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Cyclin-dependent kinase 4 is a novel target in micoRNA-195-mediated cell cycle arrest in bladder cancer cells

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ABSTRACT

miRNAs are a class of small-noncoding RNAs capable of negatively regulating gene expression. Here, we found that miR-195 is down-regulated in human bladder cancer tissue versus normal adjacent tissue. To better characterize the role of miR-195 in bladder cancer, we conducted gain of function analysis by transfecting bladder cancer cell line T24 with chemically synthesized miR-195 mimic. We identified CDK4, an early G1 cell cycle regulator, as a novel target of miR-195. Selective over-expression of miR-195 could induce G1-phase arrest in T24 cells, and subsequently inhibit T24 cell growth. These findings indicate that miR-195 could be a potential tumor suppressor in bladder cancer.
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1. Introduction

Urinary bladder cancer ranks nineth in worldwide cancer incidence [1], and it has been listed as the most common genitourinary tract malignancy in China [2]. It is believed that tumorigenesis and progression of bladder cancer would involve various genetic changes, such as chromosomal anomalies, genetic polymorphisms, genetic and epigenetic alterations [3]. However, the exact mechanisms of bladder tumorigenesis are still not well understood. Understanding these genetic alterations is crucial to identify novel prognostic biomarkers and therapeutic targets.

MicroRNAs (miRNAs) are small (~22 nucleotides), endogenous, noncoding RNAs, which make up a novel class of gene regulators [4]. They induce post-transcriptional gene repression by blocking the translation and/or accelerating the degradation of their target mRNAs, and are thus involved in diverse cellular processes, such as differentiation, proliferation, metabolism and apoptosis [5]. Recently, a large amount of studies have been published to illustrate the association between specific miRNA function and carcinogenesis [6,7]. Meanwhile, accumulating evidences indicate that aberrant expression of miRNAs can contribute to bladder tumorigenesis. Gottardo et al. [8] firstly investigated miRNA expression pattern in bladder cancer in 2007. They reported ten up-regulated miRNAs (miR-223, miR-26b, miR-221, miR-103-1, miR-185, miR-23b, miR-203, miR-17-5p, miR-23a and miR-205) in 25 urothelial tumor samples compared with 2 normal bladder mucosa. In the microarray study by Lin et al. [9], bladder cancer presented with a significant lower expression of miR-143, miR-145, miR-125 and miR-199b compared with normal adjacent tissues. They also validated that miR-143 could function as tumor suppressor by targeting oncogene *ras*. Ostenfeld et al. [10] confirmed the down-regulation of miR-145 in bladder cancer tissue by ISH analysis and found that ectopic expression of miR-145 induced extensive apoptosis in urothelial carcinoma cell lines. In the bladder cancer miRNA signature analysis by Ichimi et al. [11], a set of aberrantly expressed miRNAs were identified, including miR-195, which presented with proximately 80% reduction in expression level in cancer tissue. A similar down-regulated pattern of miR-195 in bladder cancer was also observed in the genome-wide miRNA expression pattern's study by Han et al. [12].

miR-195 exhibits diverse expression pattern and function differently in different cancer [13–17]. It was reported that miR-195 could inhibit hepatocellular carcinoma cell proliferation by targeting Cyclin D1, CDK6, and E2F3 [16] and promote colorectal cancer cell apoptosis by repressing Bcl-2 [17]. However, the biological function of miR-195 in bladder cancer was not well established. In our present works, we further verified miR-195 expression

Abbreviations: CDK4, cyclin-dependent kinase 4; CDK6, cyclin-depedent kinase 6; miRNA, microRNA; Rb, retinoblastoma protein

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pattern in bladder cancer tissue. Also, for the first time, we found that miR-195 could suppress growth of the human bladder cancer cell line T24. The tumor suppressive role and regulatory mechanisms of miR-195 on bladder cancer were determined.

2. Materials and methods

2.1. Reagents

miR-195 mimic (shortly named as miR-195) and FAM-conjugated negative control duplex (named as NC) lacking significant homology to all known human sequences were applied for transient gain of function study. For the in vivo and in vitro tumorigenicity assay, the duplexes with 2′-*O*-methyl modification were used. The small interference RNA targeting human CDK4 mRNA (designated as siCDK4) was designed as described before [18], which targeted nucleotides 1062–1082 according to Genbank accession NM_000075. All the RNA duplexes were chemically synthesized by GenePharma (Shanghai, China). The sequences were listed in Table 1.

