average values of miR-188-5p and miR-141-3p expressions, tissues were divided into high expression group and low expression group. Kaplan-Meier curve (Fig. 1E and F) showed that patients with higher miR-188-5p or miR-141-3p expression suffered a lower survival rate. It manifested that higher expressions of miR-188-5p or miR-141-3p indicated poor prognosis.

### *3.2. MiR-188-5p and miR-141-3p synergistically promoted the proliferation, migration and invasion of BC cells*

Since miR-188-5p and miR-141-3p presented high expressions in BC cells and tissues, inhibitory functional experiments were performed to investigate their biological functions (Fig. 2A and B). Two 5637 cells with the highest miRNA expressions were selected for in vitro cell experiment. CCK-8 experiment showed that down-regulated miR-188-5p and miR-141-3p significantly inhibited cell proliferation. The combination of half dose down-regulated miR-188-5p and half dose down-regulated miR-141-3p presented better inhibitory effect (Fig. 2C). Wound healing assay manifested that the migration ability of cells with reduced miR-188-5p or miR-141-3p expression was significantly lower than that of the control group, and the combination of the two miRNAs with half dose down-regulated expressions inhibited cell migration better than single miRNA with full low expression (Fig. 2D). TransWell assay indicated that cell invasion was significantly reduced when miR-188-5p or miR-141-3p expression was down-regulated, and the combination of half dose down-regulated miR-188-5p and half dose down-regulated miR-141-3p had better inhibitory effect on cell migration compared with single miRNA with full low expression (Fig. 2E). Thus, it can be seen that, in vitro, down-regulated expressions of miR-188-5p or miR-141-3p can inhibit the proliferation, migration and invasion of BC tissues and cells, and the combination of the two miRNAs with half dose down-regulated expressions had better inhibitory effect on BC than single miRNA. It follows that both miR-188-5p and miR-141-3p are tumor promoters which can promote BC progress, and the two miRNAs work respectively as well as synergistically.

## 4. Discussion

Our study found that miR-188-5p and miR-141-3p were over-expressed in BC tissues compared with adjacent normal tissues. MiR-188-5p and miR-141-3p were also over-expressed in BC cell lines (T24, BIU87 and 5637) compared with normal bladder epithelial cell line SV-HUC-1. Current studies have shown that miRNA promoter demethylation can activate miRNA and increase its expression [16–18]. Demethylation could be the reason why miR-188-5p and miR-141-3p were over-expressed. In addition, over expression of miR-188-5p and miR-141-3p indicated poor prognosis of BC patients. It follows that miR-188-5p and miR-141-3p promote BC progression. To investigate the mechanism of promotion, in vitro cell function experiments were performed through transient transfection in this study. In vitro experiment showed that down-regulated miR-188-5p expression or miR-141-3p expression observably inhibited the proliferation, migration and invasion of 5637 cells. A combination of half dose down-regulated miR-188-5p and half dose down-regulated miR-141-3p demonstrated a more obvious inhibition effect. The results suggested that miR-188-5p and miR-141-3p reversely regulated the function of cancer suppressors. Therefore, the two miRNAs were considered to be tumor promoters, and the two miRNAs promoted BC progression respectively as well as synergistically.

Metastatic BC patients often have poor prognosis. Therefore, new diagnostic and prognostic markers are in urgent need to assess the risk

