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Original Article

Decrement of miR-199a-5p contributes to the tumorigenesis of bladder urothelial carcinoma by regulating MLK3/NF-κB pathway

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Abstract: Aberrant miRNA expression is implicated in tumorigenesis. However, the role of miRNAs in bladder urothelial carcinoma still remains largely unknown. In this study, miR-199a-5p was validated to be significantly down-regulated in bladder urothelial carcinoma. In addition, restoring expression of miR-199a-5p inhibited the tumorigenesis of bladder urothelial carcinoma *in vitro* and *in vivo* by inducing the apoptosis and suppressing the proliferation of bladder cancerous cells. Further investigation reported that MLK3 was a direct target of miR-199a-5p. Moreover, the expression level of miR-199a-5p was conversely correlated with MLK3 in bladder cancerous cells. In addition, reintroduction of MLK3 was identified to promote the proliferation and inhibit the apoptotic rate of cells, which have been altered by miR-199a-5p through activating the NF-κB pathway. All together, decrement of miR-199a-5p contributes to the tumorigenesis of bladder cancer by directly regulating MLK3/NF-κB pathway and miR-199a-5p might be developed as a therapeutic target for treatment of the bladder urothelial carcinoma.

Keywords: miR-199a-5p, MLK3, NF-κB, bladder urothelial carcinoma

Introduction

Noncoding RNA is a functional RNA molecular which is not translated into a protein. MicroRNA is a small noncoding RNA (~22 nucleotides) which functions in post-transcriptional regulation of target gene expression by base-pairing with mRNA primarily at the 3'untranslated region (UTR) to cause mRNA degradation or translational repression [1, 2]. The alterations in miRNA expression have been associated with various cancers [1, 3].

Bladder urothelial carcinoma is one of the commonly diagnosed cancers in the world and has the highest rate of recurrence of any malignancy [4, 5]. In China, bladder cancer is the most common malignancy in the genitourinary tract and the fifth most common cancer in men [6]. Although numerous ongoing research efforts are directed to identify new strategies for effective therapy of cancer, the prospect is uncertain. New molecular mechanism needed to be clarified to identify the new potential therapeutic targets. The importance of miRNAs in the diagnosis and therapy of cancer has recently been emphasized [7, 8].

Mixed-lineage protein kinase 3 (MLK3) is a member of the mitogen-activated protein (MAP) kinase group that has been implicated in multiple signaling cascades, including the NF-κB pathway [9, 10], c-Jun NH(2)-terminal kinase (JNK) and p38 MAP kinase pathways [11, 12]. Moreover, the oncogenic activity of MLK3 has been identified in breast cancer [13, 14], prostate cancer [15], ovarian Cancer [16] and gastrointestinal cancer [17]. However, the mechanism of MLK3 dysregulation in cancer cells has not been fully clarified.

In this study, we found that miR-199a-5p not only was significantly down-regulated in bladder urothelial carcinoma cells but also could inhibit the tumorigenesis of bladder urothelial carcinoma *in vitro* and *in vivo* through regulating MLK3/NF-κB signaling pathway. This provides further insight into the pathogenesis of bladder urothelial carcinoma and indicates potential novel therapeutic targets for the treatment of bladder cancer.

Materials and methods

Tumor characteristics and cell lines

Bladder urothelial carcinoma and control tissue specimens were obtained from patients at General Hospital of the People's Liberation Army (Beijing, China) after surgical resection with informed consent. The tumor tissues and adjacent normal tissues were frozen in liquid nitrogen after resection. No patient in the current study received chemotherapy or radiation therapy before surgery. The institutional ethics committee of People's Armed Police Corps General Hospital approved the study, and all patients gave written informed consent. Detailed information about bladder urothelial carcinoma patients is summarized in Table S1. The tumor-node-metastasis (TNM) classification system was used for staging the bladder urothelial carcinoma. The human bladder cancer cell lines T24 and J82 were maintained in RPMI 1640 (Gibco). The medium was supplemented with 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 U/ml streptomycin. Cells were cultured at 37°C in 5% CO_2.

Transfection

The miRNA mimic, interfering RNA complex (si-MLK3) and negative control RNA duplexs (denoted NC) were synthesized by Genepharma (Shanghai, China). Cells were transfected using Lipofetamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, mixture containing the miRNA, siRNA, NC or medium (mock group) and lipofectamine 2000 was prepared and added directly to cells at a final oligonucleotide concentration of 50 nM. Total RNA or protein was extracted for qRT-PCR or western blot analysis.