2.2. Tissue samples

Paired bladder cancer tissues and adjacent non-tumorous bladder mucosal tissues were obtained from patients undergoing radical cystectomy. The samples were collected between Jan 2011 and June 2011 at the First Affiliated Hospital of Medical College, Zhejiang University (Hangzhou, P.R. China) after informed consent and Ethics Committee's approval. The demographic and cancer staging data was listed in Table s1. Tissue samples were trimmed and snap frozen in liquid nitrogen until use.

2.3. Cell culture and transfection

The human bladder cancer cell line T24 (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) in a humidified atmosphere containing 5% CO2 maintained at 37 °C. The day before transfection cells were plated to 60–70% confluency in medium without antibiotics. The transfection was performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions and the transfection efficiency was monitored by FAM-conjugated NC.

2.4. RNA isolation and real-time PCR

Small RNA was isolated from frozen samples by using RNAiso for Small RNA (TaKaRa, Japan) and reverse transcribed using One Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa, Japan), while total RNA from transfected T24 cells was extracted with RNAiso plus (TaKaRa, Japan) and transcribed into cDNA with PrimeScript RT reagent Kit (TaKaRa, Japan). The resulting cDNA was quantified by real-time RT-PCR using SYBR Premix Ex Taq (TaKaRa, Japan). The relative expression level of miR-195 and CDK4 was calculated and quantified with the $2^{-\Delta\Delta Ct}$ method after normalization with reference to expression of U6 small nuclear RNA and GAPDH, respectively. All the primers were listed in Table 1.

2.5. Cell growth/cell viability assay

Cells were seeded in 96-well plate at a density of 4×10^3/well. After overnight incubation, the cells were treated with RNA duplexes (5–100 nM) for 48–72 h. After incubated for indicated time, cell-counting solution (WST-8, Dojindo Laboratories, Tokyo, Japan)

Table 1
The oligonucleotides used in this study.

Name[a]	Sequence(5′->3′)[b]
miR-195 mimics (sense)	UAGCAGCACAGAAAUAUUGGC
NC (sense)	ACUACUGAGUGACAGUAGA
siCDK4 (sense)	AACCCACACAAGCGAAUCUCU
miR-195 F	GATAGCAGCACAGAAATATTGGC
U6 F	TGCGGGTGCTCGCTTCGGCAGC
CDK4 F	CTACCTCTCGATATGAGCCAGT
CDK4 R	CATCTGGTAGCTGTAGATTCTG
GAPDH F	AAGGTGAAGGTCGGAGTCA
GAPDH R	GGAAGATGGTGATGGGATTT
CDK4-utr F	TCGA**GAGCTC**TGGAGTGGCTGCCATGGAAGGA
CDK4-utr R	TCGA**GTCGAC**AGGGACAAGAGGGAACATACCCCT
CDK4-mut F	TTCTACAGAGATTAC<u>AAACGACG</u>CTTAATGACATTC
CDK4-mut R	AGATGGAGGAGGACCCTCCATAGCC

[a] F, forward primer; R, reverse primer.
[b] Restriction sites are in bold; Mutated sites are underlined.

was added to each well and incubated for an additional 2 h. The absorbance of the solution was measured spectrophotometrically at 450 nm with MRX II absorbance reader (Dynex Technologies, Chantilly, VA, USA).

2.6. In vitro colony formation assay

Twenty-four hours after transfection with 2′-*O*-Methyl modified duplexes (50 nM), cells were harvest. Five hundreds of transfected cells were seeded in a fresh six-well and kept in culture undisturbed for 10 days, during which time the surviving cells spawned a colony of proliferating cells. Colony formation was analyzed by staining the cells with 0.1% crystal violet. The rate of colony formation was calculated with the following equation: colony formation rate = (number of colonies/number of seeded cells) × 100%.

2.7. In vivo tumorigenicity assays

Animal study was performed according to institutional guidelines. Male BALB/c-nude mice aged 4 weeks were used for human tumor xenograft model (supplied by the Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai, China). The mice were injected s.c. with either NC or miR-195 transfected cells (5×10^7 in 50 μl PBS). Tumor size was monitored and evaluated every 3 days. When the tumor became palpable, the tumor growth curves were depicted. The volume of the tumor was calculated from the formula length × width^2 × 0.52 [19], where length and width were tumor diameters measured with calipers in mutually perpendicular directions.

