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miR-615 Inhibits Prostate Cancer Cell Proliferation and Invasion by Directly Targeting Cyclin D2

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Previous studies have reported that miR-615 exerts a tumor suppressor role in some tumors, such as esophageal squamous cell carcinoma and non-small cell lung cancer. However, the role of miR-615 in prostate cancer has not been defined. Here we found that miR-615 was downregulated in prostate cancer tissues and cell lines. Overexpression of miR-615 in PC-3 cells significantly inhibited cellular proliferation, migration, and invasion. Moreover, overexpression of miR-615 delayed tumor growth in vivo. In terms of mechanism, we found that cyclin D2 (CCND2) is a target gene of miR-615 in prostate cancer. We showed that miR-615 could bind to the 3′-UTR region of CCND2 mRNA and inhibit its expression. There was a negative correlation between the expression of miR-615 and CCND2 in prostate cancer tissues. Moreover, restoration of cyclin D2 abolished the inhibitory effects of miR-615 on the proliferation, migration, and invasion of prostate cancer cells. Taken together, our study identified miR-615 as a tumor suppressor by targeting cyclin D2 in prostate cancer.

Key words: miR-615; Prostate cancer; Proliferation; Migration; Cyclin D2

INTRODUCTION

Prostate cancer is one of the most malignant and common cancers in the male population worldwide and leads to a third of cancer-related deaths in male patients[1]. Nowadays, the main therapeutic strategy for prostate cancer treatment is surgery combined with chemotherapy[2,3]. Although the survival of patients with localized prostate tumor is high, the 5-year survival rate in prostate cancer patients with distant metastatic tumors is lower than 30%[4]. Therefore the current therapeutic method is unsatisfactory. There is an urgent need to determine the molecular mechanism of prostate progression and develop novel effective therapies for prostate cancer treatment.

MicroRNAs (miRNAs) are a class of small noncoding RNAs with a length of 18–25 nucleotides[5]. miRNAs have been demonstrated to regulate gene expression by targeting the 3′-untranslated regions (3′-UTR) of specific mRNAs[6]. Accumulating evidence has indicated that miRNAs are involved in the regulation of various biological processes, including cell differentiation, migration, invasion, proliferation, and apoptosis[7,8]. In many human cancers, miRNAs are shown to be abnormally expressed. miRNAs are also important regulators in the development and progression of prostate cancers. For example, Guo et al. reported that miR-20b promotes cellular proliferation and migration by directly regulating phosphatase and tensin homolog in prostate cancer[9]. Liu and colleagues showed that miR-193a-3p inhibits cell proliferation in prostate cancer by targeting cyclin D1[10]. Therefore, it is necessary to explore the functions of miRNAs in prostate cancer, which will contribute to the development of novel diagnostic or prognostic biomarkers.

Previous studies indicated that miR-615 was a great regulator in human cancers, such as pancreatic adenocarcinoma[11] and hepatocellular carcinoma[12]. However, the function of miR-615 in prostate cancer remains largely unknown. In the present study, we identified miR-615 as a tumor suppressor in prostate cancer. We found that the expression of miR-615 was significantly downregulated in prostate cancer tissues and cell lines. Overexpression of miR-615 inhibited the proliferation, migration, and invasion of prostate cancer cells by targeting CCND2

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encoding an important cell cycle regulating protein cyclin D2. Altogether, our findings, for the first time, demonstrated the key role of a miR-615/cyclin D2 axis in the progression of prostate cancer.

MATERIALS AND METHODS

Patient Samples

A total of 35 pairs of prostate cancer tissues and adjacent normal prostate tissues were obtained from Yantai Municipal Laiyang Central Hospital (Shandong Province, P.R. China). The patients with prostate cancer did not receive chemotherapy or radiotherapy prior to prostatectomy. All samples were immediately snap frozen in liquid nitrogen until RNA extraction. The histological diagnosis of each sample was confirmed simultaneously by two pathologists using hematoxylin and eosin staining. Written informed consent was obtained from each patient, and the present study was approved by the Ethics Committee of Yantai Municipal Laiyang Central Hospital.

