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Original Article

MiR-124 retards bladder cancer growth by directly targeting CDK4

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MicroRNAs (miRNAs) are a class of non-coding small RNAs that act as negative regulators of gene expression by binding to the 3′-untranslated region (3′UTR) of target mRNAs. In order to investigate the physiological role of miR-124 in bladder cancer, target genes of miR-124 were predicted by the TargetScan software, and cyclin-dependent kinase (CDK4), which has been implicated as a regulator of cell cycle, was chosen for further study. MiR-124 could significantly repress CDK4 expression by targeting its binding site in the 3′UTR of CDK4 *in vitro*. In both bladder cancer cell lines and tissues, the expression of miR-124 was significantly down-regulated, while CDK4 expression was up-regulated. Ectopic expression of miR-124 in transplanted HT1197 cells resulted in the retardation of tumor growth in mouse tumor xenografts. And the expression of miR-124 and CDK4 showed an obvious inverse correlation in these xenograft tissues, which was also observed in human bladder cancer tissue samples. Taken together, our results strongly suggest that miR-124 can arrest cell cycle and restrain the growth of bladder cancer by targeting CDK4 directly.

Keywords miR-124; bladder cancer; CDK4

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Introduction

MicroRNAs (miRNA) are 21–25 nt long non-coding RNA molecules that inhibit gene expression at transcriptional and post-transcriptional level by binding to the 3′-untranslated region (3′UTR) of target mRNAs. MiRNAs can bind to partially complementary recognition sequences of mRNA, subsequently causing mRNA degradation or translation inhibition, thus effectively silencing their target genes [1–3]. Bioinformatic studies suggested that a third of all known genes may be regulated by miRNAs. MiRNAs have been reported to participate in many important cellular processes, such as apoptosis, cell differentiation and proliferation, tumor suppression, development, and metabolism. In recent years, more and more miRNAs have been detected by microarray analysis or other advanced technologies [4–7].

MiR-124 has been reported as a tumor suppressor in human glioma, breast cancer and many other malignant cancers [2,3,8,9]. However, there is no evidence to show that miR-124 can suppress the growth of human bladder cancer. To understand the molecular mechanisms associated with tumorigenesis of bladder cancer, the identification of regulatory target genes of miR-124 is critical. Target genes of miR-124 were predicted by TargetScan. The potential target gene cyclin-dependent kinase (*CDK4*) which can promote cell proliferation was chosen in this study. CDK4, a catalytic subunit of the protein kinase complex, is important for cell cycle G1 phase progression. The activity of the kinase complex is restricted to the G1-S phase, which is controlled by the regulatory subunits D-type cyclins and CDK inhibitor p16 (INK4a). This kinase was shown to be responsible for the phosphorylation of retinoblastoma gene product (Rb).

In this study, we showed that miR-124 could directly target CDK4 by targeting its 3′UTR in bladder cancer cell lines. MiR-124 expressed at a lower level in bladder cancer cell lines (HT-1197, HT-1376, J82, and 5637) than in normal bladder cells. On the contrary, the expression level of CDK4 was abnormally up-regulated in these cancer cells. And the expression of CDK4 and miR-124 showed a negative correlation in 27 human clinical bladder cancer tissues in the progress of bladder cancer. The cell cycle of bladder cancer cells was arrested significantly after the transfection of miR-24. Our results strongly suggest that miR-124 can suppress bladder cancer growth by targeting CDK4 both *in vitro* and *in vivo*.

Materials and Methods

Clinical cancer tissue samples

Human bladder cancer tissue samples were obtained from the Department of Urology, Second Hospital of Tianjin Medical University (Tianjin, China) under ethical assessment. The clinicopathological characteristics of 27 bladder cancer patients are summarized in Table 1.

Cell culture

Human bladder cancer cell lines (HT1197, HT1376, J82, and 5637) were obtained from the American Type Culture Collection (Manassas, USA). Human bladder epithelial cells were purchased from Sciencell (San Diego, USA). Human bladder cancer cell lines were maintained in Eagle's Minimum Essential Medium (Gibco, Gaithersburg , USA), except for the 5637 cell line which was cultured in RPMI-1640 medium. The complete medium was supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37°C in 5% CO_2.

Construction of recombinant expression vectors

The 3′UTR of CDK4 bearing the predicted binding site of miR-124 was cloned into the pGL3 vector (Promega, Madison, USA) and designated as pGL3-CDK4-3′UTR. pGL3-CDK4-3′UTR/mut plasmid containing the mutated binding site was also constructed. The coding region of CDK4 was cloned into pcDNA3.1 and pEGFP-C1 plasmid named as pcDNA3.1-CDK4 and pEGFP-CDK4, respectively. The sequences of miR-124 in the genome with ~200 bp redundancies were cloned into pEGFP-C1 named pEGFP-miR-124. The sequences of primers used in this study are shown in Table 2.

