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# MicroRNA-490-5p inhibits proliferation of bladder cancer by targeting c-Fos

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ABSTRACT

MicroRNAs (miRNAs) are non-protein-coding sequences that play a crucial role in tumorigenesis by negatively regulating gene expression. Here, we found that miR-490-5p is down-regulated in human bladder cancer tissue and cell lines compared to normal adjacent tissue and a non-malignant cell line. To better characterize the function of miR-490-5p in bladder cancer, we over-expressed miR-490-5p in bladder cancer cell lines with chemically synthesized mimics. Enforced expression of miR-490-5p in bladder cancer cells significantly inhibited the cell proliferation via G1-phase arrest. Further studies found the decreased c-Fos expression at both mRNA and protein levels and Luciferase reporter assays demonstrated that c-Fos is a direct target of miR-490-5p in bladder cancer. These findings indicate miR-490-5p to be a novel tumor suppressor of bladder cancer cell proliferation through targeting c-Fos.



## 1. Introduction

Urinary bladder cancer is the most common urogenital malignant tumor which ranks ninth in worldwide cancer incidence, with a projected 386,300 new cases and 150,200 deaths in 2008 [1,2]. Surgical operation, radiotherapy and chemotherapy are the dominating treatments. However, high recurrence and progression rates as well as limited therapeutic response impel us to figure out the underlying molecular mechanisms of bladder tumorigenesis.

MicroRNAs (miRNAs) are small (~22 nucleotides), endogenous, non-coding RNAs that induce post-transcriptional gene repression by partly binding to complementary sequences in the 3′ untranslated regions (3′-UTR) of target mRNAs [3]. Aberrant expression of miRNA has been identified in various kinds of cancers including bladder cancer [4] and a number of evidences indicate that some miRNAs play important roles in tumor proliferation, apoptosis, differentiation, invasion and metastasis [4,5]. For example, miR-143, miR-195, miR-26a, miR-23b, miR-590-3p have been regarded as tumor suppressor genes by inhibiting the proliferation of bladder cancer [6–10]. In the miRNA signature analysis, Han et al. [11] identified a set of aberrant expressed miRNAs, including miR-490-5p, which was the most significantly downregulated one. However, there was no relevant study about miR-490-5p before and its functions and targets remain unknown.

In our study, we verify the down regulation of miR-490-5p in human bladder cancer tissues and cells. Moreover, for the first time we find that miR-490-5p could suppress the proliferation of bladder cancer cells via targeting c-Fos.

## 2. Materials and methods

### 2.1. Reagents

The miR-490-5p mimic (named as miR-490-5p, sense; 5′-CCA UGGAUCUCCAGGUGGGU-3′) and negative control duplex (named as NC, sense; 5′-ACUACUGAGUGACAGUAGA-3′) lacking any significant homology to known human sequences were used for transient gain of function study. The small interfering RNA targeting human c-Fos mRNA (named as siFos, sense; 5′-CUGUCAACGCGCAGGA CUU) was used for RNAi study. All the RNA duplexes were chemically synthesized by GenePharma (Shanghai, China).

### 2.2. Tissue samples

Tissue samples from bladder cancer and the adjacent non-tumorous urothelium were gained from patients receiving radical cystectomy for bladder cancer in the Department of Urology at the First Affiliated Hospital of Zhejiang University in China. The samples were obtained between January and April 2011 with informed consent and Ethics Committee's approval. All the samples were kept in liquid nitrogen before RNA extraction.

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### 2.3. Cell culture and transfection

The human bladder cancer cell lines T24 and UM-UC-3 and non-tumor urothelial cell line SV-HUC-1 used in our study (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin and 50 U/ml penicillin under a humidified atmosphere with 5% $CO_2$ at 37 °C. Cells were plated to about 60% confluency in medium without antibiotics 24 h before transfection. Transfections were performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) in accord with the manufacturer's instructions.

