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MiR-26b Acts as a Tumor Suppressor microRNA in Prostate Cancer*

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Abstract microRNAs (miRNAs/miRs) are a class of single-stranded non-coding RNA molecules of 19-24 nucleotides in length. *Via* specific mRNA complementary paring of target genes, miRNAs are able to regulate the expression of mRNA levels or inhibit protein translation following transcription. miRNAs can act as oncogenes or tumor suppressors. We have previously reported that miR-26b was expressed at a lower level in PCa cells compared to normal prostate cells and it inhibited autophagy. Here, we further revealed the role of miR-26b in prostate cancer cells. We found that over-expression of miR-26b suppressed prostate cancer cell proliferation, invasion and migration *in vitro* and inhibited the growth of prostate xenograft tumor *in vivo*. We have processed a gene expression microarray assay to investigate the concrete mechanism of miR-26b inhibition on prostate cancer cells proliferation and migration. We found that miR-26b significantly up-regulated 57 genes expression level, and simultaneously down-regulated 55 genes expression (fold change >2; $P < 0.05$) in PC-3. The differential genes were most associated with the regulation process of cell proliferation, apoptotic process, protein phosphorylation and ubiquitination respectively, and enriched in multiple pathways including TNF signaling pathway and TGF-β signaling pathway. Among these filtered genes, CEACAM6 was significantly down regulated by miR-26b with a 2.17-fold. We identified a putative miR-26b binding site on 3′UTR region of CEACAM6 and validated that miR-26b bound to the 3′UTR region of CEACAM6 mRNA, suggesting that CEACAM6 is a direct target of miR-26b. Our results suggest that miR-26b suppresses cell proliferation by targeting CEACAM6 in PCa cells and miR-26b may be a candidate tumor-suppressor in prostate cancer.

Key words miR-26b, prostate cancer cells, cell proliferation and migration, expression microarray assay, CEACAM6
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microRNAs (miRNAs/miRs) are a class of single-stranded non-coding RNA molecules of 19–24 nucleotides in length. miRNAs play important roles in significant biological processes such as proliferation, cell cycle, apoptosis, differentiation, migration and metabolism. *Via* specific mRNA complementary paring of target genes, miRNAs are able to regulate the expression of mRNA levels or inhibit protein translation following transcription[1]. miRNAs can act as oncogenes and tumor suppressors and several miRNAs are associated with cancer development and progression through the modulation of multiple cellular processes[2]. For example, in prostate cancer, the downregulated microRNAs including miR-23b-5p, miR-139-5p, miR-205-5p, miR-221-3p, miR-375-3p, miR-382-5p and miR-384-5p, could result in abnormal expression of target gene Runt related transcription factor (Runx), and contribute to tumor progression[3]. Also, over-expressed oncogenic miRNA-4534 plays critical roles in prostate tumorigenesis by targeting Phosphatase and tensin homolog (PTEN) mRNA[4]. Thus, the dysregulated miRNAs and their target genes could be regarded as attractive therapeutic targets for prostate cancer treatment.

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microRNA-26b (miR-26b), at a length of 22nt, is important in the regulation of cell biology processes. Growing evidences indicates that miR-26b is downregulated in human cancers, including nasopharyngeal carcinoma[5], breast cancer[6], parathyroid tumors[7] and non-small cell lung cancer[8]. miR-26b inhibits cell invasion by directly regulating La-related protein 1 (LARP1), an oncogene in PCa cells [9]. In addition,Verghese *et al*. have established that miR-26b was down-regulated in carcinoma-associated fibroblasts from ER-positive breast cancers, leading to enhanced cell migration and invasion [10]. However, the function of miR-26b in prostate tumorgenesis remains unclear.

CEACAM6 is a member of carcinoembryonic antigen-related cell adhesion molecules family. It has been reported to affect the cell adhesion, migration, invasion and metastatic behavior of tumor cells [11-13]. CEACAM6 is abnormally overexpressed in a wide variety of human cancers including pancreatic cancer, colon cancer, breast cancer and hepatocellular carcinoma [14-16]. Over-expression of CEACAM6 promotes pancreatic cancer progression by stimulating angiogenesis [17]. CEACAM6 also plays a crucial role in several biological process including proliferation[18-19].

In this study, we found that over-expression of miR-26b suppressed prostate cancer cell proliferation, invasion and migration *in vitro* and inhibited the growth of prostate xenograft tumor *in vivo*. In the microarray analysis we found that miR-26b significantly up-regulated 57 genes expression level, and simultaneously down-regulated 55 genes expression (fold change > 2; $P < 0.05$) in PC-3 cells. Among these filtered genes, CEACAM6 was significantly down regulated by miR-26b with a 2.17-fold. We identified a putative miR-26b binding site on 3′UTR region of CEACAM6 and validated that miR-26b bound to the 3′UTR region of CEACAM6 mRNA, suggesting that CEACAM6 is a direct and potential target of miR-26b. Our results suggest that miR-26b suppresses cell proliferation by targeting CEACAM6 in PCa cells and miR-26b may be a candidate tumor-suppressor in prostate cancer.