Dual-luciferase reporter assay

HEK-293T cells were seeded in 24-well plates and transfected with 400 ng luciferase vector by Lipofectamine™ 2000 (Life Technologies, Carlsbad, USA) at a confluence

Table 1. The information of 27 bladder cancer patients

No.	Gender	Age (years)	Histologic type	Primary tumor	Lymph gland	Migration
1	F	56	Transitional cell carcinoma	T3	N2	M0
2	M	56	Transitional cell carcinoma	T2B	N0	M0
3	M	55	Transitional cell carcinoma	T3	N2	M0
4	F	48	Transitional cell carcinoma	T2A	N0	M0
5	F	86	Transitional cell carcinoma	T2A	N1	M0
6	F	57	Transitional cell carcinoma	T2A	N1	M0
7	M	63	Transitional cell carcinoma	T2	N0	M0
8	M	54	Transitional cell carcinoma	T3	N2	M1
9	F	72	Transitional cell carcinoma	T2A	N1	M0
10	M	68	Transitional cell carcinoma	T2A	N1	M0
11	M	78	Transitional cell carcinoma	T1	N0	M1
12	M	66	Transitional cell carcinoma	T1	N0	M0
13	F	52	Transitional cell carcinoma	T1B	N0	M0
14	M	54	Transitional cell carcinoma	T1B	N0	M0
15	F	58	Squamous cell carcinoma	T2B	N0	M0
16	F	64	Transitional cell carcinoma	T1B	N1	M0
17	M	67	Transitional cell carcinoma	T1B	N0	M0
18	F	52	Transitional cell carcinoma	T1B	N0	M0
19	M	80	Transitional cell carcinoma	T1B	N1	M0
20	F	56	Transitional cell carcinoma	T1B	N1	M0
21	F	54	Transitional cell carcinoma	T2A	N1	M0
22	F	72	Transitional cell carcinoma	T2B	N0	M0
23	M	68	Transitional cell carcinoma	T1B	N1	M0
24	F	78	Transitional cell carcinoma	T2A	N1	M0
25	M	66	Transitional cell carcinoma	T2A	N1	M0
26	F	45	Transitional cell carcinoma	T2A	N0	M0
27	M	63	Transitional cell carcinoma	T1B	N1	M0

Table 2. Primer sequences used in this study

Name	Primer sequences	Restriction enzyme
pGL3-CDK4-3′UTR	Forward: TGC**TCTAGA**AGAGATGTTTCGTCGAAAGC	*Xba*I
	Reverse: TGC**GATATC**CCCCAAATATAAAGGTAGGG	*Eco*RV
pGL3-CDK4-3′UTR/mut	Forward: ACAGAGATTACTTTGCAGGCATTATGACATTCCCCT CCCACCTCTCCTTTTGAGGCTTC	
	Reverse: GAAGCCTCAAAAGGAGAGGTGGGAGGGGAATGT CATAATGCCTGCAAAGTAATCTCTGT	
pcDNA3.1-CDK4	Forward: CCG**CTCGAG**ATGACTGAATATAAACTTGTGGTAG	*Xho*I
	Reverse: TGC**GATATC**CCCCAAATATAAAGGTAGGG	*Eco*RV
pEGFP-CDK4	Forward: CCG**CTCGAG**CAATGACTGAATATAAACTTGTGGTAG	*Xho*I
	Reverse: CCG**GAATTC**CCCCAAATATAAAGGTAGGG	*Eco*RI
pEGFP-miR-124	Forward: CCG**CTCGAG**TCGCTGTTATCTCATTGTCTG	*Xho*I
	Reverse: CCG**GAATTC**CCCTGAGTCTGTTTGCATCTC	*Eco*RI
For qRT-PCR		
18S	Forward: AGGAATTCCCAGTAAGTGCG	
	Reverse: GCCTCACTAAACCATCCAA	
U6	Forward: CTCGCTTCGGCAGCACA	
	Reverse: AACGCTTCACGAATTTGCGT	
miR-124	Forward: TAAGGCACGCGGTGAATGCC	
	Reverse: Uni-miR qPCR Primer	
CDK4	Forward: AGTTCGTGAGGTGGCTTTA	
	Reverse: GGGTGCCTTGTCCAGATA	