Quantitative reverse transcription PCR

Total RNA was extracted from cells or tissues using Trizol (Invitrogen) according to the manufacturer's protocol. For cDNA synthesis, 1 μg of RNA was mixed with 500 ng of olig (dT) (Promega) or miRNA specific primers (invitrogen). Samples were reverse transcribed using M-MLV reverse transcriptase (Promega). The qPCR reaction mixture contained 12.5 μl of 2×SYBR green PCR mix (Fermetas), 0.3 μM of gene-specific forward and reverse primers, and 1 μl of cDNA template, made up to a final volume of 25 μl with distilled water. Cycling parameters were set as follows: initial activation step at 95°C for 10 min, denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 15 s. Melting curve analysis was performed at from 58°C to 95°C with stepwise fluorescence acquisition at every 1°C s^{-1}. The levels of gene expression were calculated by relative quantification using GAPDH or U6 snRNA as the endogenous reference genes. All samples were amplified in triplicate and the data analysis was carried out using the MxPro qPCR system software (Stratagene).

Western blotting analysis

The cell pellets were lysed in RIPA Lysis Buffer (50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% TritonX-100, 1% Sodium deoxycholate) for 30 min on ice. Lysates were centrifuged (12,000 g, 40 min, 4°C). Proteins at the same amount were separated by 15% SDS polyacrylamide gel electrophoresis and transferred electrophoretically to Hybond-ECL nitrocellulose membrane (Amersham Biosciences). Membranes were probed with mouse anti-MLK3 or Actin in 5% non-fat dry milk for 1 h at 37°C. After washing in PBS with 0.5% Tween 20 (PBST), the membrane was incubated in a 1:5,000 solution of HRP-conjugated goat anti-mouse secondary antibody at room temperature for 1 h. After further washing with PBST, the membrane was assayed by the enhanced chemiluminescence (ECL) Western blotting detection system.

Vector construction and luciferase reporter assay

To create a luciferase reporter construct, 3'UTR fragment of MLK3 containing putative binding sites for miR-199a-5p was inserted downstream of firefly luciferase in pGL3. Mutant 3'UTR, which carried the mutated sequence in the complementary site for miR-199a-5p, was generated using the fusion PCR method inserted downstream of firefly luciferase in pGL3. Cells were cotransfected with miRNA and 3'UTR or mutant 3'UTR luciferase reporter, using pRL-TK as control vector. At 48 h after transfection, Luciferase activity was measured using the Dual-Luciferase Assay kit (Promega) with a beta-counter luminometer. Relative luciferase activity was calculated as ratio of the raw firefly luciferase activity and the renilla luciferase activity.

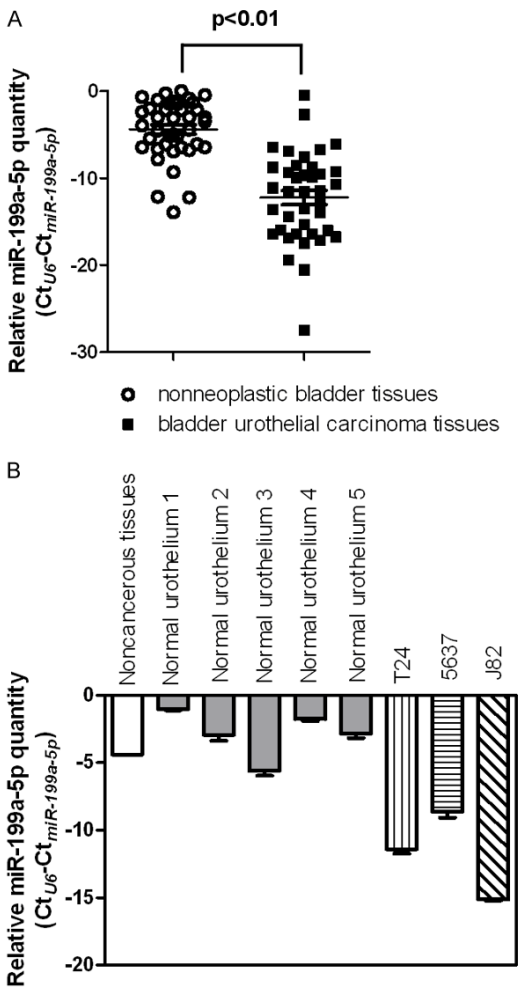

Figure 1. miR-199a-5p is decreased in bladder urothelial carcinoma cells. A. miR-199a-5p expression was analyzed in 40 bladder carcinoma tissues and matched nonneoplastic bladder tissues by qRT-PCR. miR-199a-5p in bladder carcinoma was significantly down-regulated compared with nonneoplastic tissues. Significant differences between groups were evaluated using a two-tailed Student's t test. B. The miR-199a-5p expression was analyzed in 5 biopsies of normal urothelium obtained from healthy individuals and 3 tumorigenic bladder urothelial carcinoma cell lines T24, 5637, and J82. It was found that the level of miR-199a-5p in the normal urothelium closely approximated the nonneoplastic bladder tissues matched to the tumors. Nevertheless, the miR-199a-5p levels in tumorigenic cell lines were significantly higher than in the normal urothelium.