1 Materials and methods

1.1 Reagents

The antibody for CEACAM6 (ab134074) was obtained from Abcam (Shanghai, China). Goat Anti-Rabbit IgG, Goat Anti-Mouse IgG and BCA Protein Assay kit were obtained from CoWin Biotechnology (Beijing, China). Mouse Anti-β-actin antibody and cell lysis buffer RIPA reagent were from Beyotime Biotechnology (Beijing, China). RNAiso-Plus Reagent, cDNA Reverse Transcription Kit, SYBR® PrimeScript™ RT-PCR Kit Ⅱ, and restriction enzymes were from TaKaRa (Dalian, China). Lipofectamine 2000 Reagent was from Life Technology (Grand Island, NY, USA). Dual-Luciferase Reporter Assay System (E1910) kit was from Promega (Madison, WI, USA).

1.2 microRNAs and DNA plasmid construction

miR-26b mimics, CEACAM6 siRNA and their respective control were acquired from Gene Pharma (Shanghai, China). The DNA sequences contained the wild-type or mutant regions of the CEACAM6 3′UTR were respectively cloned into *Xho* Ⅰ-*Not* Ⅰ-digested psiCHECKTM-2 vectors. The mutant CEACAM6 3′UTR sequence was prepared with five nucleotides mutated in the seed region for miR-26b. The primers used for CEACAM6 were as follow: CEACAM6 3′UTR-wt forward, 5′ AGG AAG ACT GGC AGA TTG 3′; reverse, 5′ AAT ACA AGA ACA GTG GG 3′. The site-directed mutagenesis was performed with the following primer sets: CEACAM6 3′ UTR-mut forward, 5′ TAT GTA GTT AGC ATA ATA CAG AAG TCC CCT 3′; reverse, 5′ TAG ACT GGA ATC TCC TGT GTT CTT T 3′.

1.3 Cell culture and transfection

Human prostate cancer cell lines C4-2 and PC-3 were purchased from the China Institute of Basic Medicine in Peking Union Medical College. Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (Thermo Scientific, USA) and penicillin/streptomycin (100 U/ml penicillin with 100 mg/L streptomycin). Cells were incubated at 37℃ with 5% CO_2. For transient transfection, Lipofectamine 2000 reagent (Life Technologies, USA) was used according to the manufacturer's protocol.

1.4 Cell growth and cell viability assays

PC-3 and C4-2 cells were plated on 24-well plates at a density of 2×10^4 per well and cultured until attachment. Then the cells were transfected with 50 pmol of miR-26b or miR-NC and cultured for 1 to 6 days. Cell growth curve was assessed by trypan blue exclusion assay. Cell viability assay was also performed to investigate the effect of CEACAM6 knockdown on PC-3 and C4-2 cells. The cells were

plated on 96-well plates at a density of 2×10^3 per well until attachment, then transfected the cells with correlated miRNA mimics or CEACAM6 siRNA plasmids. The cell viability was stained with CCK-8 kit and the live cell absorbance was measured with microplate reader.

1.5 Cell clonogenic assay

Logarithmic growth phase PC-3 and C4-2 cells were plated on 6-well plates and cultured until attachment. Cells were transfected with 100 pmol of miR-26b or miR-NC. 48 h later, cells were harvested and counted, and 1×10^3 cells were replated on 60 mm culture dishes. Growth was allowed to continue for 14 days until clone formation was assessed by crystal violet staining.

1.6 Wound healing assay

Logarithmic growth phase PC-3 and C4-2 cells were plated in 10 cm plates and cultured until 90% confluence in the incubator. Cells were transfected with 100 pmol of miR-26b or miR-NC. 48 h post transfection, a straight scratch was made in the middle of the sub-confluent layer of cells. All the wells were loaded with 2 ml fresh medium containing 50 μmol/L mitomycin C and photographed using an inverted phase microscope equipped with a camera. The plates were put back in the incubator and images were then captured again after 24 h and 48 h to monitor the migration ability of the cells to the wound.

1.7 Mouse xenograft model and management

Nude mice (BALB/c, aged 4 weeks) purchased from Shanghai Changling Biological Technology Co. Ltd (SCXR2013-0018) were maintained in laminar flow cabinets under pathogen free condition. PC-3 cells (4×10^6 cells) with stably expressing miR-NC or miR-26b were diluted in D-Hanks solution, and then injected under the right armpit skin of nude mice. At the 14th day after injection, the mice were weighed and the tumors were measured every three days with calipers. For calculation of tumor volumes, the formula was used as $V=\pi/6\times L\times W\times W$ where L and W were length and width respectively. After 31 days, the mice were sacrificed and the tumors were collected for weighting and correlated testing. Total RNA isolated from the tumor tissues were used for analysis of expression levels of miR-26b. The tumor tissue were fixed in formaldehyde, routinely processed and paraffin embedded. Five micron-thick sections were prepared, and hematoxylin-eosin staining or immunohistochemical staining with streptavidin-biotin immunoperoxidase assay was performed using antibodies to Ki-67(1∶1 000, Santa Cruz Biotechnology, USA).

1.8 Microarray hybridation

PC-3 cells were plated in 6-well plates and cultured until attachment, then transfected with 100 pmol of miR-26b or miR-NC using Lipofectamine 2000 reagent according to the manufacturer indications. 48 h post transfection, cells were collected in RNAiso Plus (A9907-1) for microarray hybridation using lncRNA4.0 Array (Beijing Capital Bio Technology). lncRNA V4.0 containing about 34 235 mRNA and 40 916 lncRNA oligo nucleotide probes.