of 60%−70%. Reporter assays were performed at 36 h post-transfection using the dual-luciferase assay system (Promega). To determine the transfection efficiency, 20 ng of pRL-SV-40 (Promega) was cotransfected as the control.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from bladder cells or bladder cancer tissues using TRIzol reagent (Bio Basic, Inc., Toronto, Canada) according to the manufacturer's instructions. Reverse transcription was performed using the PrimeScript™ RT reagent kit (Takara, Dalian, China). U6 snRNA and the house-keeping gene 18S rRNA were used as the endogenous control for miRNA and mRNA, respectively. All the samples were treated under the same conditions and analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR Premix Ex Taq™ (Takara) according to the manufacturer's protocol. The sequences of primers are shown in Table 2.

Western blot analysis

Protein samples were subject to sodium dodecyl sulfate-polyacryl-amide gel electrophoresis and then transferred to polyvinylidene difluoride membrane. The membrane was blocked in Tris-buffered saline-Tween buffer containing 5% low-fat milk for 50 min with gentle shaking. After being incubated with primary and secondary antibodies, membranes were developed using the chemiluminescence reagent kit (Thermo Scientific, Waltham, USA). Protein bands were quantified by densitometric analysis using Image Lab analysis software and normalized to GAPDH. The primary antibodies used were rabbit anti-CDK4 (1 : 1000) and rabbit anti-GAPDH (1 : 1000). All antibodies were purchased from Cell Signaling Technology (Beverly, USA).

Cell proliferation assay

Cell growth was determined by using a tetrazolium salt (MTS) cell proliferation assay kit (Promega). Twenty-four hours after transfection, the transfected cells were seeded into 96-well plates and incubated at 37°C for 3 days. Absorbance at 490 nm was determined using a microplate reader (OrionL Microplate Luminometer, Pforzheim, Germany).

Cell cycle analysis

Cells grown to 70%−90% confluence were detached by trypsinization, and fixed in 70% ethanol for 12 h at 4°C. After being washed with phosphate-buffered solution, cells were treated with RNase A (50 μg/ml) and stained with propidium iodide (PI; 25 μg/ml) for 30 min at 37°C. Samples were analyzed using a MoFlo XDP flow cytometer (Beckman Coulter, Pasadena, USA), and the distribution of cell cycle phases was determined using FlowJo software. The phase ratio (%) was calculated as the percentage of cells in the G1/S/G2 phase.

Subcutaneous tumor assay

Six-week-old nude mice BALB/c-A were purchased from the animal center of the Cancer Institute of Chinese Academy of Medical Science (Beijing, China). All experimental procedures were carried out according to the regulations and internal biosafety and bioethics guidelines of Tianjin Medical University and the Tianjin Municipal Science and Technology Commission. The HT-1179 subcutaneous model was established as described in [10]. The mice were randomly divided into two groups (five mice per group): miR-124 over-expression group and CDK4 over-expression group. Stable miR-124 or CDK4 expressing HT1197 cells were injected into the mice. Treatment was conducted every 4 days, until the end of the experiment. The tumor volume was measured with a caliper every 4 days and calculated using the formula, volume = length × width^2/2. At the end of the 24-day observation period, the mice were sacrificed and the tumor tissues were collected for formalin fixation and preparation of paraffin-embedded sections for immunohistochemistry analysis.

Immunohistochemistry analysis

Dissected tissues were fixed in 10% formalin and embedded in paraffin. Sections of 30 μm thickness were cut and subject to hematoxylin and eosin staining and immunohistochemistry. For immunohistochemistry, the sections were deparaffinized with xylene, rehydrated in ethanol, and boiled in 10 mM citrate buffer (pH 6.0) for 30 min for antigen retrieval. Endogenous peroxidase was blocked by treatment with 3% H_2O_2 in methanol for 15 min. The slides were blocked in serum for 30 min at room temperature, incubated with the anti-CDK4 primary antibody at a dilution of 1 : 80 at 4°C overnight followed by incubation with anti-rabbit secondary antibodies for 1 h, and visualized with Envision System (DAKO Corporation, Carpinteria, USA) and diaminobenzadine (DAB kit; Invitrogen, Carlsbad, USA). The slides were counterstained with hematoxylin. Negative controls were obtained by omission of primary antibodies.

Statistical analysis

Results were expressed as means ± standard error of the mean (SEM), and analyzed using GraphPad Prism 5 software, using *t*-tests for two-group comparisons and one-way analysis of varinace for three or more group comparisons. *P* value of <0.05 was considered to be statistically significant.