Assessment of apoptosis

The transfected cells were collected by trypsinization and washed in PBS. Then cells were resuspended at a concentration of 1 × 10^6 cells/ml in Binding Buffer (0.01 M HEPES/NaOH, pH 7.4, 14 mM NaCl, 0.25 mM $CaCl_2$). 500 μl aliquots of cells were added into FACS tubes and mixed with 25 ng/ml fluorescein isothiocyanate-labeled annexin V and 10 mg/ml propidium iodide (PI) to incubation for 15 min at room temperature in the dark. Then the cells were analyzed immediately by flow cytometry.

In vitro proliferation assay

Cells were plated at 1 × 10^5 cells per well in complete RPMI 1640 medium and transfected by RNA duplex. Cell proliferation was quantitated by pulsing cells during the last 24 hours culture with 0.5 μCi per well of [^{3}H]-thymidine at liquid scintillation counter (Perkin Elmer).

Tumorigenicity assays

The experimental procedure involving animals was performed in accordance with the Guide for the Care and Use of Laboratory Animals National Institutes of Health and according to the institutional ethical guidelines for animal experiments. MiR-199a-5p- or NC-transfected cells (2 × 10^6) were suspended in 200 μl PBS and then injected subcutaneously into either side of the posterior flank of the male BALB/c athymic nude mouse at 6-8 weeks of age. 10 nude mice were included in each group and tumor growth was examined every 10 days. Tumor volume (V) was monitored by measuring the length (L) and width (W) of the tumor with calipers and was calculated with the formula V = (L × W^2) × 0.5.

Statistical analysis

Two-tailed Student's t test was used for comparisons of two independent groups. Significant correlations were verified using two-sided Pearson correlations. $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed with GraphPad Prism software.

Results

miR-199a-5p is consistently decreased in bladder urothelial carcinoma samples as compared with matched nonneoplastic bladder tissues

We have applied microarray to carry out the variation of miRNA expression profile in 25