1.9 Luciferase assay

PC-3 and C4-2 cells were plated in 24-well plates and cultured until attachment, then co-transfected with 50 pmol of miR-26b and 50 ng of psiCHECKTM-2-CEACAM6 3′ UTR or psiCHECKTM-2-CEACAM6 3′ UTR mutant plasmids. Cells were harvested 48 h after transfection, and luciferase activity was analyzed by the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) according to the manufacturer's instructions.

1.10 Western blot analysis

Parental, miRNA mimics transfected or plasmids transfected PC-3 and C4-2 cells were washed three times with prechilled phosphate-buffered saline (PBS). The cells were collected and then lysed with RIPA reagent (Beytime, Beijing). A total of 30 mg of lysates were subjected to SDS-PAGE on a 10% SDS-polyacrylamide gel. Separated proteins were transferred to PVDF membranes (Merck Millipore, USA) and incubated with primary antibodies for human CEACAM6 (1∶1 000 dilution) or β-actin (1∶1 000 dilution) followed by incubation with an HRP- conjugated secondary antibody. The bands were detected using the Immubilon ECL system (Merck Millipore, USA) and ChemiDoc XRS + imaging system (Bio-Rad, USA). Band densities were determined using Image J software.

1.11 Statistical analysis

GraphPad Prism 6.0 (GraphPad Software Inc, USA) was used for all calculations. All values are represented by the mean ± *SD*. Statistical comparisons between the two groups were performed using Student's *t*-test. Differences among groups were determined by a two-way ANOVA followed by Dunnett's test. The threshold for statistical significance was set at $P < 0.05$.

2 Results

2.1 miR-26b inhibits cell proliferation and migration of PCa cells

To investigate the inhibitory effect of miR-26b on the cell proliferation and migration of prostate cancer cells, we treated the PC-3 and C4-2 cells with miR-26b mimics for a time gradient of 6 days, and we counted the live cells every day. As shown in Figure 1a and 1b, miR-26b had significantly inhibited the cell proliferation of PC-3 and C4-2 cells. Notably, miR-26b treatment for 48 h resulted in 27% inhibition of PC-3 cell growth and 22% inhibition of C4-2 cell growth compared to the miR-NC-treated group.

To further confirm the inhibitory effect of miR-26b on PCa cell proliferation, we performed clonogenic assays. As shown in Figure 1c, the clone diameter of miR-26b overexpressed cells were smaller than the miR-NC transfected cells. Overexpression of miR-26b in PC-3 and C4-2 cells could significantly reduce the number of clones formed by PC-3 and C4-2 cells compared to the non-control group.

In addition, to verify the miR-26b inhibitory on cell invasion and migration of prostate cancer cells, we performed wound healing assays. As shown in Figure 1d, the relative wound width in the miR-26b overexpressed cells group was wider than miR-NC transfected cells group at the same time point, indicating that miR-26b treatment significantly inhibited the closure of the wound. These results suggest that miR-26b could reduce the cell invasion and migration ability of prostate cancer cells.

Taken together, our data investigate that miR-26b efficiently inhibits cell proliferation, invasion and migration of prostate cancer cells.