Cell Lines and Cell Culture

All cell lines used in this study were purchased from the Shanghai Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, P.R. China). The cell lines included the DU-145, LNCap, and PC-3 human prostate cancer cell lines, as well as the RWPE-1 normal prostate epithelium cell line. All cell lines were cultured in RPMI-1640 medium (Takara Biotechnology Co., Ltd., Dalian, P.R. China) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Bovine pituitary extract (0.05 mg/ml; Thermo Fisher Scientific, Inc.) and human recombinant epidermal growth factor (5 ng/ml; Thermo Fisher Scientific, Inc.) were added to the culture medium of the RWPE-1 cells. The cell culture environment was thermostatic at 37°C with constant humidity and 5% CO_2. The cells were mainly seeded into six-well plates at a density of 4×10^5 cells; a lower density was used depending on certain experiments. Once the cells reached 60–70% confluency, all transfections were performed with Invitrogen Lipofectamine® 2000 Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, with the synthesized RNA mimic or negative control (NC). Transfected cells were cultured for 48 or 72 h at the same conditions described above.

Xenograft Tumor Formation

We purchased 6-week-old male BALB/c nude mice from HFK Biosciences and maintained them under pathogen-free conditions with the approval of Yantai Municipal Laiyang Central Hospital. For tumor propagation analysis, 2×10^6 miR-615 overexpressed cells or control cells were subcutaneously injected into BALB/c nude mice. Six mice were used for each group. Tumor weight was measured on day 30 after injection. Animal experiments were performed in accordance with relevant guidelines and regulations of the Institutional Animal Care and Use Committee at Yantai Municipal Laiyang Central Hospital.

Cell Proliferation

Each well of a 96-well plate contained about 5,000 transfected cells. Cell viability was assessed using a cell counting kit-8 (CCK-8) assay (WST-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Following incubation for 24, 48, and 72 h, 10 µl of CCK-8 was added to each well and incubated for 2 h. The absorbance value at 450 nm was determined using a multimode microplate reader (Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany).

Transwell Assay

Cells were suspended in serum-free medium, and 3×10^4 cells (100 µl) were plated into the upper chambers of Transwell inserts (8.0-µm pore size; Corning Incorporated, Corning, NY, USA) for invasion (with Matrigel) or migration (without Matrigel) assays. The inserts were placed in 24-well plates containing 600 µl of media with 10% FBS. After incubation for 24 h for migration and 48 h for invasion, cells were fixed for 20 min and stained with 1% crystal violet. The number of migrated or invaded cells were imaged and counted under a microscope from five random fields.

Real-Time Quantitative Polymerase Chain Reaction (PCR)

Total RNA was extracted from prostate cancer cells and normal cells with standard TRIzol® solution (Invitrogen; Thermo Fisher Scientific, Inc.). For miRNA expression, RT reactions were performed with a One Step PrimeScript miRNA cDNA Synthesis kit (Takara Biotechnology Co., Ltd.), followed by PCR with SYBR® Premix Ex Taq (Takara Biotechnology Co., Ltd.). For mRNA, cDNA was synthesized from the total RNA using a PrimeScript RT Reagent Kit (Takara Biotechnology Co., Ltd.). qPCR amplification reactions were performed with SYBR® Premix Ex Taq II with ROX (Takara Biotechnology Co., Ltd.).

Statistical Analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences version 20.0 software (SPSS Inc., Chicago, IL, USA). Survival curves were calculated using the Kaplan–Meier method and were analyzed using the log rank test. For comparisons, one-way analyses of variance and two-tailed Student's *t*-tests were performed, as appropriate. A value of $p<0.05$ was considered statistically significant.

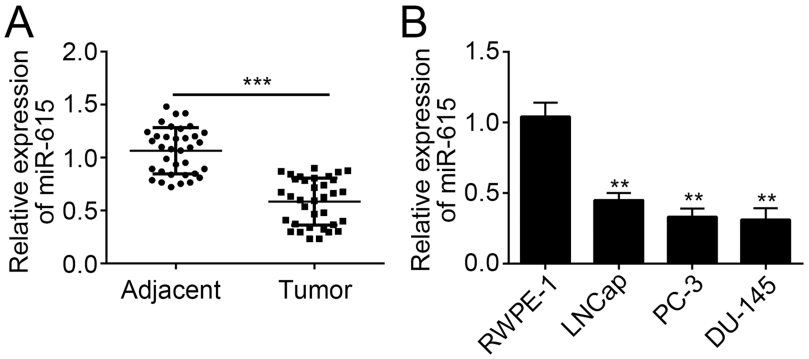

Figure 1. MicroRNA-615 (miR-615) was downregulated in prostate cancer cells. (A) Real-time quantitative PCR (RT-qPCR) was used to check the expression of miR-615 in prostate cancer tissues and adjacent normal tissues. (B) RT-qPCR analysis for the expression of miR-615 in human prostate cancer cell lines and normal prostate epithelium cell line. $^{**}p<0.01$ and $^{***}p<0.001$ by two-tailed Student's *t*-test.