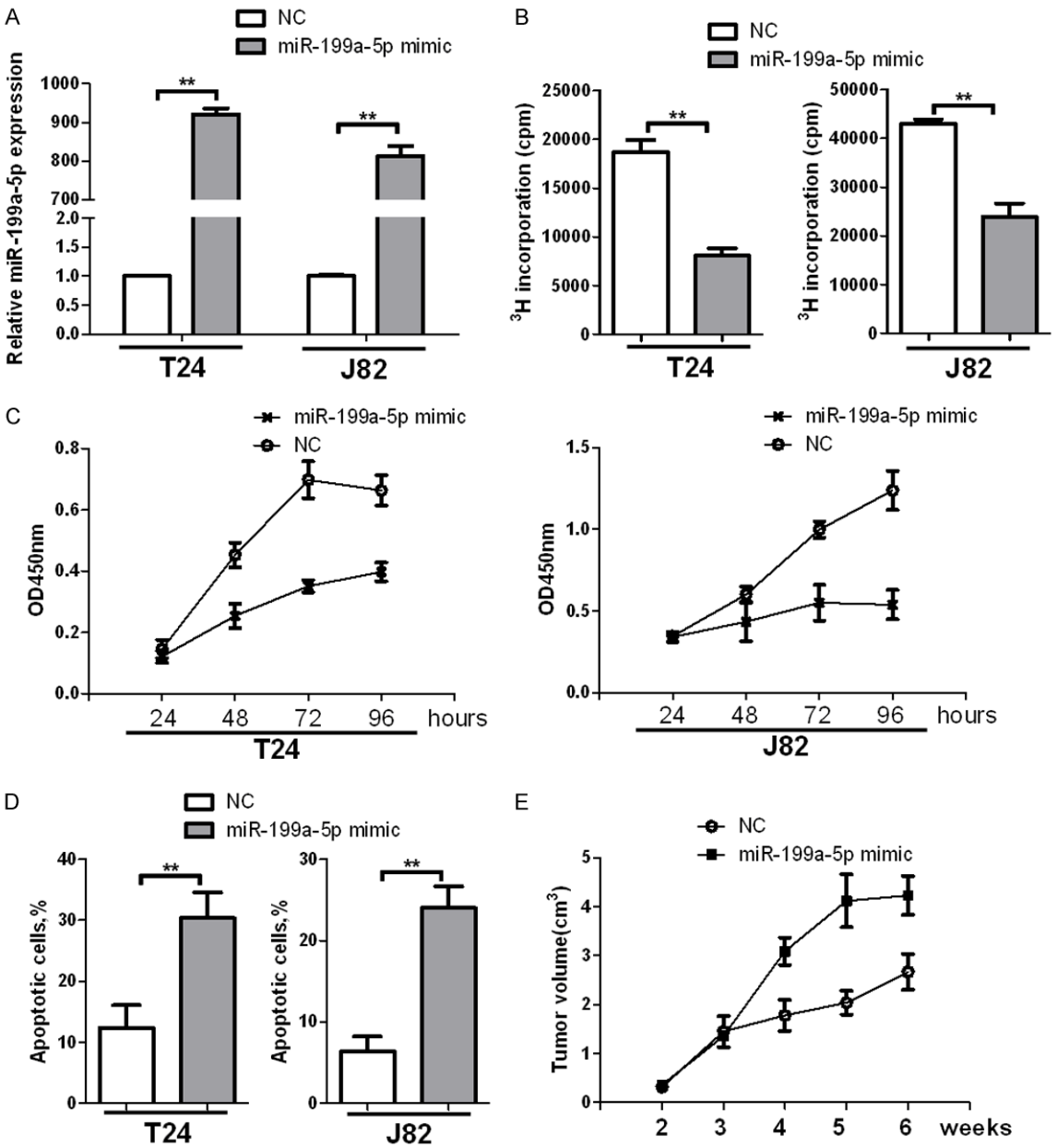

Figure 2. miR-199a-5p inhibits the tumorigenesis of bladder urothelial carcinoma *in vitro* and *in vivo*. A. Detection of the level of miR-199a-5p using qRT-PCR after cells transfected with miRNA mimic/NC in T24 cells and J82 cells. Data are representative of three independent experiments and shown as the mean ± SD. Significant differences between groups were evaluated using a two-tailed Student's t test. B. The proliferation of cells after transfection was detected by ^{3}H-thymidine incorporation. Data are representative of three independent experiments and shown as the mean ± SD. Significant differences between groups were evaluated using a two-tailed Student's t test. C. The proliferation of cells after transfection was detected by CCK8 assay. Data are mean ± SD of triplicate wells. D. The apoptotic rate of cells was detected by flow cytometry. Data are representative of three independent experiments and shown as the mean ± SD. Significant differences between groups were evaluated using a two-tailed Student's t test. E. Effect of miR-199a-5p restoration on the tumor growth. The cancerous cells T24 were subcutaneously transplanted into the host nude mice. Following transplantation, the tumors were allowed to grow for 3 weeks, and then the miR-199a-5p mimic was injected intratumorally. The results showed that the growth of the tumor was significantly inhibited following the increased level of miR-199a-5p.

bladder urothelial carcinoma and their matched nonneoplastic tissues and differential expressed miRNAs were validated by real-time quantitative PCR technique [18]. In this study,