### 2.4. RNA isolation and real-time PCR

The miR-490-5p and c-Fos expression levels were determined by quantitative real-time RT-PCR. MicroRNAs were extracted from frozen tissues or cancer cell lines using the RNAiso for small RNA kit (Takara, Dalian, China) and reverse transcribed into cDNAs using the One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Dalian, China).Total RNA was extracted using TRIzol reagent (Takara, Dalian, China) and reverse transcribed into cDNA before detection using the PrimeScript RT reagent Kit (Takara, Dalian, China). The resulting cDNAs were quantified by SYBR Green (Takara, Dalian, China) using an ABI 7500 fast real-time PCR System (Applied Biosystems, Carlsbad, USA). We used small nuclear RNA U6 and GAPDH mRNA as internal controls to determine the relative expression levels of miR-490-5p and c-Fos, respectively. The qPCR primers were provided by Sango Biotech (Shanghai, China) and the sequences are as follows: miR-490-5p (forward: 5′-CCATGGATCTC-CAGGTGGGT-3′), U6(forward: 5′-TGCGGGTGCTCGCTTCGGCAGC-3′), c-Fos (forward: 5′-CCGGGGATAGCCTCTCTTACT-3′; reverse: 5′-CCAGGTCCGTGCAGAAGTC-3′), and GAPDH (forward: 5′-AAGGT-GAAGGTCGGAGTCA-3′; reverse: 5′-GGAAGATGGTGATGGGATTT-3′).

### 2.5. Cell growth/cell viability assay

Each 96-well plates were plated with approximately 5000 T24 or UM-UC-3 cells. After overnight incubation, these cells were treated with RNA duplexes (NC, miR-490-5p, siFos) for 48–72 h and the concentration of miR-490-5p ranged from 5 to 100 nM. After different times the medium were removed and Cell Counting solution (WST-8, Dojindo Laboratories, Tokyo, Japan) was added to each well and incubated at 37 °C for another hour. The absorbance of the solution was measured spectrophotometrically at 450 nm with MRX II absorbance reader (Dynex Technologies, Chantilly, VA, USA).

### 2.6. Colony formation assay

T24 and UM-UC-3 cells were harvest 24 h after treated with 2′-O-Methyl modified duplexes. 500 of each transfected cells were seeded in new six-wells and went on culture for 10 days without any disturbance. Cells were stained with 0.1% crystal violet in order to observe and analyze the colony formation. The following equation was used to calculate the rate of colony formation: colony formation rate = (number of colonies/number of seeded cells) × 100%.

### 2.7. Cell cycle analysis by flow cytometry

48 h after the transfection, cells were harvest and washed with PBS and fixed in 75% ethanol at −20 °C for the following night. Before cell cycle analysis, they were washed with PBS and treated with DNA Prep Stain (Beckman Coulter, Fullerton, CA) for 30 min. Cell cycle analysis was performed by BD LSRII Flow Cytometry System with FACSDiva software (BD Bioscience, Franklin Lakes, USA). The data were analyzed by ModFit LT software package and the distribution of cell cycle was showed as the percentage of cells in G1, S, and G2 populations.

### 2.8. Protein extraction and Western blotting

The cells were lysed in cell lysis buffer 48 h after transfected with RNA duplexes. The BCA Protein Assay kit (Pierce) was used to calculate the total protein concentration in every lysate. Equivalent amounts of protein samples were separated by 10% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h and then incubated overnight with primary antibody. After washing, they were incubated with the corresponding secondary antibody for 1 h and then detected by Chemi-luminescence (ECL). The primary antibodies used were: anti-Fos, anti-Cyclin D1, anti-CDK4, anti-E2F1, anti-phosphorylated Rb (pT356) and anti-GAPDH (Epitomics, Burlingame, CA).

### 2.9. Luciferase assays

In order to construct luciferase reporter vectors, a pair of oligonucleotide which contained the desired miR-490-5p target region was designed (forward: 5′-TGTTCTGACATTAACAGTTTTC-CATGAAAACGTTTTATTGTGTTTTTg-3′; reverse: 5′-tcgacAAAAACA-CA ATAAAACGTTTTCATGGAAAACTGTTAATGTCAGAACAgagct-3′) (Sangon, Shanghai, China). The oligonucleotide pair was annealed at 90 °C for 3 min and then transferred to 37 °C for 15 min before being inserted into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA) between the *Sac*I and *Sal*I sites. Meanwhile, the mutant miRNA target region was designed, annealed and inserted into pmirGLO Dual-Luciferase Vector in the same way (forward: 5′-cTGTTCTGACATTAACAGTTTAGGTACT AAACGTTTTATTGTGTTTTTg-3′; reverse: 5′-tcgacAAAAACACAATA AAACGTTTAGTACCTAAACTGTTAATGTCAGAACAgagct-3′) (Sangon, Shanghai, China). Both insertions were verified by sequencing.