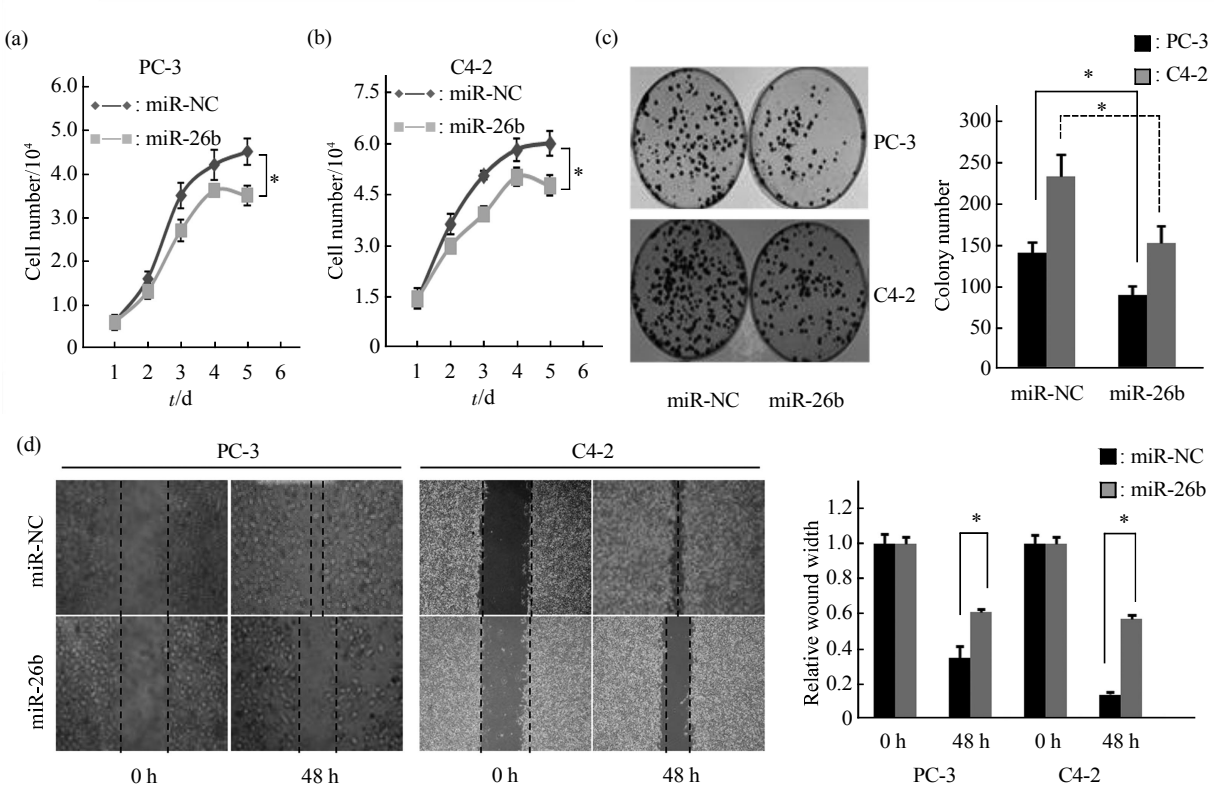

Fig. 1 miR-26b inhibits the growth of PCa cells

(a, b) Cell growth was assessed by trypan blue exclusion assay every day for 6 days in PC-3 and C4-2 cells transfected with miR-26b or miR-NC. (c) Effect of miR-26b on colony formation. 48 h post transfection with miR-26b or miR-NC, 1×10^3 cells were harvested, replated in new petri dishes and cultured for 14 days. Clone formation was assessed with crystal violet staining. (d) Wound healing assay was assessed on PC-3 and C4-2 cells transfected with miR-26b or miR-NC. The graph shows the relative wound width in the miR-26b-treated cells and miR-NC-treated cells. Data are expressed as the mean ± SD of three independent experiments, $^*P < 0.05$.

2.2 miR-26b inhibits tumor growth in a xenograft nude mouse model

Next, to further verify the role of miR-26b and determine its inhibition on prostate tumor growth *in vivo*, we established prostate cancer mouse xenograft model using miR-26b overexpressed PC-3 or miR-NC

overexpressed PC-3 cells. For the both models, miR-26b or miR-NC mimics were transfected to the PC-3 cells for 48 h before injecting cells under the right armpit skin of the mice. Tumor sizes were measured every third day and tumor volumes were calculated ($V=\pi/6\times L\times W\times W$, L: length, W: width) until the mice were sacrificed. As shown in Figure 2b, miR-26b overexpression resulted in a significant reduction of the subcutaneous tumor volume compared with the miR-NC control group. The average weight of miR-26b tumors were about 30% less than the control group (Figure 2c). Real-time PCR analyses confirmed that miR-26b expression was significantly increased in miR-26b overexpressed xenograft tumor (Figure 2d). We next performed H&E staining and IHC staining to evaluate the pathological changes in each of the prostate xenograft tumors. After overexpressing miR-26b, the Ki-67 expression had been inhibited in tumors, and densities of nuclei were sparser than the miR-NC transfected tumors (Figure 2e). Collectively, these data were consistent with the *in vitro* cell proliferation assay results and confirmed the anti-proliferative effect of miR-26b. These results show that miR-26b may serve as a potential tumor suppressor in prostate cancer *in vivo* and *in vitro*.

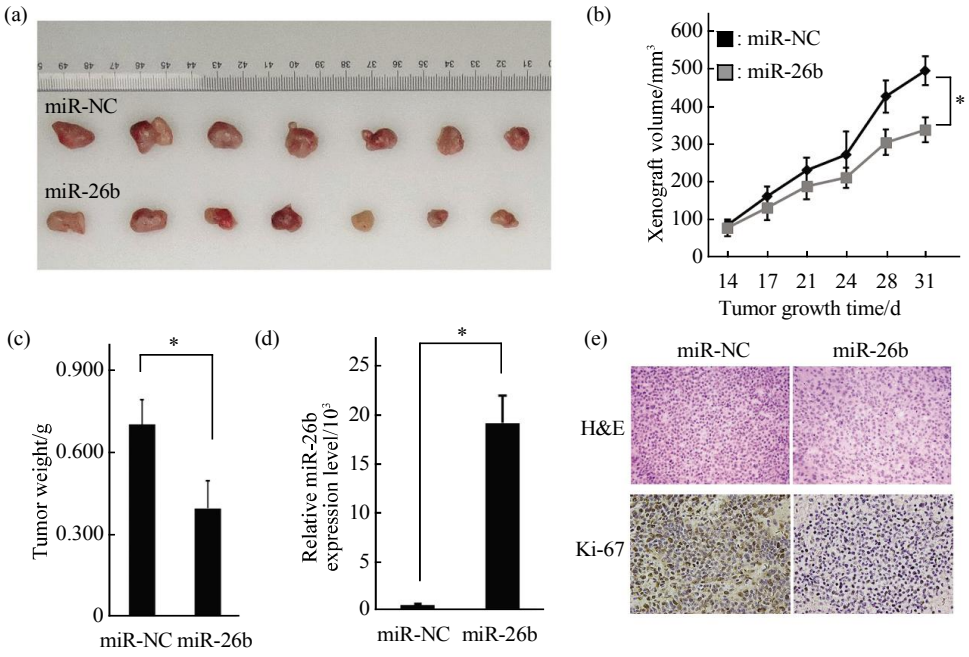

Fig. 2 miR-26b inhibits tumor growth in a xenograft model

(a) Growth curves of control and miR-26b tumors. Nude mice (BALB/c, aged 4 weeks) were injected with PC-3 cells (4×10^6 cells) stably expressing miR-26b or miR-NC. (b) Tumor sizes were measured every third day after two weeks to calculate tumor volumes. (c, d) Tumors collected for weighting and analyses of miR-26b expression. The average weight of miR-26b tumors were about 43% of the control tumors. MiR-26b expression was significantly increased in miR-26b tumors measured by real-time PCR. (e) H&E and Ki-67 Immunohistochemistry stained tissue from miR-26b tumors and miR-NC tumors. Data are expressed as the mean ± *SD* of three independent experiments. **P* < 0.05.