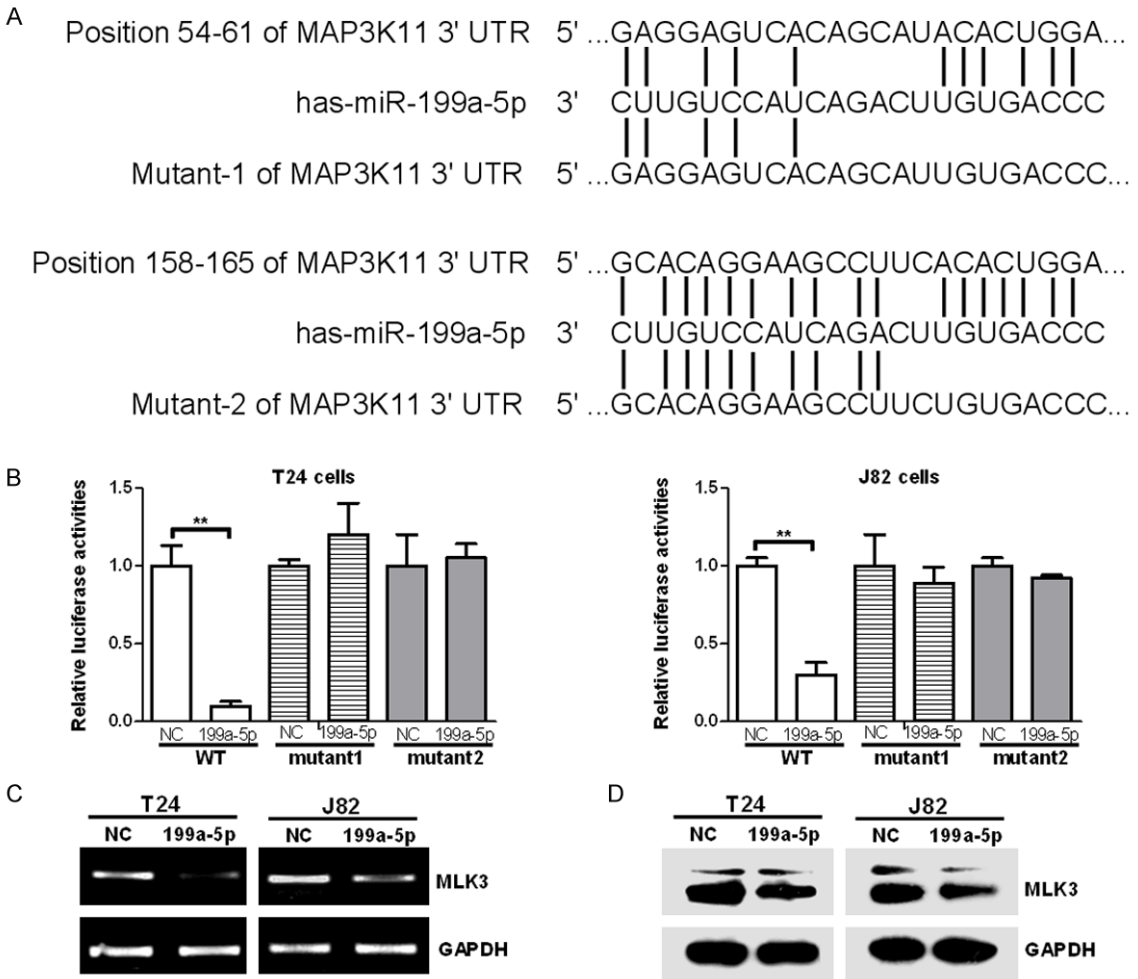

Figure 3. MLK3 is a direct target of miR-199a-5p in bladder cancerous cells and contributes to tumor-suppressing effect of miR-199-5p. A. The predicted miR-199a-5p binding sites in the 3'UTR of MLK3 mRNA were analyzed. The mutations on the "seed" sequences were designed as below. B. The relative luciferase activity was measured 48 h after T24 and J82 cells transfected by NC/miRNA mimic and WT/mutant1/mutant2 luciferase reporters. Data are representative of three independent experiments and shown as the mean ± SD. Significant differences between groups were evaluated using a two-tailed Student's t test. C. The MLK3 and GAPDH (internal control) mRNA levels were detected by RT-PCR. D. The MLK3 and GAPDH (internal control) protein levels were detected by western blotting.

we further found that miR-199a-5p expression was frequently decreased in 40 bladder urothelial carcinoma tissues as compared with distal nonneoplastic bladder tissues (**Figure 1A**). Furthermore, the miR-199a-5p expression was analyzed in 5 biopsies of normal urothelium obtained from healthy individuals and 3 tumorigenic bladder urothelial carcinoma cell lines T24, 5637, and J82. It was found that the level of miR-199a-5p in the normal urothelium closely approximated the nonneoplastic bladder tissues matched to the tumors (**Figure 1B**). On the contrary, in the tumorigenic cell lines miR-199a-5p expression was significantly decreased (**Figure 1B**). These results suggested that the reduced miR-199a-5p might contribute to the pathogenesis of bladder urothelial carcinoma.

MiR-199a-5p inhibits the tumorigenesis of bladder urothelial carcinoma in vitro and in vivo

To investigate whether the reduced miR-199a-5p is important in the development of bladder urothelial carcinoma, the miR-199a-5p mimic was transfected to the bladder urothelial carcinoma cell lines T24 and J82. qRT-PCR result confirmed that the level of miR-199a-5p was restored (**Figure 2A**). It was also found that