Results

MiR-124 directly targeted 3′UTR of *CDK4* mRNA

TargetScan is a kind of software widely used to predict target genes of miRNAs. In the present study, we used it to predict the miR-124 target genes. Results revealed a potential target site of miR-124 in the 3′UTR of CDK4 (**Fig. 1A**). To confirm this, pGL3-CDK4-3′UTR and pGL3-CDK4-3′UTR/mut containing miR-124 binding sites and a mutant binding site were constructed, respectively (**Fig. 1A,B**). The luciferase activity from pGL3-CDK4-3′UTR was obviously inhibited by miR-124 mimics but not by miR-NC, a negative control miRNA. However, miR-124 mimics exhibited no inhibition on pGL3-CDK4-3′UTR/mut (**Fig. 1C**). To further investigate whether miR-124 affects the expression of CDK4

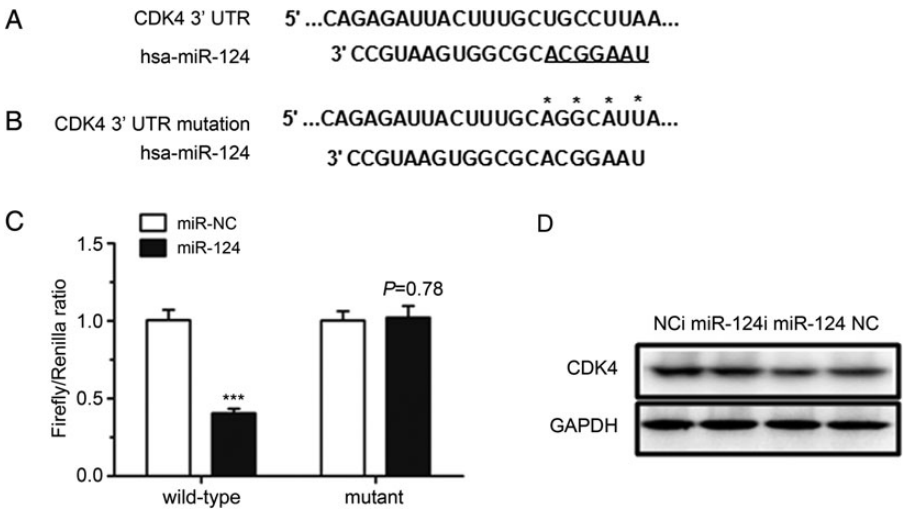

Figure 1. MiR-124 directly targeted *CDK4* gene by interaction with the 3′UTR (A) miR-124 binding sequence in the 3′UTR of CDK4. (B) Mutation of CDK4 3′UTR was generated on the complementary site for the seed region of miR-124. *Indicates the mutant nucleotide. (C) Luciferase activity of wild-type or mutant 3′UTR reporter gene in HEK-293T cells tranfected with or without miR-124 mimics. The normalized luciferase activity in the control group was used as relative luciferase activity. (D) Western blots showed the expression of CDK4 after NC, miR-124, NCi (as a control for miR-124 inhibitor), and miR-124 inhibitor transfected in HT1197 cells. GAPDH was used as an internal control. Each assay was performed in triplicate. ***$P < 0.001$.

at both post-transcription and translation levels, miR-124 mimics and miR-124 inhibitor were transfected into HT1197 cell lines, respectively. Western blot analysis showed that the expression level of CDK4 was significantly inhibited by miR-124. However, miR-NC mimics showed no repression on CDK4 expression (**Fig. 1D**). Endogenous protein level of CDK4 after miR-124 inhibitor transfection in HT1197 cell lines was also determined by western blotting. CDK4 expression was significantly up-regulated when miR-124 was inhibited (**Fig. 1D**). These data indicate that miR-124 inhibits the expression of CDK4 by directly targeting its binding site in the 3′UTR of *CDK4* mRNA.

Expression of miR-124 and CDK4 in bladder cancer cell lines and tissue samples

To investigate whether miR-124 was correlated with CDK4 in bladder cancer cells, qRT-PCR analysis was performed to detect the expression level of miR-124 and *CDK4* mRNA in four bladder cancer cell lines (HT1197, HT1376, J82, and 5637) and a normal bladder cell line (HBEC). The data showed that the expression level of miR-124 was also negatively correlated with CDK4 in these cell lines. Especially, the lowest expression of miR-124 and the highest expression of CDK4 were obtained in the HT1197 cell line (**Fig. 2A,B**). Expressions of miR-124 and CDK4 were also detected in 27 tissue samples from patients with bladder cancer. The expression of CDK4 in stage pT3 samples was higher than that in pT1 stage tissue samples. MiR-124 expression was obviously negatively correlated with CDK4 expression in tissue samples (**Fig. 2C,D**). Then the correlation analysis was performed for the miR-124 and CDK4 in tissue samples. The r value is -0.57, which demonstrates a negative correlation of miR-124 with CDK4 (**Fig. 2E**).