HEK 293T cells were plated on a 24-well plate for 24 h and then co-transfected with 50 nM of either miR-490-5p mimics or NC oligos and 200 ng reporter plasmid carrying either wild-type or mutant 3′-UTR. The Dual-Luciferase Reporter Assay System (Promega, USA) was used to measure the relative luciferase activity 48 h after transfection.

### 2.10. c-Fos rescue experiments

By inserting the human c-Fos coding sequence which lacked the 3′-UTR into the pIRES-EGFP vector, the pIRES-EGFP-Fos plasmid was constructed (GENECHEM) and verified by sequencing. The cells were co-transfected with either miR-490-5p mimics or NC oligos with pIRES-EGFP-Fos or empty pIRES-EGPF vector. The cells were harvest 48 h after the transfection and the flow cytometry was used to analyze the cell cycle. In addition, the c-Fos expression was determined by Western blotting.

### 2.11. Statistical analysis

All data were expressed as the mean ± standard deviation (SD) for three independent experiments. Differences between samples were analyzed by *t*-tests using SPSS V17.0 software. $P < 0.05$ was regarded to be statistically significant.

## 3. Results

### 3.1. miR-490-5p is down-regulated in bladder cancer

It has been pointed out by the previous microarray analysis that miR-490-5p was down-regulated in human bladder cancer [11]. In order to further verify the expression pattern of miR-490-5p in human bladder cancer, we used real-time RT-PCR to quantify and compare the expression levels of miR-490-5p in eleven pairs of human bladder cancer tissues versus adjacent non-tumor tissues as well as in three kinds of bladder cancer cell lines versus SV-HUC-1 cell (a normal transitional epithelial cell line). It turned out that compared with the corresponding normal tissues, miR-490-5p expression levels in tumorous tissues were decreased markedly. Ten out of eleven tumorous tissues presented down-regulated pattern of miR-490-5p, with six exhibiting with more than 50% reduction (Fig. 1A). Moreover, the observation of three kinds of bladder cancer lines showed the similar miR-490-5p expression pattern compared with SV-HUC-1 cell (Fig. 1B). Therefore, we conjectured that miR-490-5p might be a tumor suppressor in bladder cancer.

### 3.2. miR-490-5p suppresses bladder cancer cell proliferation, inhibits colony formation and induces G1-phase arrest

The low expression level of miR-490-5p in bladder cancer led us wondering about its function in bladder cancer and in order to find out whether it would function as a tumor suppressor, T24 and UM-UC-3 cells were transfected with miR-490-5p mimics. Apparently, miR-490-5p presented an effect of suppressing the growth of bladder cancer cell. MiR-490-5p at a concentration of 25 nM could reduce cell viability by 28% and 35%, respectively, at 48 or 72 h after transfection in T24 cell. The corresponding results of UM-UC-3 were 37% and 46% (Fig. 1C). Similarly, the colony formation ability was inhibited in both of the bladder cancer lines. The colony formation rates of miR-490-5p transfected cells were much lower compared with those transfected with NC (Fig. 1D).

To better understand the potential mechanisms of miR-490-5p-mediated growth suppression, we used flow cytometry to observe the distribution of the cell cycle of bladder cancer lines after transfected with miR-490-5p. The cell cycle demonstrated that there was a significant G1-phase arrest in both of T24 and UM-UC-3 cells (Fig. 1E and F). Furthermore, the regulators of