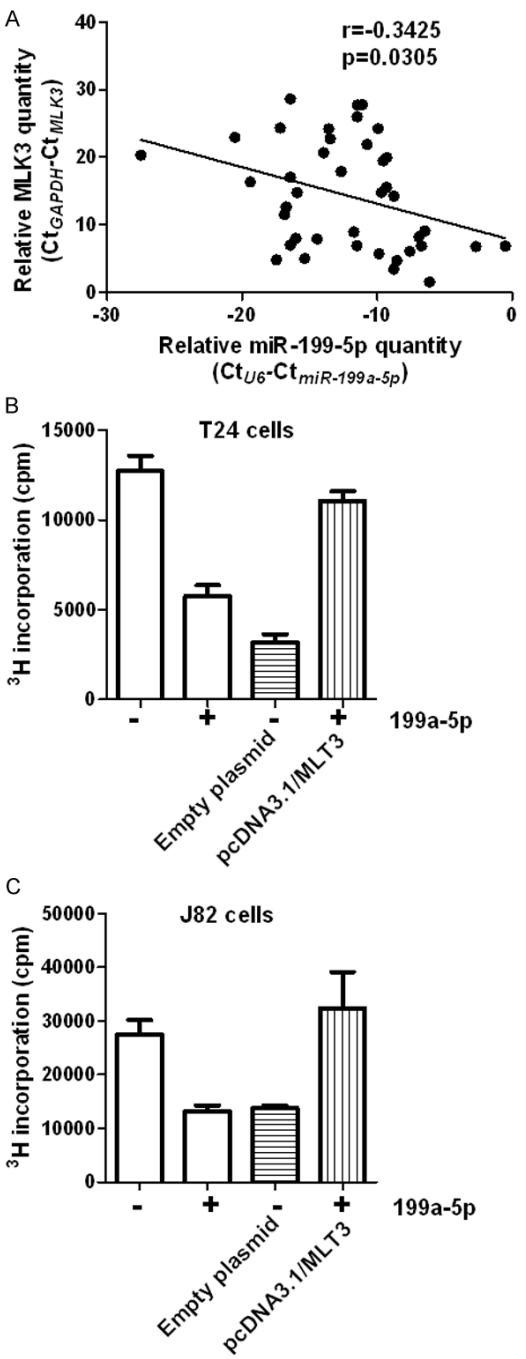

Figure 4. MLK3 contributes to tumor-suppressing effect of miR-199-5p. (A) The level of miR-199a-5p is significantly negatively associated with the level of MLK3 mRNA in the bladder urothelial carcinoma tissues. Significant correlations were verified using two-sided Pearson correlations. (B, C). The proliferation of T24 (B) and J82 (C) cells after transfection was detected by ^{3}H-thymidine incorporation. Data are mean ± SD of triplicate wells.

accompanied by elevated miR-199a-5p expression, the proliferation of bladder urothelial carcinoma cells T24 and J82 was markedly decreased (**Figure 2B**, **2C**) and the apoptotic rates of cells were substantially increased (**Figure 2D**). In addition, the role of miR-199a-5p was investigated *in vivo*. The cancerous cells T24 were subcutaneously transplanted into the host nude mice. Following transplantation, the tumors were allowed to grow for 3 weeks, and then the miR-199a-5p mimic was injected intratumorally. The results showed that the growth of tumor was significantly inhibited following the increased level of miR-199a-5p. Overall, the miR-199a-5p could be identified as a tumor suppressor in bladder urothelial carcinoma.

MLK3 is a direct target of miR-199a-5p in bladder cancerous cells

Functional mechanism of miRNAs depends on identification of their direct targets [1]. To investigate the tumor-suppressing mechanism of miR-199a-5p in bladder carcinoma, the direct targets of miR-199a-5p were further predicted by bioinformatics tools targetscan, pictar and miRanda. Noticeably, MLK3 (mixed lineage kinase-3), also known as MAP3K11, was identified within all three softwares, and its distinguishing feature is that MLK3 is involved in the cell proliferation [9-12]. To clarify the role of MLK3 in the miR-199a-5p inhibited cell proliferation, we determined whether MLK3 was a direct target of miR-199a-5p. Computational screening showed that there were two potential target sites of miR-199a-5p in the 3'UTR of MLK3 (**Figure 3A**). Then the 3'UTR of MLK3 harboring two potential binding sites for miR-199a-5p was inserted downstream of the luciferase gene and transfected into T24 and J82 cells together with miR-199a-5p mimic or negative-control (NC) and pRL-TK to normalize transfection. The result showed that miR-199a-5p could significantly down-regulate the luciferase activity of the reporter (**Figure 3B**). In addition, mutants of MLK3 3'UTR were constructed by deleting the miR-199a-5p target sites (**Figure 3A**) and cotransfected into T24 and J82 cells together with miR-199a-5p mimic or NC. The luciferase expression of mutants of MLK3 3' UTR was no longer subject to be regulated by miR-199a-5p (**Figure 3B**).