2.3 Microarray analysis of mir-26b regulated genes expression

To investigate the concrete mechanism of miR-26b inhibition on prostate cancer cells proliferation and migration, we applied mRNA expression microarray to comparatively quantify the differential genes regulated by miR-26b. In total, 21543 non-redundant genes were identified, of which 121 genes displayed quantifiable differential expression level in response to miR-26b. Differential genes clustered as shown in Figure 3a. Among these genes, 57 genes displayed more than or equal to 2-fold increased expression level, and 55 genes were down-regulated by miR-26b more than 2-fold ($P < 0.05$) (Figure 3b). Differential genes were list in Table 1. Differential genes set was used for gene ontology and pathways annotation and enrichment analysis. We used three gene ontology categories: biological process, molecular function, and cellular compartment. As shown in Figure 3c, the differential genes were most

associated with the regulation process of cell proliferation, apoptotic process, protein phosphorylation and ubiquitination respectively. In point of molecular function annotation, the miR-26b could regulate genes which were essential for ATP binding or protein serine/threonine kinase activity. These data indicate that miR-26b can be involved in regulation of cell proliferation and apoptosis *via* participating in protein modification and serious kinase pathways.

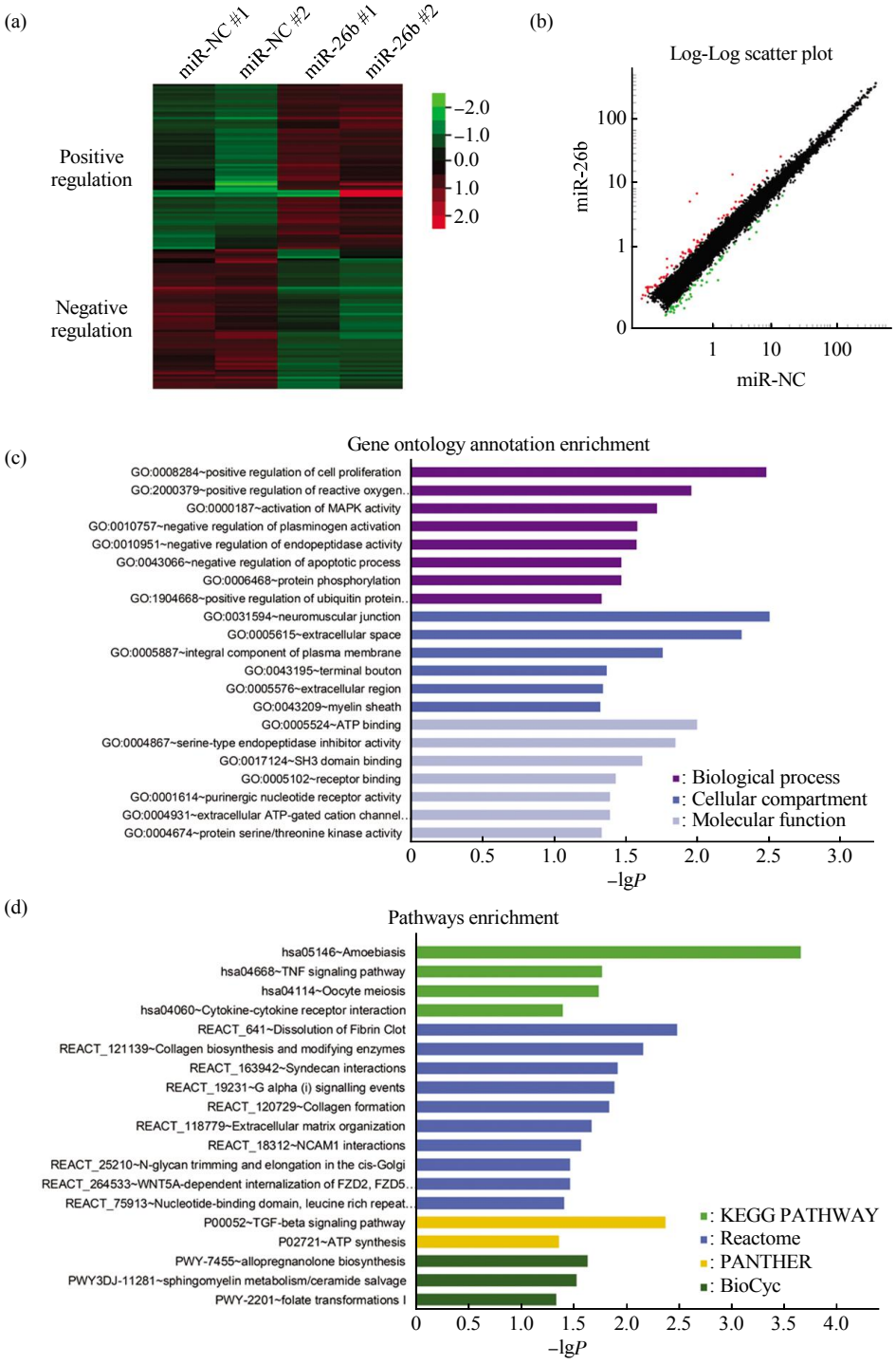

Fig. 3 Gene expression microarray data analysis

(a) Cluster analysis of differentially expressed gene mRNAs in cells-treated with miR-26b compared to the control miR-NC. Each row represents a single gene. Colorbar indicated the fold change. Red, genes upregulated by miR-26b; green, genes decreased. (b) Scatter plot indicating differing mRNA expression between cells-treated with miR-2b and miR-NC control. (c) and (d) Gene ontology annotation and pathways enrichment of the differential genes according to multiple database.