RESULTS

miR-615 Was Downregulated in Prostate Cancer Cells

To investigate the function of miR-615 in human prostate cancer, we performed RT-qPCR analysis and found that the expression of miR-615 was significantly downregulated in prostate cancer tissues ($n=35$) compared to paired adjacent normal tissues ($n=35$) (Fig. 1A). We also evaluated the expression patterns of miR-615 in prostate cancer cell lines by RT-qPCR. Results indicated that the expression of miR-615 was markedly downregulated in prostate cancer cell lines (LNCap, PC-3, and DU-145 cells) compared with RWPE-1 cells (Fig. 1B).

Overexpression of miR-615 Inhibited the Proliferation of Prostate Cancer In Vitro and In Vivo

In order to investigate the function of miR-615 in prostate cancer cells, we overexpressed miR-615 in PC-3 cells with miR-615 mimic. As shown, miR-615 was significantly upregulated in PC-3 cells after transfection with miR-615 mimic (Fig. 2A). Then we performed CCK-8 assay and colony formation assay to evaluate the effect of miR-615 on prostate cancer cell proliferation. We found that overexpression of miR-615 significantly inhibited cellular proliferation in PC-3 cells and suppressed the number of formed clones (Fig. 2B and C). To determine whether miR-615 regulated cellular

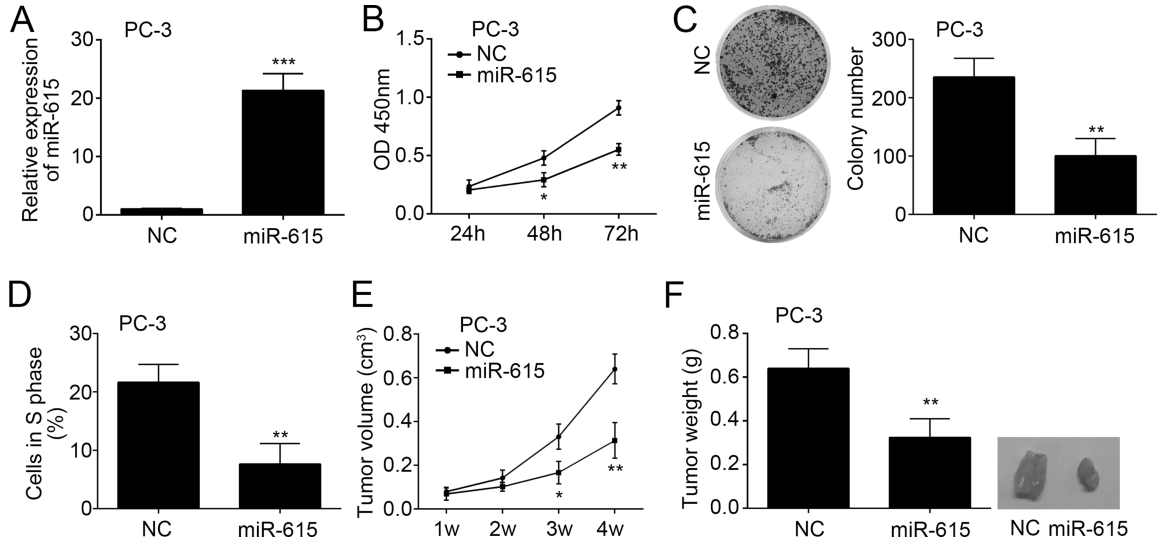

Figure 2. Overexpression of miR-615 inhibited the proliferation of prostate cancer in vitro and in vivo. (A) RT-qPCR analysis for miR-615 expression in PC-3 cells transfected with miR-615 mimics or controls. (B, C) Cellular proliferation was assessed by cell counting kit-8 (CCK-8) assays and colony formation assays with PC-3 cells transfected with miR-615 mimics or controls. (D) Cell cycle distribution was analyzed by FACS. (E) Tumor volumes were determined at the indicated time points. (F) Tumor weights were measured at the endpoint of the xenograft experiment. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.01$ by two-tailed Student's *t*-test.