To further confirm that MLK3 was a target of miR-199a-5p, the effect of miR-199a-5p on the endogenous expression of MLK3 was deter-

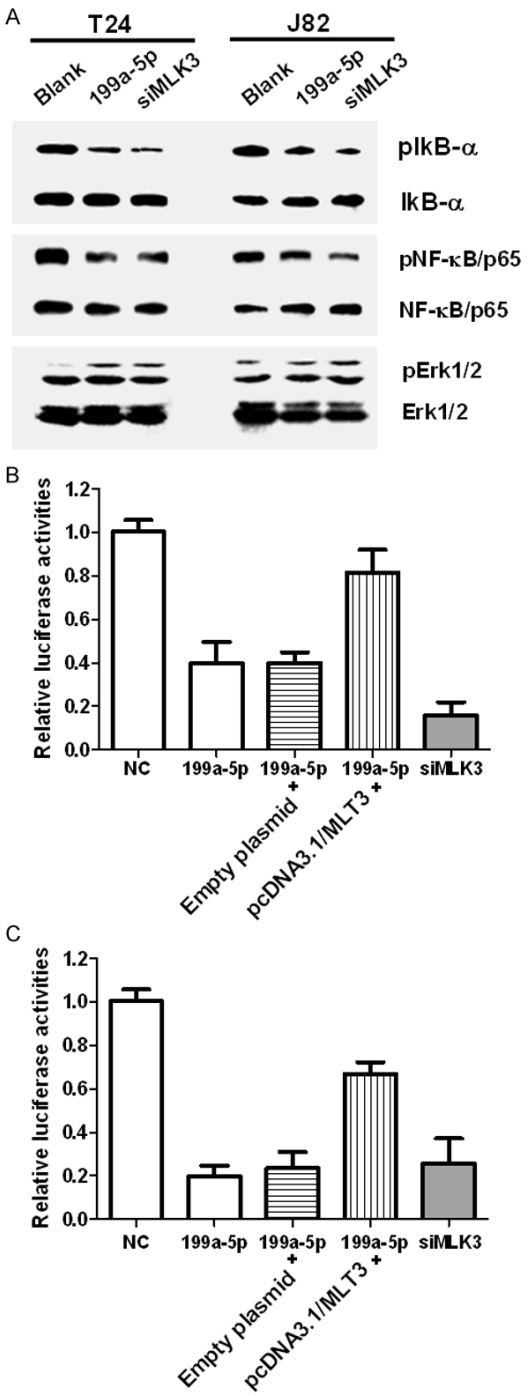

Figure 5. miR-199a-5p inhibits MLK3/IκB/NF-κB pathway in bladder urothelial carcinoma. (A) Examination of the phosphorytion of I IκB-a, NF-κB/p65 and Erk1/2 after cells transfected by miR-199a-5p mimic or siMLK3. Values under each lane indicate the expression intensity ratio of phospho-protein (pIκB-a, pNF-κB/p65 or pErk1/2) relative to total protein (IκB-a, NF-κB/p65 or Erk1/2). (B, C) The NF-κB-response-element-Luc reporter was cotransfected into T24 (B) and J82 (C) cells with miR-199a-5p (with or without MLK3 restoration) or siMLK3.

mined. miR-199a-5p mimics and NC were transfected into T24 or J82 cells respectively. The total protein was extracted from the cells at 72 h after the transfection. Compared to NC-transfected cells, the expression levels of both mRNA and protein of MLK3 were significantly reduced after transfected with miR-199a-5p mimic (**Figure 3C**, **3D**).

MLK3 contributes to the tumor-suppressing effect of miR-199-5p

Moreover, the mRNA level of MLK3 was detected in the bladder urothelial carcinoma tissues by qRT-PCR and further analysis showed that the level of miR-199a-5p is negatively associated with the level of MLK3 mRNA ($r = -0.6389$, $p < 0.001$) (**Figure 4A**). These results suggested that MLK3 was a target of miR-199a-5p in bladder cancerous cells.

To verify whether miR-199a-5p inhibits tumorigenesis through MLK3, the MLK3 expression was restored by constructing MLK3 expression plasmid pcDNA3.1/MLT3 without its 3'UTR and thereby cotransfecting pcDNA3.1/MLT3 with miR-199a-5p mimic to T24 and J82 cells. The results showed that the miR-199a-5p reduced proliferation was increased in the cells transfected by pcDNA3.1/MLT3 compared with empty plasmid (**Figure 4B**, **4C**). All together, these results suggest that miR-199a-5p suppresses the tumorigenesis of bladder urothelial carcinoma via directly inhibiting MLK3.

MiR-199a-5p inhibits MLK3/IκB/NF-κB pathway in bladder urothelial carcinoma

MLK3 is known as an activator of multiple MAPK signaling pathways and also has a function in phosphorylating IkB kinase and mediating the activation of the transcriptional factor NFκB, suggesting a role for dysregulated MLK3 in tumorigenesis [19]. We further determined which signal pathway was regulated by miR-199a-5p via targeting MLK3 accordingly. As shown in **Figure 5A**, both elevated expression of miR-199a-5p and inhibition of MLK3 suppressed the phosphorylation of IκB-a and NF-κB/p65 in both T24 and J82 cells (**Figure 5A**), while the phosphorylation of Erk1/2, a key regulator of MAPK signaling pathway, was not affected (**Figure 5A**). In addition, the luciferase activity of NF-κB activity reporter was decreased in T24 and J82 cells after transfected by miR-

199a-5p mimic and siMLK3 (**Figure 5B**). Moreover, further restoration of MLK3 could rescue the activation of NF-κB which have been reduced by miR-199a-5p mimic (**Figure 5B**, **5C**). Together, the results suggest that miR-199a-5p impacts NF-κB signaling pathway to suppress the tumorigenesis of bladder urothelial carcinoma via directly targeting MLK3.