Table 1 Summary of identified differential genes regulated by miR-26b in prostate cancer cell line PC-3

	Unregulated genes	Downregulated genes
miR-26b group compared to NC group	THBS1, PIGS, MAN1A1, KRT80, COLGALT2, STK35, KIAA1467, LIMCH1, DDAH1, GNA14, SGMS2, EHD1, KLHL4, FRMD5, STARD7, SFXN1, HCAR1, ACVR2B, TAB3, MAGT1, ATP5A1, AVEN, PRSS35, SERPINE2, KRTAP4-9, LOC100506885, CLDN1, CHIC1, SYNGR1, BMP3, TMEM164, NRM, CSRNP3, PMEPA1, PTPRJ, EPHA7, PPP6C, CDC42SE1, MANSC4, GMFB, COL5A2, PLK1, ATP8A1, ADAM19, CPA4, LINC00920, TLL1, CPEB3, NEDD9, ROR1, SFXN2, TTC6, LOC401220, PAPL, ERN2, LIME1, SLC29A4	EVI2B, LOC285095, IL13RA2, SERPINB4, SERPINB3, ARNT2, VTCN1, LOC102723946, FA2H, TRABD2B, HPSE, DNER, FAM83A, CYP27B1, EFCAB2, GDNF, PID1, IL17F, C1orf54, SH2D2A, SERPINB2, NQO2, LINC01219, AKR1C1, FLRT1, CAMK2A, TRPV3, NMU, CCL20, CEACAM6, TLE6, SAPCD2, RGS4, EPGN, FAM25A, LOC102477328, UBE2S, SYN1, GAS2L3, FTMT, KIF14, MTHFD2L, DBF4, CABYR, P2RX7, TROAP, IL24, SPATA12, TSNAX, TAGLN3, C12orf76, CXCL3, NEK2, RPS26, PTPN22

We then performed a pathway clustering analysis through four pathways annotation database, including Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome dababase, Protein Analysis Through Evolutionary Relationships (PANTHER), and BioCyc database. As shown in Figure 3d, miR-26b mediated multiple pathways including TNF signaling pathway and TGF-β signaling pathway, suggesting that miR-26b may be involved in cell invasion and migration. miR-26b also regulated genes participated in ATP synthesis and extracellular matrix organization, suggesting that miR-26b may be associated with exocytosis and cell invasion process.

In general, the mRNA expression microarray analysis data showe that miR-26b may be involved in multiple cell process of tumorigenesis in prostate cancer cells *via* dysregulating plenty of genes expressed in cellular process associated with prostate cancer cell proliferation, invasion and migration.

2.4 CEACAM6 is a target of miR-26b

Based on microarray and bioinformatics analysis, CEACAM6 emerged as a candidate target for miR-26b binding. We analyzed the sequence of CEACAM6 and identified a putative miR-26b binding site (1200-GGAGAUUCCAGUCUACUUGAG-1221)(Figure 4a) and performed promoter-binding luciferase report assays to examine the miR-26b binding on 3′UTR of CEACAM6 mRNA. We constructed luciferase report plasmids as followed: psiCHECKTM2-CEACAM6-UTR plasmid containing wild type 3′UTR region of CEACAM6 and psiCHECKTM2-CEACAM6-UTR-mut plasmid containing 5 nucleotides mutant of 3′UTR region of CEACAM6. The dual-luciferase report assay result showed that miR-26b remarkably suppressed the luciferase expression promotion on the downstream of wildtype CEACAM6 3′UTR region, but have no effect on the mutant CEACAM6 3′UTR region both in PC-3 and C4-2 cells (Figure 4b). MiR-26b could also suppress the CEACAM6 mRNA expression level and protein levels in both PC-3 and C4-2 cells (Figure 4c and 4d), suggesting that CEACAM6 was the direct binding target of miR-26b in prostate cancer cells. To verify the inhibition effect of miR-26b on CEACAM6, we constructed CEACAM6 knockdown in both PC3 and C4-2 cell lines. In CEACAM6 knockdown PC-3 and C4-2 cells, the protein level of CEACAM6 were both decreased (Figure 4e). Interestingly, we also observed that both PC-3 and C4-2 cells proliferation were suppressed by CEACAM6 knockdown(Figure 4f). As shown in Figure 4f, the miR-26b could inhibit cell proliferation of both PC-3 and C4-2 cells, and the CEACAM6 silence have the similar efficiency on the PC-3 and C4-2 cells. Knockdown of CEACAM6 decreased cell viability by 22.2% and 17.6% in PC-3 and C4-2 respectively compared with the group control. Our data indicate that miR-26b may inhibit prostate cell proliferation depending on a mechanism that miR-26b bind to 3′UTR region of CEACAM6 and lead the mRNA degradation of CEACAM6.