MiR-124 inhibited the growth of bladder cancer cells by inducing cell cycle arrest

To verify that miR-124 may function as a tumor suppressor in bladder cancer cells, over-expression of miR-124 mimics and pcDNA3.1-CDK4 was conducted, respectively. Western blot analysis was performed to identify the expression of CDK4 in HT1197 cells (**Fig. 3A,B**). The results showed that the expression of endogenous CDK4 was inhibited after miR-124 transfection. And the pcDNA3.1-CDK4 vector worked well. Then the HT1197 cell line transfected with miR-124 was found to grow more slowly than those cells transfected with pcDNA3.1. HT1197 cells transfected with pcDNA3.1-CDK4 showed vigorous growth. And when CDK4 and miR-124 were cotransfected, the growth of HT1197 cells showed no difference compared with cells transfected with pcDNA3.1 (**Fig. 3C**). Thus, these results indicate that ectopic miR-124 expression could inhibit the proliferation of HT1197 cell lines.

We further examined bladder cancer cell cycle distribution by flow cytometry to determine if it was accompanied with

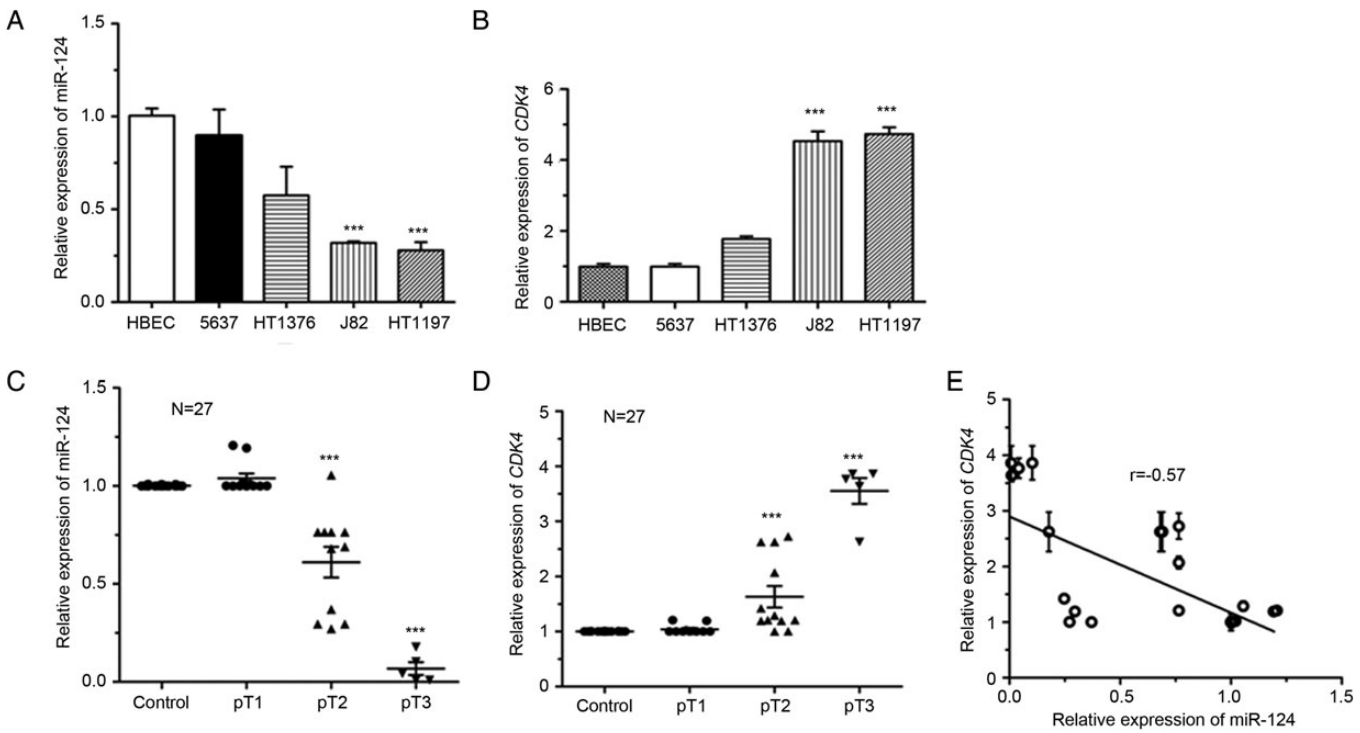

Figure 2. Expression level of miR-124 and CDK4 in bladder cancer cell lines and patient tissue samples (A, B) Expression of miR-124 and CDK4 in four bladder cancer cell lines and a normal bladder cell line (HBEC). (C, D) Expression of miR-124 and CDK4 was detected by qRT-PCR in 27 bladder cancer patient tissues, respectively. U6 and 18S were used as internal control in A, C and B, D respectively. (E) The correlation analysis was performed for the miR-124 and CDK4 in 27 tissue samples. Each assay was performed in triplicate. ***$P < 0.001$.