Discussion

It is wildly known that miRNAs are involved in the pathogenesis of many diseases [20, 21], such as cancer [22, 23]. Accordingly, miRNA molecules are entering the clinic as diagnostic and prognostic biomarkers for patient stratification and also as therapeutic targets [23]. Although increasing evidence emphasizes that miRNAs have the diagnostic and prognostic potential in bladder cancer [24-27], the pivotal miRNA which can be used as the applied therapeutic target is still unclear. Better evidence is required to test their use in these fields [28].

This study aimed to further clarify the mechanism of relevant miRNAs and their downstream target genes involved in the pathogenesis of bladder urothelial carcinoma, thus developing new therapeutic target. For the first time, we established the miR-199a-5p/MLK3/NF-κB pathways in bladder urothelial carcinoma. We identified that miR-199a-5p was significantly decreased in the bladder carcinoma tissues compared with the non-tumorous tissues, suggesting miR-199a-5p acts as the tumour-suppressor in bladder cancer. Further investigation demonstrated that the elevated expression of miR-199a-5p significantly inhibited the growth of bladder tumor *in vivo*. With one exception, the level miR-199a-5p was not found to be correlated with tumor stage, and overall survival time. Consistently, the metastasis of bladder carcinoma cell lines T24 and J82 was not altered in both transwell assay and wound healing test after the level of miR-199a-5p has been increased (data not shown). These results suggest that miR-199a-5p might be involved in the tumorigenesis of bladder urothelial carcinoma, while not in the tumor progression.

It has been demonstrated that miRNAs functions in post-transcriptional regulation of gene expression through directly binding the 3'UTR of target mRNA, thus participate in a wide variety of biological responses. A given miRNA may have multiple different mRNA targets. Here, we identified that MLK3 is the target of miR-199a-5p in bladder urothelial carcinoma. Importantly, we also confirmed that the miR-199a-5p induced cell apoptosis and reduced cell proliferation were achieved through inhibiting MLK3/NF-κB pathway. The essential function for MLK3 in tumour pathogenesis has been elucidated [19]. In addition, constitutive NF-κB activation has been found in many human malignancies, including bladder carcinoma [29]. Our data was helpful in further elucidation the aberrant NF-κB activation in tumor cells.

In conclusion, our studies suggest that decrement of miR-199a-5p contributes to the tumorigenesis of bladder cancer by directly regulating MLK3/NF-κB pathway. And miR-199a-5p might be developed as a therapeutic target in the bladder urothelial carcinoma.

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Disclosure of conflict of interest

None.

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Table S1. Clinicopathological information of bladder carcinoma patients

Patient Number	Sex	Age (years)	Primary or recurrent tumor	Grade	Stage
1	M	68	Primary	High	II
2	F	73	Primary	Low	II
3	M	50	Primary	High	III
4	M	70	Primary	Low	II
5	F	55	Primary	Low	I
6	M	66	Primary	High	III
7	M	70	Recurrent	Low	II
8	F	51	Primary	High	II
9	M	66	Recurrent	Low	II
10	M	52	Primary	High	II
11	M	47	Recurrent	Low	II
12	F	77	Recurrent	Low	I
13	M	56	Primary	Low	I
14	M	56	Primary	High	III
15	F	49	Primary	High	III
16	M	56	Primary	Low	II
17	M	77	Primary	Low	II
18	M	50	Primary	Low	II
19	M	66	Primary	Low	II
20	M	40	Primary	Low	I
21	F	45	Recurrent	Low	I
22	M	39	Primary	High	III
23	M	49	Recurrent	High	II
24	M	56	Primary	Low	II
25	M	70	Recurrent	Low	II
26	M	43	Primary	Low	I
27	M	44	Primary	Low	II
28	M	32	Primary	Low	I
29	F	49	Primary	High	II
30	M	69	Primary	High	III
31	M	76	Primary	High	III
32	M	55	Primary	Low	II
33	F	35	Recurrent	Low	I
34	M	65	Primary	Low	II
35	M	68	Primary	High	II
36	F	54	Primary	High	III
37	M	47	Primary	Low	I
38	M	56	Primary	Low	II
39	M	66	Recurrent	Low	III
40	M	50	Recurrent	Low	III