proliferation by inhibiting the cell cycle, we analyzed cell cycle distribution by FACS with PC-3 cells. As shown, overexpression of miR-615 significantly reduced the percentage of PC-3 cells in the S phase (Fig. 2D). To further confirm the inhibitory effect of miR-615 on proliferation, we performed xenograft experiments with nude mice. We injected miR-615-overexpressing PC-3 cells or control cells into nude mice. At the indicated time points, we measured the tumor volumes and weights (Fig. 2E and F). We found that overexpression of miR-615 markedly inhibited the tumor growth in vivo.

Overexpression of miR-615 Suppressed Prostate Cancer Cell Migration and Invasion

Tumor metastasis is the main reason for the malignance and poor outcomes of prostate cancer patients. So we determined whether miR-615 regulates the metastasis of prostate cancer cells. We performed Transwell assay with miR-615-overexpressing PC-3 cells or control cells. We found that overexpression of miR-615 significantly inhibited the migration and invasion of PC-3 cells (Fig. 3A and B).

CCND2 Was a Direct Target of miR-615

miRNAs could regulate gene expression by targeting the 3′-UTR region of specific mRNAs. We then explored the downstream target gene of miR-615 in prostate cancer cells. We made a prediction with a TargetScan tool. We found that CCND2, encoding an essential regulator protein of the cell cycle, was the potential target gene of miR-615. We found that there was a potential binding site in the 3′-UTR region of CCND2 mRNA (Fig. 4A). We then constructed luciferase reporter plasmid containing the 3′-UTR region of CCND2 and a site mutant reporter plasmid (Fig. 4A). Dual-luciferase activity reporter assay indicated that overexpression of miR-615 significantly inhibited the luciferase intensity in PC-3 cells transfected with wild-type (WT) reporter plasmid but not in PC-3 cells transfected with mutant reporter plasmid (Fig. 4B), which implied that CCND2 was a direct target of miR-615 in prostate cancer cells. Moreover, RT-qPCR analysis indicated that overexpression of miR-615 significantly inhibited the mRNA and protein levels of cyclin D2 in PC-3 cells (Fig. 4C and D). Finally, we also demonstrated that the expression of miR-615 was negatively correlated with that of CCND2 in prostate cancer tissues (Fig. 4E). Taken together, our data indicated that miR-615 targeted the 3′-UTR region of CCND2 mRNA in prostate cancer cells.

Restoration of Cyclin D2 Reversed the Effects of miR-615 on Cell Proliferation, Migration, and Invasion

To further confirm that miR-615 regulated prostate cancer cell proliferation, migration, and invasion by targeting CCND2, we restored the protein level of cyclin D2 in PC-3 cells transfected with miR-615 mimics (Fig. 5A). Then we performed CCK-8 and colony formation assay to evaluate cellular proliferation in indicative cell lines. As shown, we found that overexpression of cyclin D2 significantly rescued the inhibitory effect of miR-615 on PC-3 cell proliferation and colony formation (Fig. 5B and C). Moreover, the Transwell assay indicated that restoration of cyclin D2 in miR-615-overexpressing PC-3 cells promoted cell migration and invasion (Fig. 5D and E). Our findings demonstrated that miR-615 inhibited

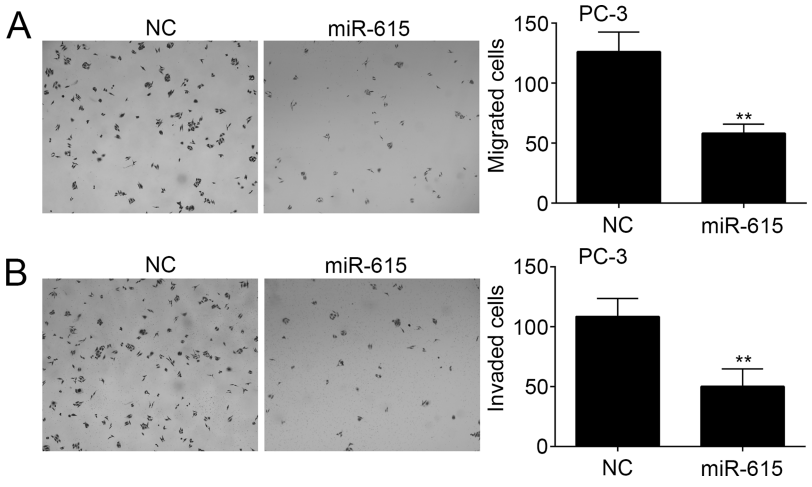

Figure 3. Overexpression of miR-615 suppressed prostate cancer cell migration and invasion. (A, B) Transwell assay was used for the evaluation of cell migration and invasion in PC-3 cells transfected with miR-615 mimics or controls. $^{**}p<0.01$ by two-tailed Student's *t*-test.