3 Discussion

Recent studies have demonstrated that miR-26b was frequently downregulated in various tumors, including breast cancer[20], lung cancer[21], and colorectal cancer [22] suggesting a potential role of miR-26b in tumor suppression. It has been reported to be a critical regulator in carcinogenesis and tumor progression by acting as a tumor suppressor gene in various types of cancer[20, 23-24]. Here, we focused on the role of miR-26b in prostate carcinogenesis. Consistent with these reports, we have previously reported that miR-26b was expressed at a lower level in PCa cells compared to

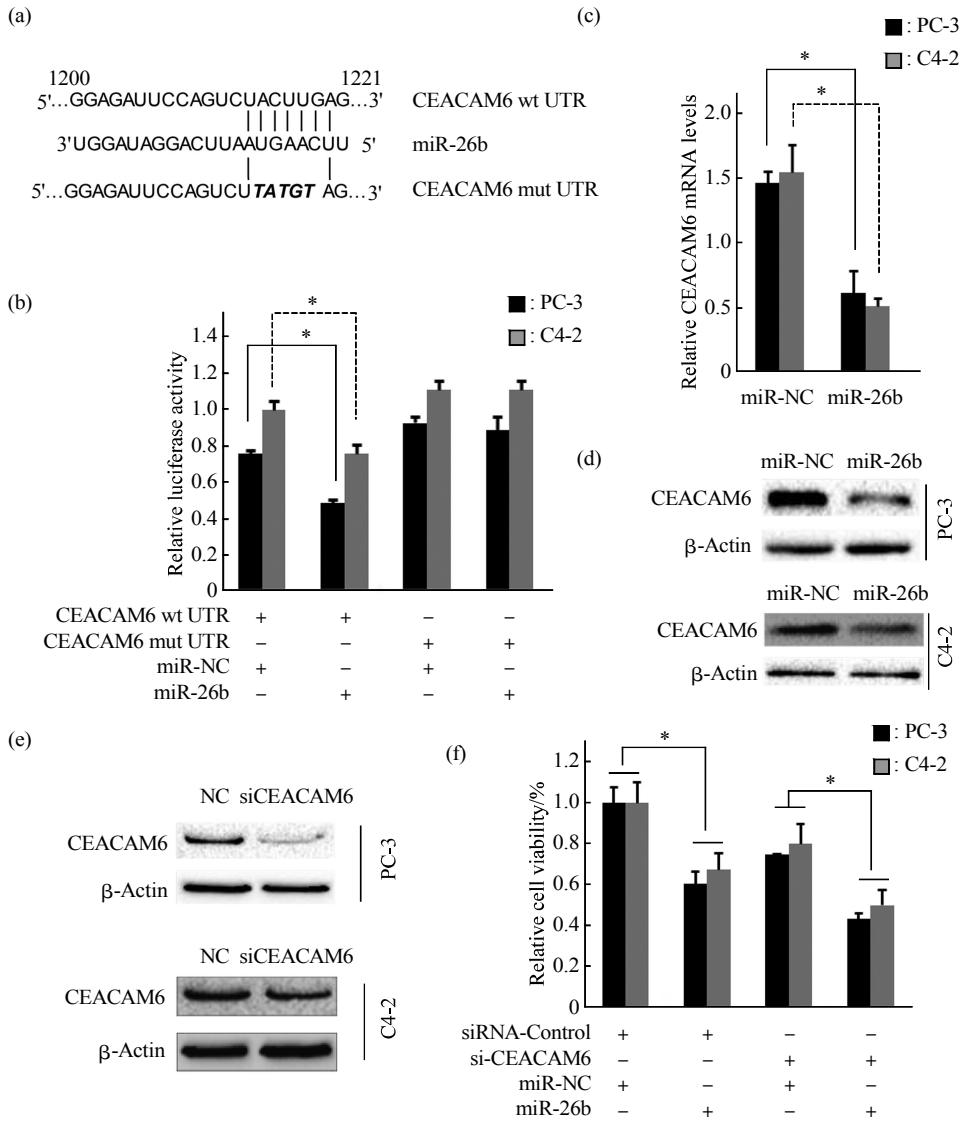

Fig. 4 CEACAM6 is identified as a potential target of miR-26b

(a) Prediction of miR-26b targets was done in silico and CEACAM6 was selected as one of the top candidates. The CEACAM6 gene is predicted to contain miR-26b binding sites in its 3'UTR, starting at positions 1200. The bottom line shows the mutations introduced into the two putative miR-26b binding sites. (b) The effect of miR-26b on reporters of psiCHECKTM2-CEACAM6 and psiCHECKTM2-CEACAM6 -mut in PC-3 and C4-2 cells was analyzed with a luminometer to capture luciferase activity. The activity of Renilla luciferase was normalized to that of firefly luciferase. (c, d) The mRNA and protein levels of CEACAM6 were examined in PC-3 and C4-2 cells transfected with miR-26b by RT-PCR and Western blot. (e, f) Knockdown of CEACAM6 transfected together miR-26b on cell viability was assessed by CCK-8 in PC-3 and C4-2 cells. β-actin was used as an internal control. Data are expressed as the mean ± *SD* of three independent experiments.*$P < 0.05$.

normal prostate cells and it inhibited autophagy in PC-3 and C4-2 cells through down regulation of ULK2 expression[25].