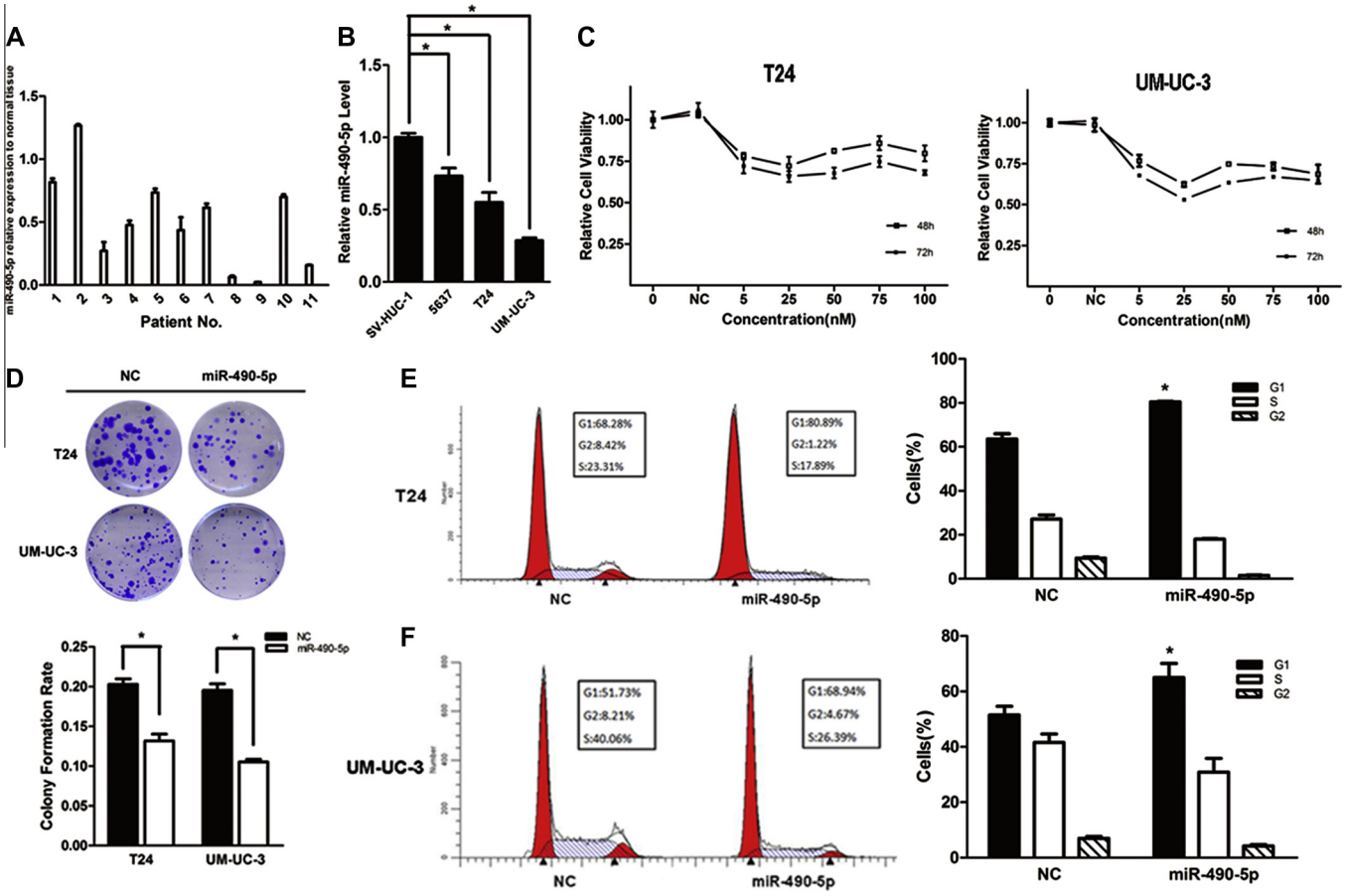


**Fig. 1.** MiR-490-5p is down-regulated in bladder cancer tissues and cell lines and over-expression of miR-490-5p inhibited bladder cancer proliferation. Expression levels for miR-490-5p by real-time PCR analysis were normalized with U6. (A) The miR-490-5p levels in 11 bladder cancer tissue samples were standardized according to the levels in the corresponding adjacent normal bladder tissue, which were appointed a value of 1.0. (B) The miR-490-5p levels in bladder cancer cell lines (5637, T24 and UM-UC-3) were detected and compared with non-tumor urothelial cell line SV-HUC-1 (*$P < 0.05$). (C) Cell growth/cell viability assay. The relative cell viability of the miR-490-5p treated groups of T24 and UM-UC-3 cells were lower than that of NC treated (cell viability of 0 nM was regarded as 1.0), respectively (*$P < 0.05$). The function of miR-490-5p made the best at a concentration of 25 nM in both of the cell lines. (D) Colony formation assay (Representative wells were presented). The colony formation rate was lower for miR-490-5p transfected groups compared with NC transfected groups (*$P < 0.05$). (E and F) Cell cycle distribution in T24 and UM-UC-3 cell lines. Over-expression of miR-490-5p induced significant G1-phase arrest in both of the cell lines (representative histograms are shown above. The indicated percentages are the average of triplicate experiments) (*$P < 0.05$).