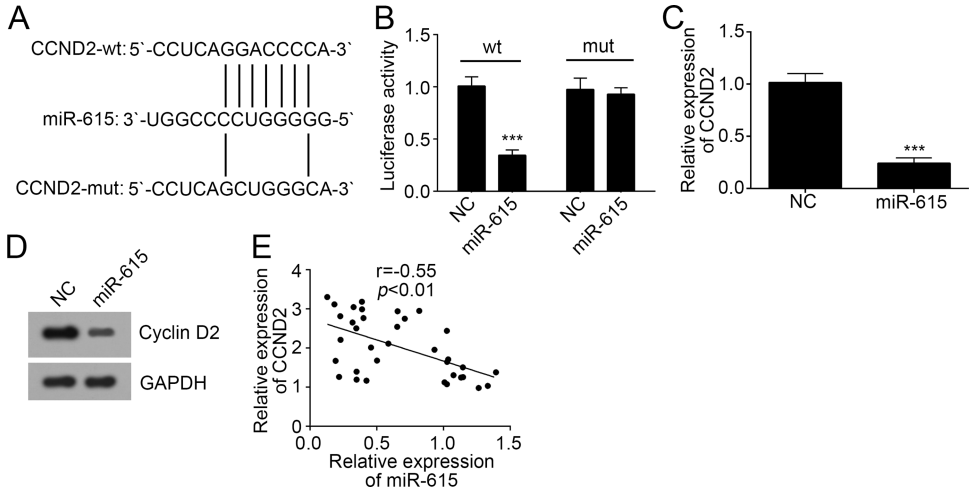

Figure 4. Cyclin D2 (CCND2) was a direct target of miR-615. (A) A diagram for the binding site of miR-615 with the 3′-untranslated region (3′-UTR) of CCND2. (B) Dual-luciferase reporter assay was used to verify the interaction of miR-615 with the 3′-UTR of CCND2 in PC-3 cells. (C, D) Overexpression of miR-615 significantly inhibited the (C) mRNA and (D) protein levels of CCND2 in PC-3 cells. (E) There was an inverse correlation between the expression of miR-615 and CCND2 in prostate cancer tissues. ***$p < 0.001$ by two-tailed Student's *t*-test.

the proliferation, migration, and invasion of prostate cancer cells by directly inhibiting cyclin D2.

DISCUSSION

miRNAs have been demonstrated to be great regulators and promising diagnostic and prognostic biomarkers in cancers[13]. Abnormal expression of miRNAs is accompanied by tumor occurrence, including prostate cancer[14,15]. A report indicated that miRNAs are involved in the regulation of tumor cell proliferation, migration, and metastasis[16]. For instance, miR-378 suppresses the proliferation, migration, and invasion of colon cancer cells by inhibiting SDAD1[17]. miR-143 acts as a suppressor of hemangioma growth by targeting Bcl-2[18], while