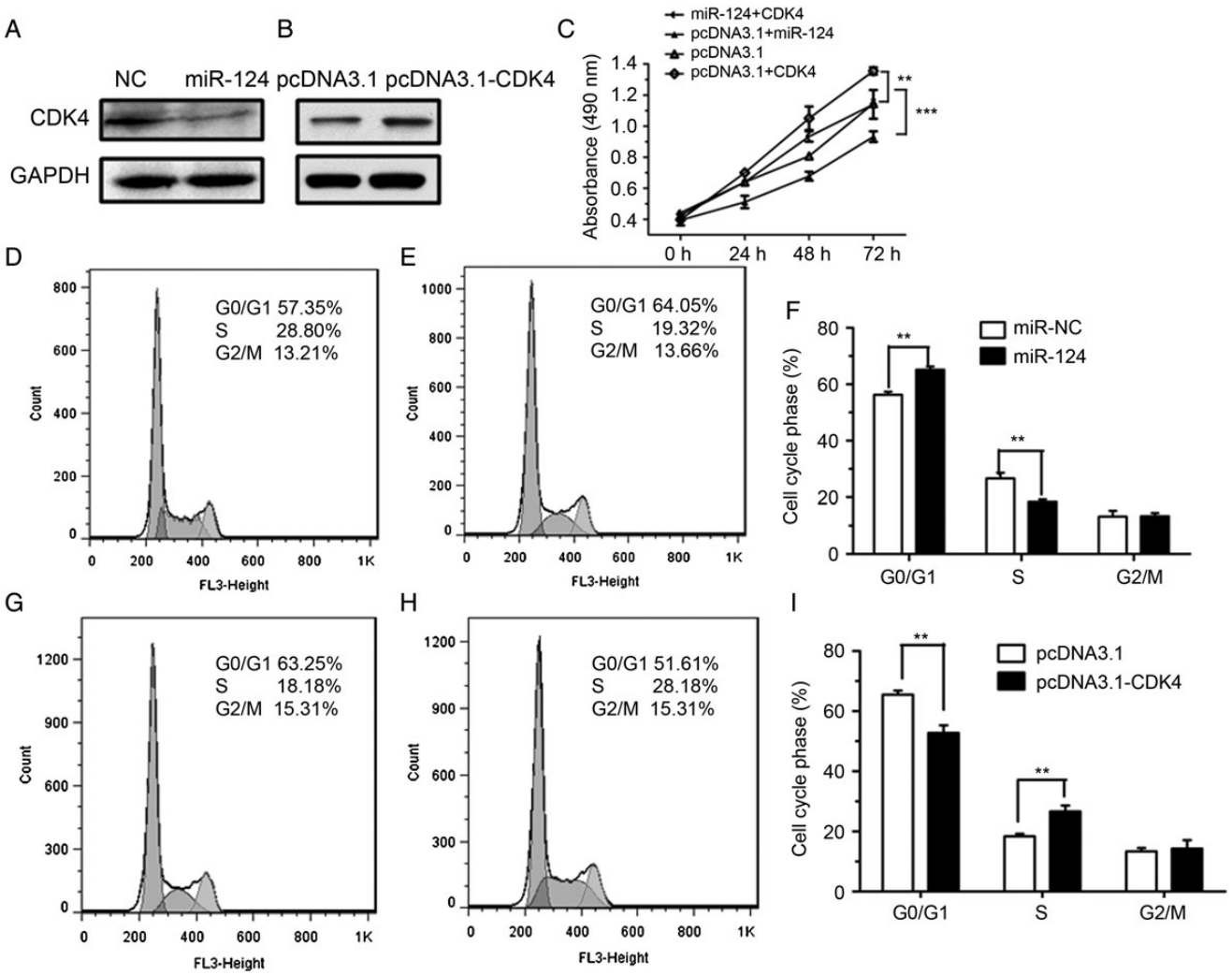

Figure 3. MiR-124 inhibited the proliferation of HT1197 cells by retarding the cell cycle targeting CDK4 (A, B) Western blots showed the expression level of CDK4 in the transfected HT1197 cell. GAPDH was used as an internal control. (C) HT1197 cells were transfected with miR-124 or CDK4. After re-seeding, cell numbers were measured with MTS at 0, 24, 48, and 72 h. (D,E) HT1197 cells were transfected with miR-NC or miR-124 mimics, respectively. (F) The phase ratio (%) of cells transfected with miR-NC or miR-124 mimics. (G,H) HT1197 cells were transfected with pcDNA3.1 or pcDNA3.1-CDK4, respectively. (I) The phase ratio (%) of cells transfected with pcDNA3.1 or pcDNA3.1-CDK4. Data were obtained by FlowJo software analysis and the mean of three experiments was shown. $^{**}P < 0.05$.

cell growth inhibition. Compared with the miR-NC group (**Fig. 3D**), HT1197 cells in the G0/G1 phase increased by 12% after miR-124 transfection (**Fig. 3E,F**). It is worth noting that the percentage of HT1197 cells in the S phase also reduced by ~10% compared with that of the miR-NC group ($P < 0.01$). On the contrary, HT1197 cells in the G0/G1 phase significantly decreased after pcDNA3.1-CDK4 transfection (**Fig. 3G–I**). Our results indicate that miR-124 could induce cell cycle arrest, which might contribute to the cell proliferation inhibition.

MiR-124 inhibited tumor growth *in vivo*

To investigate whether miR-124 suppresses tumor progression *in vivo*, HT1197 cells were transfected with pEGFP-miR-124 or pEGFP-CDK4, and then G418 was added into the medium. One month later, HT1197 cells stably expressing miR-124 or CDK4 were obtained. Then the cells were implanted subcutaneously into nude mice to generate tumor xenograft models. Four days post-implantation, all the control animals developed palpable tumors, while no tumors were detected in the miR-124 over-expressed mice. At the end of the experiment, the tumor growth rate was substantially lower in miR-124 over-expressing animals than in the control as judged by the tumor volume (**Fig. 4A,C**). On the 24th day after implantation, the average tumor volume of the miR-124 over-expressing group was much smaller than that of the control group. When tumor sections were stained with an anti-CDK4 antibody, the expression of CDK4 was