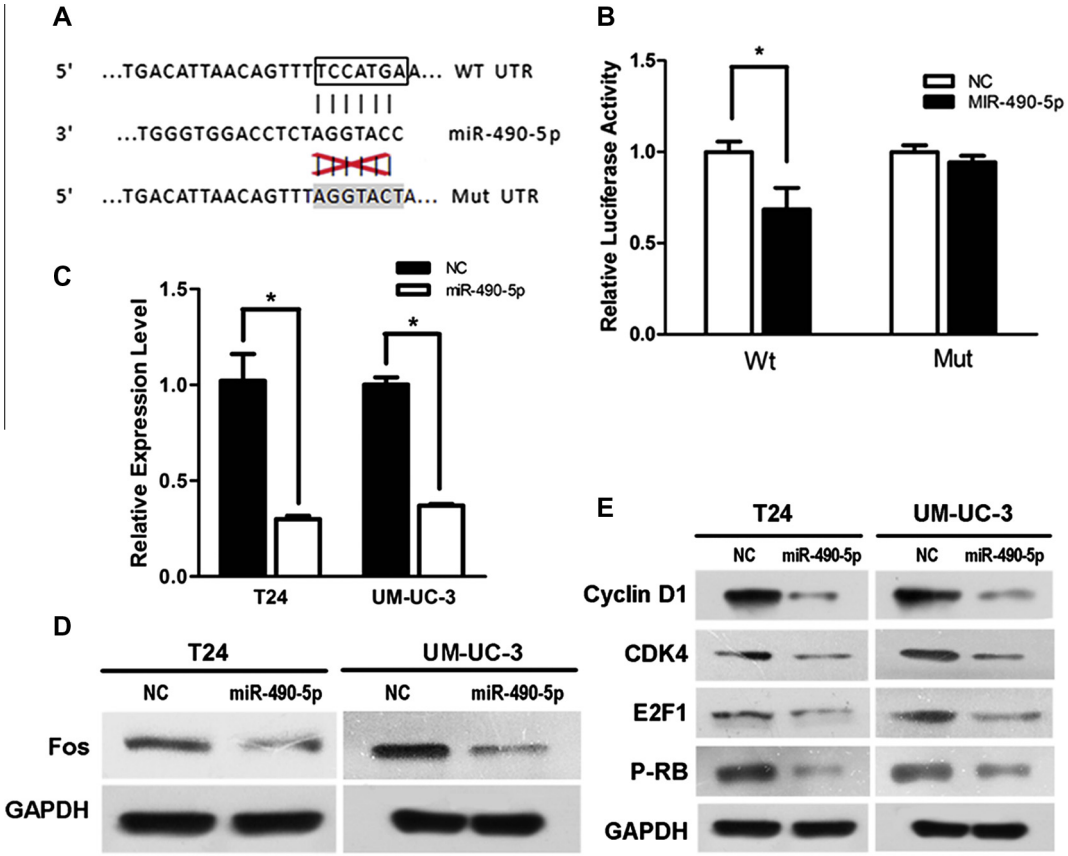


**Fig. 2.** C-Fos is a direct target of miR-490-5p. (A) A predicted seed region in the 3′-UTR of c-Fos was shown (top). The mutated sequence used was highlighted in grey (bottom). (B) 293T cells were co-transfected with 50 nM of either miR-490-5p mimics or NC oligos and 200 ng plasmid carrying either Wt or Mut 3′-UTR of c-Fos. The relative firefly luciferase activity normalized with Renilla luciferase was measured 48 h after transfection (*$P < 0.05$). (C) Over-expression of miR-490-5p reduced c-Fos expression levels of T24 and UM-UC-3 cells significantly (*$P < 0.05$). (D) Western blot analysis was performed to detect the expression of c-Fos or GAPDH following treatment of T24 and UM-UC-3 cells with miR-490-5p mimics. (E) Western blot analysis of G1/S transition regulators in T24 and UM-UC-3 cell lines. MiR-490-5p induced change of G1/S transition regulators (cyclin D1, CDK4, E2F1, p-RB). GAPDH was also blotted and served as a normalizer.

positive G1/S transition including cyclin D1, CDK4, E2F1, p-RB showed obvious change (Fig. 2E).

The above results indicated that miR-490-5p could suppress the growth of bladder cancer cells by arresting G1 phase.