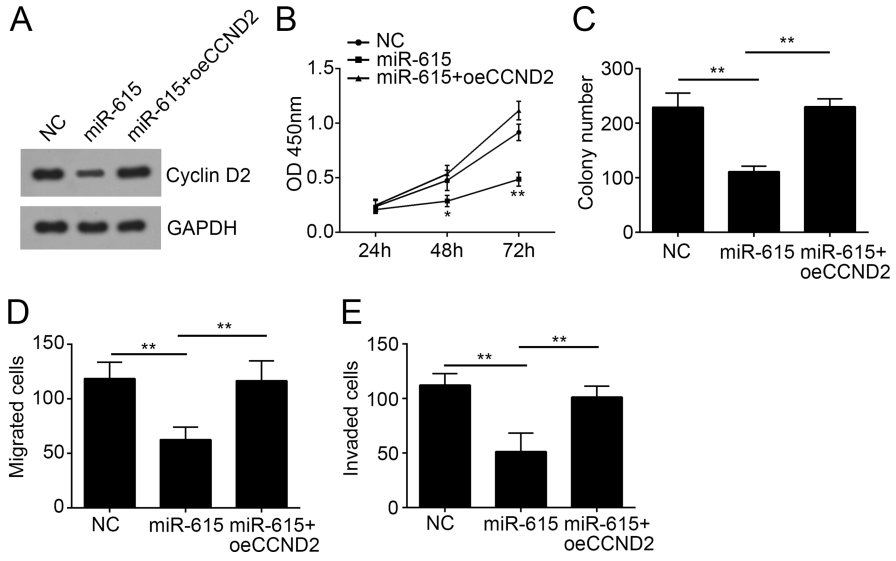

Figure 5. Restoration of cyclin D2 reversed the effects of miR-615 on cell proliferation, migration, and invasion. (A) Western blot was used to check the protein level of cyclin D2 in PC-3 cells transfected with miR-615 and cyclin D2 expressing plasmid. (B, C) CCK-8 and colony formation assays indicated that overexpression of cyclin D2 rescued the proliferation of PC-3 cells. (D, E) Transwell assays indicated that restoration of cyclin D2 promoted the migration and invasion of PC-3 cells transfected with miR-615. **$p < 0.01$ by two-tailed Student's *t*-test.

miR-125a-5p upregulation suppresses the proliferation and induces the cell apoptosis of lung adenocarcinoma by targeting NEDD9[19]. Thus, it will be of great importance to explore the functions of miRNAs in cancers. In this study, we investigated the role of miR-615 and identified it as a tumor suppressor in prostate cancer.

Previous studies have reported that miR-615 acts as a tumor suppressor in some human cancers. For instance, Yang et al. reported that miR-615-5p targets insulin-like growth factor 2 and exerts the tumor-suppressing functions in human esophageal squamous cell carcinoma[20]. Pu et al. identified miR-615-3p as a novel tumor suppressor in non-small cell lung cancer[21]. Chen et al. showed that KDM4B-mediated epigenetic silencing of miR-615-5p augments RAB24 to facilitate malignancy of hepatoma cells[22]. Jiang and colleagues reported that CDX2 inhibits pancreatic adenocarcinoma cell proliferation via promoting the tumor suppressor miR-615-5p[11]. In addition, Bai et al. reported that miR-615 inhibited cell proliferation and cell cycle of human breast cancer cells by suppressing AKT2 expression[23]. However, the function of miR-615 in prostate cancer remains largely unknown. In this study, we found that the expression of miR-615 was significantly upregulated in prostate cancer tissues and cell lines. Moreover, we performed CCK-8, colony formation, and xenograft assays and demonstrated that overexpression of miR-615 inhibited prostate cancer cell proliferation in vitro and in vivo. Overexpression of miR-615 suppressed the migration and invasion of PC-3 cells. Our data suggested that miR-615 had a profound effect on the cell cycle and proliferation in prostate cancer.

Subsequently, we explored the molecular mechanism underlying the miR-615-mediated regulation of cellular proliferation, migration, and invasion. We identified CCND2 as one of the target genes of miR-615 in prostate cancer cells. CCND2 encodes cyclin D2, a member of the cyclin proteins that regulate the progression of cells through the cell cycle. Cyclin D2 has been reported to regulate the development and progression of many cancers, including prostate cancer. For instance, Zhu et al. reported that miR-154 inhibits prostate cancer cell proliferation by targeting CCND2[24]. In addition, Dong et al. showed that miRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2[25]. Therefore, we chose CCND2 for further investigation. By dual-luciferase reporter assay, we verified that CCND2 was a direct target gene of miR-615. We found that overexpression of miR-615 inhibited the mRNA and protein levels of CCND2 in prostate cancer cells. Moreover, RT-qPCR analysis indicated that there was an inverse correlation between the expression of miR-615 and CCND2. Finally, through functional experiments, we demonstrated that restoration of cyclin D2 at least partially rescued the inhibitory effects of miR-615 on the proliferation, migration, and invasion of prostate cancer cells.

In summary, our study demonstrated that miR-615 acts as a tumor suppressor in prostate cancer by targeting CCND2, which suggested that miR-615 may be a promising therapeutic target for prostate cancer treatment.

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