2.8. Cell cycle analysis by flow cytometry

Cells were harvested 48 following transfection, washed with PBS and fixed in 75% ethanol at −20 °C. After overnight fixation, cells were washed with PBS and stained 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 cell cycle distribution was presented as the percentage of cells in G1, S, and G2 populations. Data was analyzed with ModFit LT software package.

2.9. Western blotting analysis

Briefly, cells were harvested at 48 h following oligo treatment as described above, lysed and quantified. Equivalent quantities (30–50 μg) of protein were separated by 10% SDS-polyacrylamide gels and trans-

ferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk and then incubated overnight with the appropriate primary antibody at dilutions specified by the manufacturer. They were next washed three times in 15 ml TBS-Tween and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at 1:2 500 dilution in TBS-Tween for 1 h. Bound secondary antibody was detected using an enhanced chemiluminescence (ECL) system (Pierce Biotechnology Inc., Rockford, IL). Primary immunoblotting antibodies were: anti-β-actin, anti-phosphorylated Rb (S795), anti-phosphorylated Rb (S780) (Cell Signaling Technology, Beverly, MA), anti-CDK4, anti-CDK6, anti-Cyclin D1(Epitomics, Burlingame, CA) and anti-E2F3 (Santa-Cruz Biotechnology, Santa Cruz, CA).

2.10. Luciferase assays

To construct the luciferase reporter vectors, the 3′-UTR (untranslated region) of CDK4, which contained putative binding sites for miR-195, was amplified from genomic DNA (primer set in Table 1). The amplified fragment was inserted into pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA) between the SacI and SalI sites. In addition, mutant 3′-UTR, which carried a mutated sequence in the seeding region of miR-195, was mutated (primer set in Table 1) within pMD-18 T vector (TaKaRa, Japan) by the MutanBest Kit (TaKaRa, Japan) and then subcloned into pmirGLO Dual-Luciferase Vector. Both insertions were verified by sequencing. T24 cells plated in a 24-well plate were cotransfected with 50 nM of either miR-195 mimic or negative control oligo and 200 ng reporter comprising wildtype or mutant 3′-UTR. The relative luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega, USA) 48 h after transfection.

2.11. Statistical analysis

Data were expressed as mean ± standard deviation (SD) of three independent experiments. All analyses were performed using GraphPad Prism version 5 for Windows and $P < 0.05$ was considered to be statistically significant with either the student's *t*-test or Two-way ANOVA.

3. Results

3.1. miR-195 is down-regulated in bladder cancer

To determine the expression pattern of miR-195 in human bladder cancer, we quantified the expression levels of miR-195 in 12 pairs of human bladder cancer tissues and adjacent normal mucosal tissues by real-time RT-PCR. We found that miR-195 expression levels were generally lower in cancerous tissues comparing with their non-cancerous counterparts with exception of 1 pairs (Fig. 1). Furthermore, 7 out of 11 exhibited 50% reduction (Fig. 1A). This was consistent with fold change data from previous deep sequencing analysis [12]. Thus, we speculated that miR-195 might be a putative tumor suppressor in bladder cancer.

3.2. miR-195 suppresses bladder cancer cell proliferation, and inhibits clonogenicity both in vitro and in vivo

To elucidate that whether miR-195 could function as a tumor suppressor, the effects of miR-195 over-expression was evaluated in vitro with cell growth/cell viability assay. Notably, miR-195 demonstrated a potent inhibitory effect in a dose-dependent manner with a maximal inhibition at about 50 nM (Fig. 2A). miR-195 at a concentration of 50 nM could reduce cell viability by 29% and 39%, respectively, at 48 or 72 h after transfection. In parallel, miR-195 also dramatically impaired the colony forming ability of bladder cancer cells. The colony formation capability of miR-195 transfected cells was much lower than those transfected with NC (Fig. 2B). To further confirm the above findings, the tumor growths of T24 cells with or without miR-195 over-expression were examined after s.c. implantation into BALB/c mice. Over-expression of miR-195 resulted in dramatic retardation of tumor initiation and growth in vivo. In contrast, tumors in miR-195 group presented with a much slower growth pattern. (Fig. 2C). Taken together, these results showed that miR-195 negatively modulate bladder cancer cells growth.