### *3.3. miR-490-5p inhibits the expression of c-Fos via binding to its 3'-UTR*

TargetScan analysis (http://www.targetscan.org) was thought to be a useful tool to identify the downstream targets of miR-490-5p. Among all the genes that were predicted, c-Fos performed remarkable decrease in both the mRNA and protein levels when transfected with miR-490-5p mimics (Fig. 2C and D). Moreover, the knock-down of c-Fos by RNAi technique (Fig. 3E) caused the inhibition of cell growth and G1-phase arrest in both the T24 and UM-UC-3 cells just as expected (Fig. 3A–D). Meanwhile, after transfected with siFos, proteins of positive G1/S transition which had been mentioned before presented the similar change as treated with miR-490-5p (Fig. 3F). The above results all showed that the knock-down of c-Fos could phenocopy the effect of miR-490-5p.

In order to confirm that whether c-Fos was a direct target of miR-490-5p, the 3′-UTR of c-Fos was inserted into down-stream of firefly luciferase of pmirGLO Dual-Luciferase miRNA Target Expression Vector. Another vector with a mutated putative binding site was also constructed (Fig. 2A). The results showed that co-transfected with wildtype (Wt) and miR-490-5p in HEK 293T cells significantly reduced the relative luciferase activity compared with co-treated with Wt and NC as anticipated. However, co-transfected with mutant (Mut) 3′-UTR and miR-490-5p did not change the luciferase activity (Fig. 2B). Therefore we came to the conclusion that c-Fos was a direct target of miR-490-5p.

### *3.4. Restoration of c-Fos expression partially rescues miR-490-5p-induced cell cycle arrest*

We had verified that miR-490-5p could induce G1-phase arrest before and we were wondering whether forced c-Fos expression could abrogate the cell cycle arrest by miR-490-5p. For this purpose, we inserted the human c-Fos coding sequence which lacked the 3′-UTR into the pIRES-EGFP vector and presented that c-Fos expression was restored after treated with this pIRES-EGFP-Fos vector (Fig. 4A). Cell cycle was evaluated by flow cytometry after co-transfected with either miR-490-5p mimics or NC oligos with pIRES-EGFP-Fos or empty pIRES-EGPF vector into UM-UC-3 cells (Fig. 4B and C). The results demonstrated that restored c-Fos expression could partially reverse the miR-490-5p-induced cell cycle arrest.

## 4. Discussion

During the past decades, many studies concentrate more on tumor developing mechanism and more effective cancer treatments, of which microRNAs have turned out to be promising