The results presented here indicate that miR-26b could act as a tumor suppressor in prostate cancer cells. Over-expression of miR-26b suppressed cell growth *in vitro* and inhibited the tumor growth in prostate cancer xenograft *in vivo*. These findings corroborate with the work of Goto *et al*.[9] reported it in cancer cell invasion of prostate cancer, Yan *et al*.[23] in breast cancer, Xiao *et al*.[26] in lung cancer and Zhang *et al*.[27] in colorectal cancer on the role of miR-26b in tumor.

To investigate the concrete mechanism of miR-26b inhibition on prostate cancer cells proliferation and migration, we applied mRNA

expression microarray to comparatively quantify the differential genes regulated by miR-26b. Among 21543 non-redundant identified genes, 57 genes displayed 2-fold increased expression level, and 55 genes were down-regulated by miR-26b more than 2-fold ($P < 0.05$). This result suggests that miR-26 has limit inhibit activity in prostate cancer cells. After annotation enrichment of differential genes set, we found that the differential genes were most associated with the regulation process of cell proliferation, apoptotic process, protein phosphorylation and ubiquitination respectively, suggesting that miR-26b regulated the prostate cancer cell proliferation and apoptosis process *via* regulating protein modification and protein activation and degradation [28]. In point of molecular function annotation, the miR-26b could regulate genes which were essential for ATP binding or protein serine/threonine kinase activity. These data indicate that miR-26b could be involved in regulation of cell proliferation and apoptosis via participating in protein modification and serious kinase pathways.

We then performed a pathway clustering analysis and found that miR-26b mediated multiple pathways including TNF signaling pathway and TGF-β signaling pathway. miR-26b also regulated genes participated in ATP synthesis and extracellular matrix organization, suggesting that miR-26b may be associated with exocytosis and cell invasion process [29]. In general, the mRNA expression microarray analysis data showe that miR-26b may be involved in multiple cell process of tumorigenesis in prostate cancer cells *via* dysregulating plenty of genes expressed in cellular process associated with prostate cancer cell proliferation, invasion and migration.

Among all the down-regulated genes, we found that miR-26b directly targeting CEACAM6 3′ UTR. As it has been reported CEACAM6 is involved in several biological processes such as cell proliferation, migration and known to playing a crucial role in human cancer such as gastric cancer [30], pancreatic cancer[31]. We further studied the role of CEACAM6 in the proliferation inhibition effect of miR-26b in PCa cells. MiR-26b clearly down-regulate CEACAM6 expression to inhibit PCa cells. CEACAM6 is overexpressed in a wide variety of carcinomas such as pancreatic, colon, breast, gastric, and hepatocellular carcinomas, knockdown of CEACAM6 has been shown to reverse anoikis resistance and inhibit the metastatic potential in pancreatic cancer mouse xenograft models *in vivo* by enhancing caspase-3-mediated apoptosis. Also, knockdown of CEACAM6 caused a significant inhibition of PC-3 and C4-2 cell growth compared to the control group, confirming that CEACAM6 contribute to the anti-proliferation of miR-26b in PCa cells. We identified a putative miR-26b binding site on 3'UTR region of CEACAM6 and validated that miR-26b bound to the 3′UTR region of CEACAM6 mRNA, suggesting that CEACAM6 is a direct and potential target of miR-26b. In general, our results suggest that miR-26b suppresses cell proliferation by targeting CEACAM6 in PCa cells and miR-26b may be a candidate tumor-suppressor in prostate cancer.

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MiR-26b 是前列腺癌中的抑癌微 RNA*

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摘要 微 RNAs(又称 miRNAs 或 miRs)是一类长度为 19～24 个核苷酸的单链非编码 RNA 分子．miRNA 通过与其靶向的 mRNA 分子序列特异性互补配对，调节 mRNA 表达水平，抑制转录后的蛋白质翻译．miRNA 在肿瘤中既可作为致癌因子也可作为抑癌因子．本研究前期已报道 miR-26b 在前列腺癌细胞系中低表达，并且抑制细胞自噬．本研究进一步全面揭示 miR-26b 对前列腺肿瘤细胞的作用．我们发现过表达 miR-26b 能够在体外抑制前列腺癌细胞的增殖和侵袭，并抑制裸鼠体内原位异种前列腺肿瘤的生长．为了探究 miR-26b 对前列腺癌细胞增殖和侵袭的潜在调控机制，我们进行了表达谱芯片鉴定 miR-26b 调控基因．表达谱芯片分析表明，在前列腺癌细胞系 PC-3 中过表达 miR-26b 后，显著上调的基因 57 个，显著下调的基因 55 个(变化倍数均大于 2，且 *P* 值小于 0.05)．差异基因的功能多与细胞增殖、凋亡调控、蛋白质磷酸化和泛素化修饰调控过程相关，并且富集在多种信号通路中，例如 TNF 和 TGF-β 信号通路．在这些筛选出的基因中，CEACAM6 表达水平下调 2.17 倍；序列分析及实验验证表明，CEACAM6 的 3′UTR 区存在 miR-26b 的互补序列，是 miR-26b 的直接靶标．本研究证明了 miR-26b 能够靶向结合抑制 CEACAM6 的表达，从而抑制前列腺癌细胞在体外和体内的细胞增殖和侵袭活性，miR-26b 是前列腺癌中的抑癌 microRNA．

关键词 miR-26b，前列腺癌细胞，细胞增殖和侵袭，表达谱芯片，CEACAM6
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