3.3. miR-195 triggers G1-phase arrest via down-regulation of CDK4

The underlying mechanisms for miR-195-suppressed tumor growth were further explored with FACS. We transfected the T24 cells with 50 nM miR-195 and assessed the impact of miR-195 on cell cycle distribution 48 h after transfection. The results indicated that miR-195 could induce G1-phase arrest (Fig. 3A). Consistent with the cell cycle arrest phenomenon, it also presented with notable change in the content of positive G1/S transition regulators, including CDK6, cyclin D1 and E2F3, after 50 nM miR-195 treatment (Fig. 4). Beside all these previously validated miR-195's targets[16], CDK4, a novel putative target of miR-195 after integrating data from targets prediction from MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and targeting genes from the miRTarBase (http://mirtarbase.mbc.nctu.edu.tw), was significantly decreased in both mRNA and protein level (Fig. 5). As a possible novel target of miR-195, the physiological function of CDK4 was further evaluated via RNAi approach. The knock-down of CDK4 yielded the anticipated cell cycle arrest, which phenocopied the effect of miR-195 over-expression (Fig. 3B). To further characterize the cell cycle arrest, the CDK4 specific Rb phosphorylation status was analyzed. Consistent with the G1-phase arrest, the phosphorylation of Rb on Ser780 and Ser795 was attenuated after either miR-195 or siCDK4 treatment (Fig. 5).

3.4. CDK4 is a novel direct target of miR-195

We next investigated whether CDK4 was a direct functional target of miR-195. The 3'-UTR of CDK 4 was cloned into down-stream of firefly luciferase of pmirGLO Dual-Luciferase miRNA Target Expression Vector. Additional vector with mutated putative binding sites was also constructed (Fig. 6A). Cotransfected of either miR-195 or NC and reporter comprising wildtype (Wt) or mutant (Mut) 3'-UTR was performed. T24 cells transiently transfected with the Wt-3'-UTR-reporter and miR-195 exhibited significantly decreased relative luciferase activity when compared with NC. However, the luciferase activity of the reporter carrying 3'-UTR with mutated binding sites was unaffected by a simultaneous transfection of miR-195 (Fig. 6B). These findings indicated that miR-195 inhibited CDK4 expression through direct binding of 3'-UTR of its transcript.

4. Discussion

It is generally believed that there is an urgent need to develop effective targeted therapeutic strategy leading to improve of survival outcome of patients with bladder cancer [20]. Thus, identification of the molecular pathogenesis of bladder is crucial. Recently, an increasing number of studies have demonstrated that deregulation of miRNAs was a common events in tumor tissues [6,21]. Experimental evidences have shown that miRNAs would be ideal tumor biomarkers or therapeutic targets [22,23]. Previous systematic review [24] have summarized that the deregulated miRNAs is common in bladder cancer. A number of studies [8,9,11,12,25,26] have created signatures for bladder cancer, which will help further

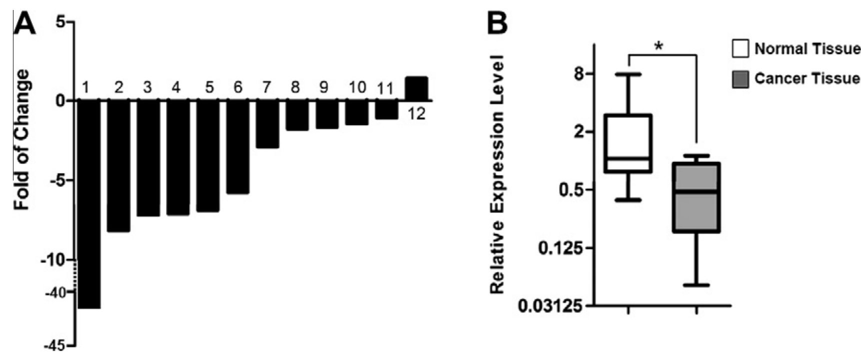

Fig. 1. Quantitative analysis of miR-195 in urinary bladder cancer tissues. The expression of miR-195 was normalized with U6 snRNA in real-time RT-PCR analysis. (A) The relative expression levels of miR-195 in individual 12 pairs of cancer tissue were presented as the fold change of miR-195 referred to the corresponding normal tissues. (B) The combined results were shown in a box and whiskers style. Box-plot lines represented medians and interquartile ranges of the normalized threshold values, and whiskers indicated10–90th percentiles. The expression level of miR-195 was significantly lower in cancer tissues ($^*P < 0.05$).