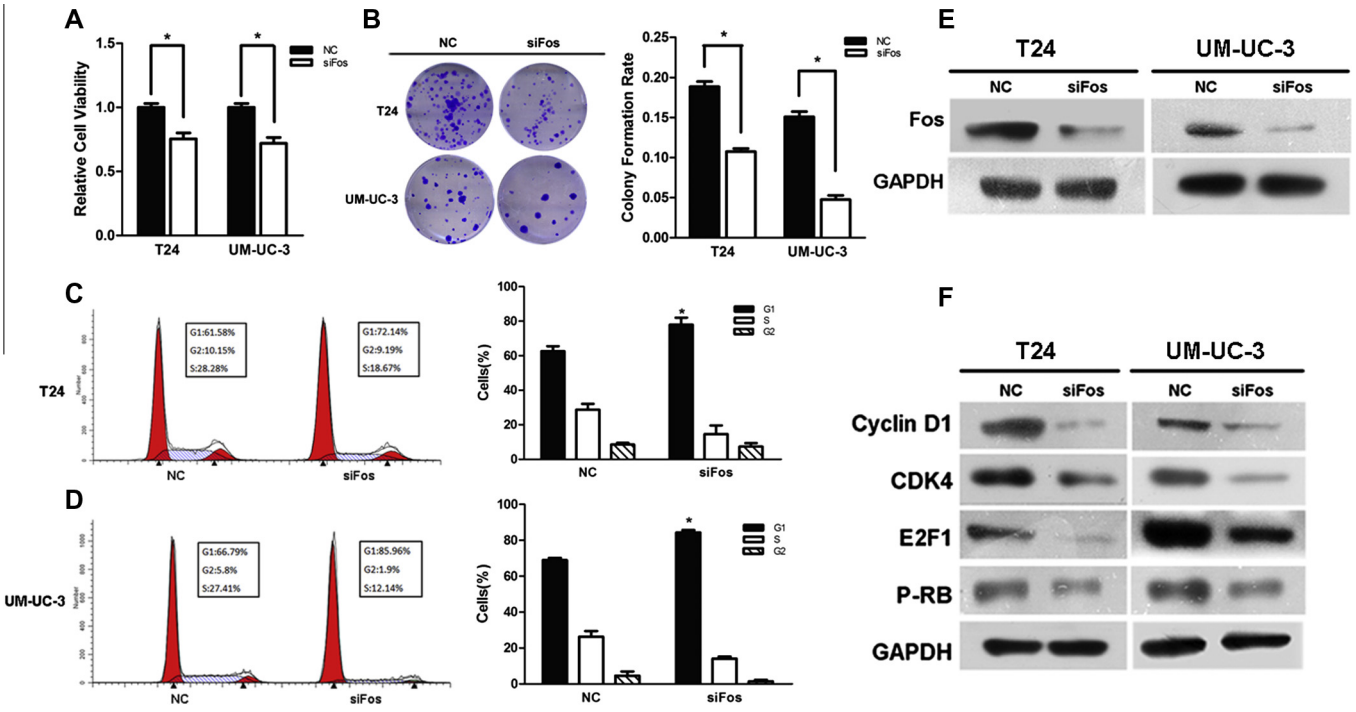


**Fig. 3.** Knock-down of c-Fos phenocopied the effect of miR-490-5p. (A) Knock-down of miR-490-5p by siFos suppressed bladder cancer cell growth. SiFos caused 24.6% and 28.1% cell viability reduction in T24 cells and UM-UC-3 cells 48 h after transfection, respectively (*$P < 0.05$). (B) SiFos reduced the colony formation rate in both of the cells (Representative wells were presented) (*$P < 0.05$). (C and D) Knock-down of c-Fos induced significant accumulation of cells in G1 phase (Representative histograms are shown above. The indicated percentages are the average of triplicate experiments) (*$P < 0.05$). (E and F) SiFos reduced the expression of c-Fos and caused change of G1/S transition regulators (cyclin D1, CDK4, E2F1, p-RB).

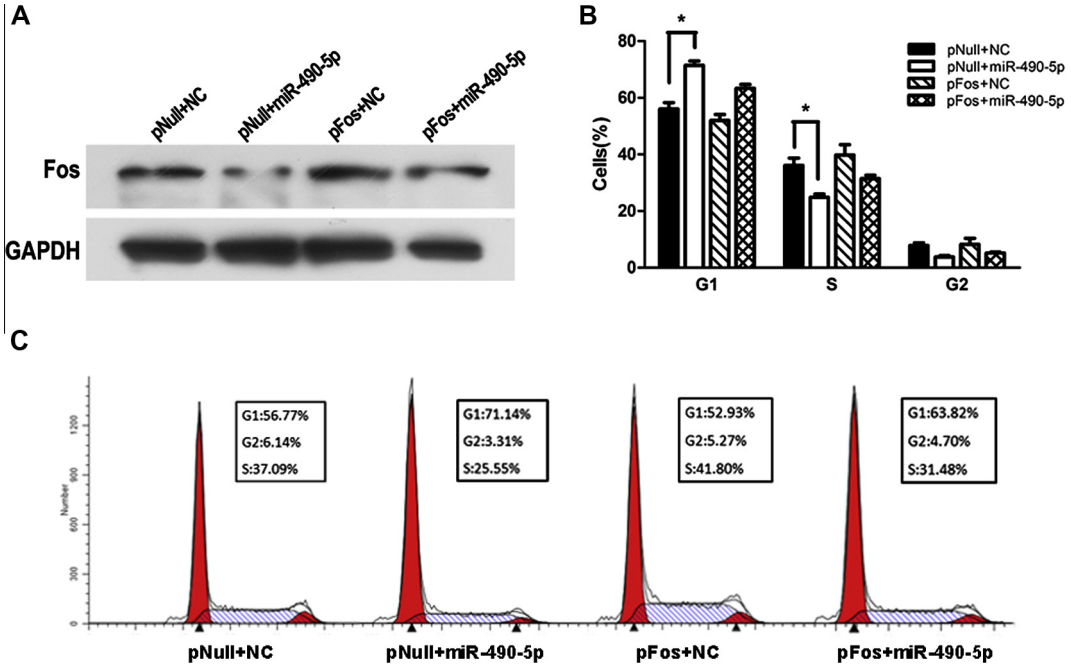


**Fig. 4.** Forced expression of c-Fos rescued miR-490-5p-dependent G1 phase arrest. (A) UM-UC-3 cells were co-transfected with either miR-490-5p mimics or NC oligos with pIRES-EGFP-Fos or empty pIRES-EGPF vector. The expression of c-Fos or GAPDH was detected by Western blot analysis. (B and C) Forced expression of c-Fos partly abrogated cell cycle arrest effect of miR-490-5p in UM-UC-3 cells (*$P < 0.05$).