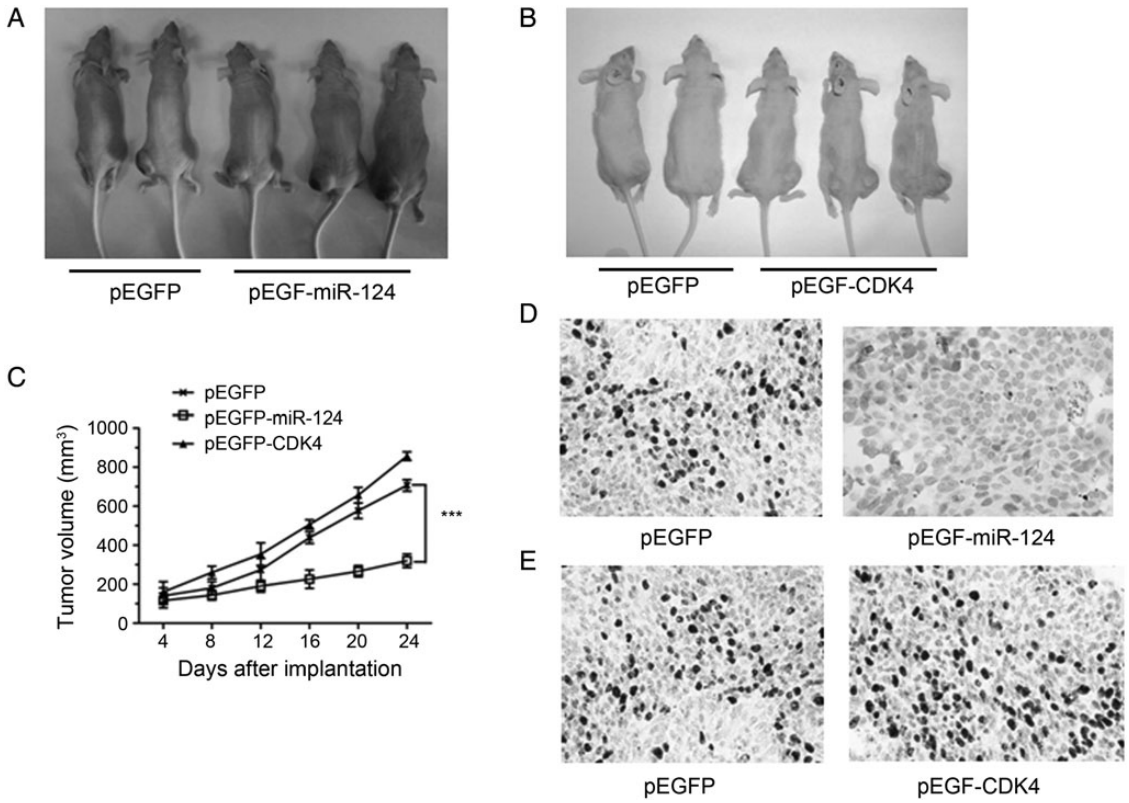

Figure 4. Tumor growth affected by miR-124 over-expression (A, B) HT1197 cells transfected with pEGFP-miR-124 or pEGFP-CDK4 were injected into nude mice. Twenty-four days after the injection, mice were photographed. (C) Tumor sizes were measured every 4 days and tumor growth curves were generated. (D, E) miR-124 inhibited the expression of CDK4 in tumor tissues. The immunohistochemistry assay showed that CDK4 were significantly down-regulated in pEGFP-miR-124 transfected tissues. And the expression of CDK4 was up-regulated in pEGFP-CDK4 transfected tumor (×200). Each assay was performed in triplicate at least. $^{***}P < 0.001$.

lower in miR-124 over-expressing tumors than in controls (**Fig. 4D**).

HT1197 cells over-expressing CDK4 were also implanted subcutaneously into nude mice to generate tumor xenograft models. All the measurement was followed as above. On the 24th day after implantation, the average tumor volume of CDK4 over-expressing group was much bigger than that of the control group. Immunohistochemical analysis consistently showed that the expression of CDK4 was up-regulated in pEGFP3.1-CDK4 transfected animals (**Fig. 4B,C,E**). These data suggest that miR-124 retards tumor development through the inhibition of CDK4 expression.

Discussion

miRNAs play major roles in regulating cell proliferation, apoptosis, and many important cellular processes [10–12]. The role of miRNA played in one particular cell line depends on the gene which the miRNA directly targeted. On this account, the same miRNA may play an opposite role in different cells. Consequently, the identification of miRNA target gene is critical. MiR-124 has been widely studied in many human malignant cancers. MiR-124 is significantly down-regulated in colorectal cancer (CRC) compared with the adjacent non-tumor colorectal tissues. Over-expression of miR-124 leads to increased apoptosis of CRC cells and reduced tumor growth *in vitro* and *in vivo*, through suppressing the expression of STAT3 by directly binding to its 3′UTR, which indicates