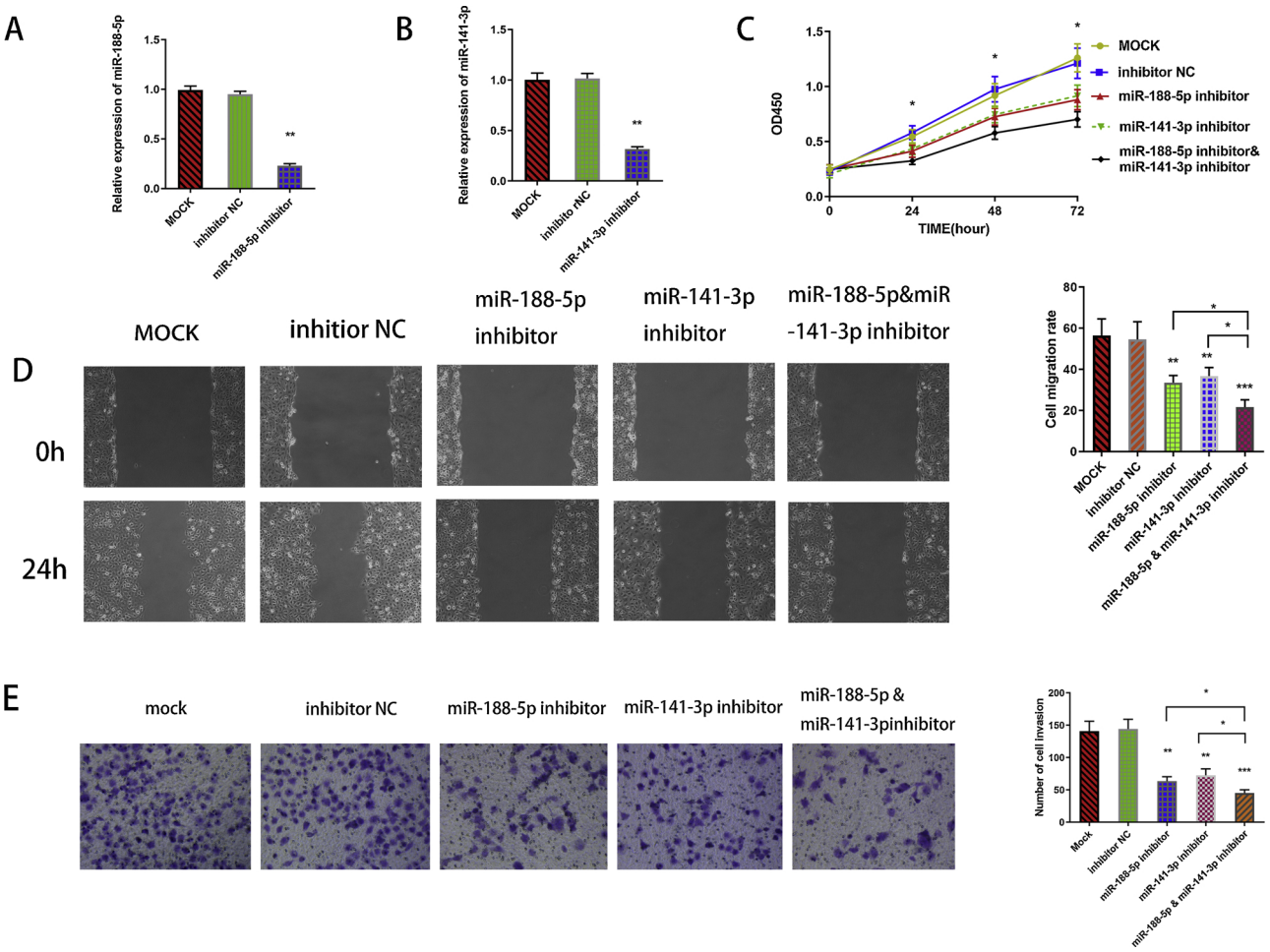


**Fig. 2.** Verification of the transfection efficiency of miRNA inhibitors. Different transfection methods result in different proliferation, migration and invasion effect of BC cells. (A) Down-regulated miR-188-5p expression after the transfection of miR-188-5p inhibitor (**P < 0.01). (B) Down-regulated miR-141-3p expression after the transfection of miR-141-3p inhibitor (**P < 0.01). (C) Down-regulated miR-188-5p or miR-141-3p expression obviously inhibit cell proliferation, and the combination of the two miRNAs with half dose down-regulated expressions had better inhibitory effect on cell proliferation (*P < 0.05). (D) Down-regulated miR-188-5p or miR-141-3p expression obviously inhibited cell migration (**P < 0.01), and the combination of the two miRNAs with half dose down-regulated expressions had better inhibitory effect on cell migration (***P < 0.0001). (E) Down-regulated miR-188-5p or miR-141-3p expression obviously inhibited cell invasion (**P < 0.01), and the combination of the two miRNAs with half dose down-regulated expressions had better inhibitory effect on cell invasion (***P < 0.0001).

of disease and develop more reasonable treatment plan. Studies have manifested that apart from regulating one single miRNA, multiple miRNAs can promote or inhibit tumor progression synergistically [19–22]. Therefore, we aimed at selecting two miRNAs to regulate the progression of BC synergistically, thus to achieve a better treatment. The above-mentioned experiments manifested that miR-188-5p and miR-141-3p promoted BC progression respectively and synergistically. Since high expressions of miR-188-5p and miR-141-3p often result in poor prognosis, the two miRNAs will not only assist the diagnosis of BC, but also predict the progression of BC as more effective joint markers. Meanwhile, in our study half dose of each inhibitor was transfected and then combined, which indicated that the total dose remained unchanged, but a greater regulation effect has been achieved. Side effects are much smaller in this way. Therefore, it is more ideal to control the progression of BC by a combination of two miRNAs than one single miRNA and regulating the two miRNAs together can control BC progression in a more effective way.

Different miRNAs can work synergistically to perform a stronger therapeutic effect while reducing side effects at the same time. However, further researches are still required to find out how multiple miRNAs play a synergistic role and whether multiple miRNAs have antagonism as well as the mechanism of antagonism. This combined gene prediction and therapy brings new ideas to the assessment and treatment of the disease. Meanwhile, more research targets will make the mechanism more complicated and pose greater challenges for researchers.

## Acknowledgement

This work was supported by 2018 Major Clinical Medicine Project of China Medical University (No. 3110118032).

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