diagnostic and prognostic molecular biomarkers as well as therapeutic target [12–15]. Until now, altered expressions of several microRNAs and their functions in bladder cancer have been observed [16,17]. It has been showed that microRNAs play important roles in bladder cancer progression and metastasis [17]. MiR-490-5p is a novel identified microRNA with decreased expression in bladder cancer according to genome-wide miRNA expression patterns in human urothelial carcinoma by deep sequencing [11]. And our quantification analysis presented the similar results. However, the expression pattern of miR-490-5p in other tumors has not

been reported yet, nor has its functions in bladder cancer and other tumors been studied. This is the first study to determine the function of miR-490-5p in cancer. Since both our tissue samples and cell lines were from patients with muscle-invasive bladder cancer, our study is probably more significant in muscle-invasive or recurrent cancer.

In order to find out the roles of miR-490-5p in bladder cancer, we conducted gain-of- function study in bladder cancer lines T24 and UM-UC-3. When transfected with miR-490-5p, both T24 and UM-UM-3 showed decrease in cell viability and colony formation rate, which indicated that miR-490-5p was probably a tumor suppressor which could inhibit proliferation of bladder cancer cells. Further analysis by FACS demonstrated that miR-490-5p could induce G1-phase arrest, which might be the potential mechanism of miR-490-5p-mediated growth suppression.

Our next step was to reveal the possible molecular mechanism responsible for miR-490-5p inducing cell growth inhibition in bladder cancer. According to our study, the real-time PCR and Western blot presented the reduced expression of c-Fos and luciferase assay further demonstrated that c-Fos is a target of miR-490-5p. Moreover, the knock-down of c-Fos caused the inhibition of cell growth and G1-phase arrest, while restored c-Fos expression could partially reverse the miR-490-5p-induced cell cycle arrest. Therefore, our study indicates that miR-490-5p is able to suppress the proliferation of bladder cancer cells by targeting, or at least partially, at c-Fos.

C-Fos (FBJ murine osteosarcoma viral oncogene homolog), which has oncogenic activity and is frequently overexpressed in tumour cells, is a part of the Fos family (c-Fos, FosB, Fra-1 and Fra-2, FosB2 and deltaFosB2). And together with Jun family members (c-Jun, JunB and JunD) they form the group of AP-1 transcription factor complex [18]. By binding to the so-called TPA-responsive elements (TRE's; TGAC/GTCA) in the promoter and enhancer regions of target genes after dimerisation, c-Fos would participate in the regulation of important regulators of proliferation, differentiation, invasion, metastasis, and survival [19]. Various studies have reported the involvement of c-Fos in many types of cancer, including skin tumors, osteosarcomas, endometrial carcinoma and some other tumors [20–23]. And there were also reported that c-Fos might play important roles in arsenic-mediated carcinogenesis of the urothelium [24,25]. Furthermore, the over-expression of c-Fos in bladder has also been reported by Oncomine™ Research Edition (https://www.oncomine.org/resource/login.html), which indicated that c-Fos might involve in the proliferation of bladder cancer. In addition, the ability of c-Fos inducing cyclin D1 expression offered a connection to the cell cycle machinery. c-Fos has been shown to directly bind to the AP-1 binding sites in the cyclin D1 promoter and regulate the cyclin D1 transcription [26]. In our study, we found the decrease expression of cyclin D1 as well as other positive G1/S transition regulators including CDK4, E2F1 and p-RB after treated with miR-490-5p and it might due to the low expression of c-Fos which was thought to be an activator of cyclin D1.

In summary, our study confirms the down-regulated of miR-490-5p in bladder cancer and for the first time reports it as a potential tumor suppressor in bladder cancer, which can induce G1-phase arrest by targeting c-Fos. Although further studies are needed to identify other targets of miR-490-5p, our experimental data demonstrates that restoration of miR-490-5p may be a novel therapeutic strategy for preventing proliferation of bladder cancer.

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