that miR-124 exhibits a tumor suppressor-like activity in CRC cells via regulation of proliferation [13]. MiR-124 was also shown to directly target the 3′UTR of rho-associated, coiled-coil-containing protein kinase 1 (*ROCK1*) gene to repress ROCK1 expression in the U87MG human glioma cell line, which suggests that miR-124 may function as an anti-migration and anti-invasion factor in glioma. Son of sevenless homolog 1(*SOS1*) was also identified as a target gene of miR-124 in human glioma [3,13–16]. In breast cancer cells, miR-124 plays a critical role in inhibiting invasive and metastatic potential by directly targeting the *CD151* genes. *CDK4* has been reported as a downstream target gene of miR-124 in human brain [6,15,17–21].

Though miR-124 has been reported as a tumor suppressor in many malignant cancers, the function and mechanism of miR-124 in human bladder cancer have not been investigated yet. In this study, our dual-luciferase reporter assay showed that miR-124 could significantly suppress CDK4

expression by targeting its binding site in the 3′UTR of CDK4 in 293T cells. Western blot analysis showed consistent results that endogenous CDK4 expression can be inhibited by miR-124 in bladder cancer cells. We detected the expression variation of miRNAs in bladder cancer cells and tissues. It was found that miR-124 expression was significantly down-regulated when compared with normal tissues in both animal model and human bladder cancer cells, which indicates that miR-124 may function as a tumor suppressor in bladder cancer. Cell cycle analysis was further performed to elucidate the mechanism of miR-124 in bladder cancer. Our results showed that the HT1197 cell line was significantly retarded in the G1 phase in miR-124 mimics transfected cells. The tumor development was also significantly retarded when the bladder cancer cell line stably expressing miR-124 was injected into mice. In conclusion, we confirm that miR-124 acts as a tumor suppressor in bladder cancer by directly targeting *CDK4* gene,

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