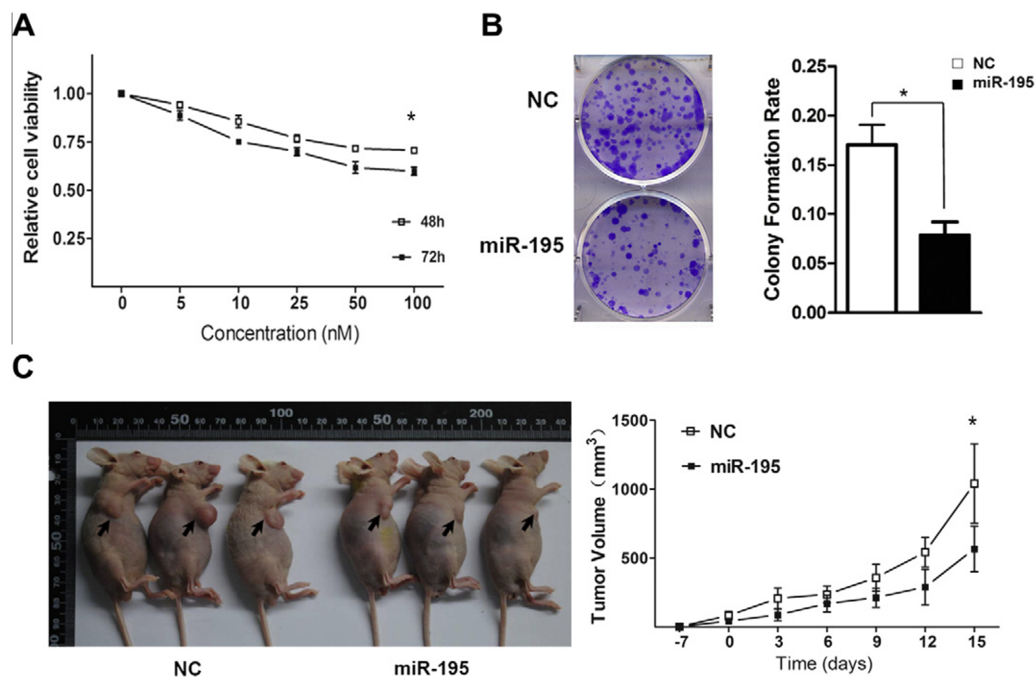

Fig. 2. Over-expression of miR-195 suppresses T24 cell growth and tumorigenicity. (A) Cell growth/cell viability assay. The relative cell viability of the miR-195 transfected group was lower than that of NC transfected (regarded as 1.0). miR-195 reduced the cell viability in dose- and time- dependent manner (Two-way ANOVA, $^*P < 0.05$). (B) Colony formation assay (Representative wells were presented). The colony formation rate was significantly lower for miR-195 treated group compared with NC treated group ($^*P < 0.05$). (C) The effect of miR-195 on tumorigenicity in nude mice. T24 cells treated with NC or miR-195 were inoculated subcutaneously into BALB/C mice. Xenograft tumors' volume were assessed every three days. The growth curves indicated that tumor in miR-195 group was in a significant slower growth pattern (Two-way ANOVA, $^*P < 0.05$).

establish molecular diagnosis, prognosis and therapy using miRNAs. However, limited studies have explored the modulatory function of miRNAs in bladder cancer. The roles of specific miRNAs in bladder are still poor understood.

Here, we revealed a decrease in expression of the newly identified tumor suppressive miR-195 in human bladder cancer tissue compared with normal adjacent tissues. Recently, studies have shown that miR-195 would be in a controversial expression pattern in different types of cancer [13–17]. However, in majority of cancers, down-regulation of miR-195 has been observed, such as prostate cancer [13], hepatic cellular carcinoma [16], colorectal cancer [17], squamous cell cancer of tongue [15]. Previous miRNA signature studies have also shown that miR-195 was down-regulated in bladder cancer [11,12]. Our quantification analysis yielded a similar expression pattern. But an additional issue still should be addressed when interpreting our results. Since our quantification data was from a set of high risk patients who subjected to radical cystectomy, the down-regulated pattern of miR-195 in bladder cancer would be probably more common in muscle-invasive or recurrent disease according to our data.

Furthermore, we validated the functional roles of miR-195 in bladder cancer cell line T24 by gain of function study. By transfecting T24 cells with miR-195 mimic, we revealed that miR-195 was a potential tumor suppressor for bladder cancer. Both cell viability assay and tumorigenicity assay demonstrated that selective over-expression of miR-195 could inhibit T24 cell growth. Moreover, FACS analysis suggested that miR-195 could disturb the cell cycle by inducing G1-phase arrest. In the target screen study, we identified a novel target of miR-195, CDK4. This findings was inconsistent with previous study by Sekiya et al. [27], most likely it was because of the difference in cell types. Our results from phenotypic analyses indicated that forced expression of miR-195 in T24 cells

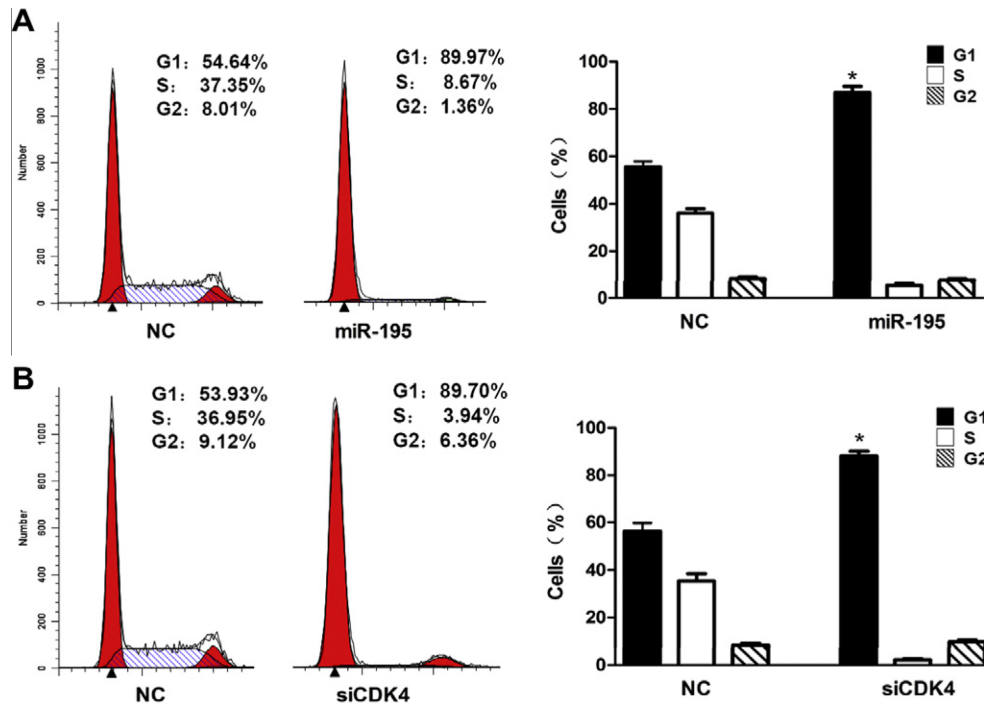

Fig. 3. Cell cycle distribution of T24 cells at 48 h after treatment with miR-195 or siCDK4 (Representative histogram are shown above. The indicated percentages are the average of triplicate experiments). Forty-eight hours after transfection, cells were subjected to flow cytometry. (A) Over-expression of miR-195 induced a significant accumulation of cells in G1-phase and blocks G1-S entry. (B) CDK4 knock-down by siCDK4 induced significant G1-phase arrest of up to 25% in T24 cells. (*$P < 0.05$)

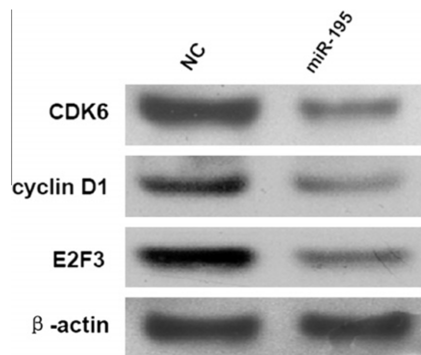

Fig. 4. Western blot analysis of G1/S transition regulators (previously validated as miR-195 targets). β-actin was also blotted and served as a normalizer. CDK6, cyclin D1 and E2F3 were suppressed by miR-195 over expression.

could abrogate Rb phosphorylation and subsequent G1/S transition by repressing multiple G1/S transition regulators, CDK4 included. Finally, luciferase assay confirm that miR-195 could specifically inhibit CDK4 by binding to a critical region located on its 3'-UTR.

As a key cell cycle regulator, serine-threonine kinase CDK4 triggers an important cascade of events in G1-phase [28]. It can efficiently catalyze Rb phosphorylation. Thus, CDK4 is regarded as a 'primary sensor' for driving cells through the R point, which marks the entry to a new round of replication. Therefore, CDK4 has been considered as a desirable target for cancer therapies [28–31]. The specific CDK4 know-down with siRNA phenocopied the cell cycle arrest effect of miR-195 over-expression, which further strengthened our findings that CDK4 is another important mediator of miR-195-induced cell cycle arrest, besides the validated E2F3, CDK6, Cyclin D1 [16] and Cyclin E [27].

In summary, our study confirms that miR-195 is frequently down-regulated in bladder cancer. miR-195, as a tumor suppressor

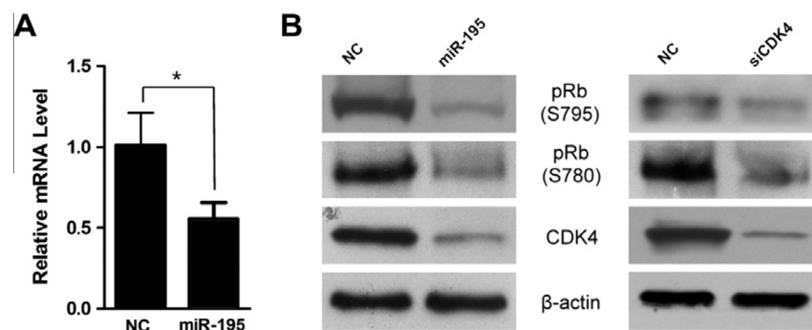

Fig. 5. The effect of ectopic expression of miR-195 on CDK4 expression and down-stream Rb phosphorylation status. (A) Real-time RT-PCR analysis indicated that the relative mRNA level of CDK4 was significantly decreased after miR-195 treatment (*$P < 0.05$). (B) Western blot analysis of CDK4 protein and Rb phosphorylation status. β-actin was also blotted and served as a normalizer. CDK4 was suppressed by miR-195 over-expression. siCDK4 effectively silenced CDK4. The phosphorylation of Rb on Ser780 and Ser795 was attenuated by either miR-195 over-expression or CDK4 silence.

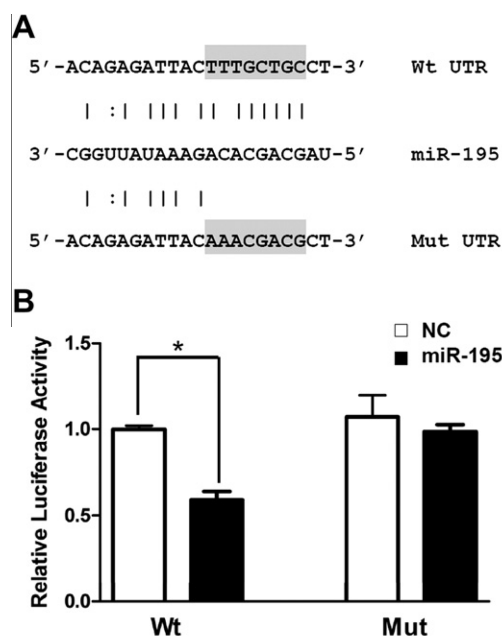

Fig. 6. Validation of CDK4 as the direct targets of miR-195. (A) A schematic of the MicroCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) predicted seed region in the 3' UTRs of CDK4 was shown, as well as the mutated sequences used in this study. (B) T24 cells were co transfected with 50 nM of either miR-195 or NC and 200 ng pmirGLO Dual-Luciferase miRNA Target Expression Vector comprising Wt or Mut 3'-UTR of CDK4. The relative firefly luciferase activity normalized with renilla luciferase was measured 48 h after transfection. miR-195 significant suppressed the firefly luciferase activity of construct with Wt 3'-UTR of CDK4. (*$P < 0.05$)

in bladder cancer cells, can induce G1-phase arrest by targeting the novel target CDK4. Our experimental data suggests an important role of miR-195 in bladder tumorigenesis and implicates restoration of miR-195 could be a potential therapeutic strategy for bladder cancer therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.01.027.

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