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miR-186-5p Inhibits Talignant Phenotypes of Prostate Cancer by Regulating CALB1

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Keywords:

Prostate cancer; miR-186-5p; CALB1

Abbreviations:

CALB1: Calbindin 1; CCK-8: Cell Counting Kit-8; DEGs: Differential expressed genes; GEO database: Gene Expression Omnibus database; mCRPC: Metastatic castrate-resistant prostate cancer; miRNA: MicroRNA; mRNA: Message RNA; OD: Optical density; PCa: Prostate cancer; PI: Propidium iodide; PVDF membrane: Polyvinylidene fluoride membrane; qRT-PCR: Real-time Quantitative Polymerase Chain Reaction

1. Abstract

1.1 Background: miRNA-186-5p plays a tumor-suppressor specific role in multiple cancers. However, the function of miRNA-186-5p in prostate cancer remains unclear.

1.2. Objective: We explore the effects of miR-186-5p on talignant phenotypes of prostate cancer.

1.3. Methods: Bioinformatics analysis was used to screen miRNA and downstream target gene. the expression level of the miR-186-5p and CALB1 in prostate cancer cells was detected by qRT-PCR and immunohistochemistry. The effects of miR-186-5p in prostate cancer were measured by CCK-8 assay, apoptosis assay and transwell assay. A series of rescue experiments were applied after si-CALB1 and miR-186-5p inhibitor were co-transfected into 22RV1 and DU145 cell lines. qRT-PCR and β-Galactosidase assay were performed to further specific the relationship between CALB1 and apoptosis.

1.4. Results: miR-186-5p was lowly expressed in prostate cancer tissues and cells. The expression of miR-186-5p suppressed the proliferation, migration and invasion and promoted the apoptosis in prostate cancer cells. Additional, CALB1 was a target of miR-186-5p. CALB1 played a cancer-promoting role in prostate cancer and CALB1 silencing reversed the promoting effects of miR-186-5p inhibitor. Besides, CALB1 was closely related to cells apoptosis of prostate cancer.

1.5. Conclusion: Our study revealed that there is a negative correlation between miR-186-5p and CALB1, and miR-186-5p can significantly inhibit the proliferation, migration and invasion of prostate cancer cells and promote the apoptosis of prostate cancer cells by targeting the expression of CALB1.

2. Introduction

Prostate cancer (PCa) is the fourth most common cancer in the world, and it ranks second among the male cancer incidence rate. According to the statistics of GLOBOCAN database, the number of new cases of PCa in the world in 2020 was about 1.4 million, second only to lung cancer among men, and the number of deaths was about 380000, which was the fifth leading cause of cancer-related deaths in men [1]. At present, radical surgery is the main clinical method for early PCa, but most of the patients are in the middle and late stage when they are first diagnosed, and it will eventually progress to metastatic castrate-resistant prostate cancer (mCRPC), for which there is no satisfactory treatment, and the average survival time is only about 18 months [2].

MicroRNA (miRNA) is a short-stranded, evolutionarily conserved, endogenous non-coding RNA, about 22nt length, which regulates about 60% of human mRNA gene through post-transcriptional regulation [3]. The abnormal expression of miRNAs play an important role in many diseases such as cancer, cardiovascular disease and hepatitis [4]. Moreover, miRNAs have significant tissue specificity and disease specificity. Studies have shown

that the expression of miRNAs is not only significantly different in cancer patients and normal people, but also in different tumor types, and different stages and grades of the same tumor [5, 6]. Therefore, the expression profile of miRNAs holds promise as a powerful molecular biomarker for the diagnosis, treatment and prognosis of different cancers, including PCa.

We downloaded the GEO dataset GSE36802 for transcriptome miRNA analysis, including 21 normal samples and 21 disease samples, and found that miR-186-5p was low expressed in prostate cancer. miRNA-186-5p is derived from miRNA-186, which is located at human 1q31.1, and miRNA-186-5p is finally obtained after two cleavage. However, at present, there is no final conclusion about the expression of miRNA-186-5p in PCa and its effect on the biological behavior of PCa.

In our study, we confirmed that miRNA-186-5p is down-expressed in prostate cancer, and through a series of in vitro experiments, we found that miRNA-186-5p can inhibit the proliferation, migration and invasion of prostate cancer cells, and promote the apoptosis of prostate cancer cells. What's more, we identified CALB1 as the downstream target gene of miRNA-186-5p, and miR-186-5p affects talignant phenotypes of prostate cancer by negatively regulating the expression of CALB1.

3. Materials and Methods

3.1. Bioinformatics Analysis

Expression microarray GSE36802 which contains all miRNA related with prostate cancer was downloaded from GEO database (http://www.ncbi.nlm.nih.gov/geo/). Transcriptome miRNA analysis was performed to identify the miRNAs differentially expressed between the prostate cancer cell lines and normal prostate cells with "edgeR" package. At first, |logFC|>1, pvalue<0.05 was set as screening threshold, and then it was further adjusted to |logFC|>2, pvalue<0.05 to narrow fiter range. Data from GEPIA database (http://gepia.cancer-pku.cn/) and TargetScan database (http://www.targetscan.org/) was downloaded respectively to find target genes for miR-186-5p, and common target genes identified using Venn diagram were regarded as candidates.

3.2. Cell Culture

Prostate cancer cell lines DU145, 22RV1, LNCaP, PC3 and normal prostate stromal immortalized cells WPMY-1 were all purchased from Wuhan Procell Life Sciences Co. 22RV1 and LNCaP were cultured in RPMI-1640 complete culture medium; PC3 and normal prostate stromal immortalized cells WPMY-1 were cultured in DMEM complete culture medium; DU145 was cultured in MEM complete culture medium. All cells were incubated at 37°C in a 5% CO2 culture incubator.

3.3. Cell Transfection

miRNA-186-5p mimic, miRNA-181b-5p inhibitor and NC were purchased from GenePharma (Shanghai, China). Three si-CALB1(siCALB1-1,2,3) were purchased from GenePharma (Shanghai, China). Cells were transfected with them respectively using Lipofectamine®2000 reagent in accordance with the manufacturer protocol.

3.4. qRT-PCR

Total RNA was extracted from tissues and cell lines by using TRIzol Reagent (MRC Global), then concentration and purity of total RNA were calculated for further reverse transcription. cDNA was reverse-transcribed using SureScript-First-strand-cDNA-synthesis-kit (GeneCopoeia), and subsequently, qRT-PCR was performed to analysis relatived level of miR-186-5p and CALB1 with BlazeTaq™ SYBR® Green qPCR Mix 2.0(GeneCopoeia). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as controls for mRNA and miRNA detection, respectively. The sequences of PCR primers were shown as Table 1.

Table 1: The sequences of PCR primers.

name	sequences of primer
miR-186-5p	5'-CAAAGAATTCTCCTTTTGGGCT- 3'
CALB1	Forward：5'-GTCATCCCTCATCACAGCC- 3'
	Reserve：5'-TCCAGGTAACCACTTCCGT- 3'
GADPH	Forward：5'-CCCATCACCATCTTCCAGG- 3'
	Reserve：5'-CATCACGCCACAGTTTCCC- 3'
miR-Reverse Primer U6	5'-GTCGTATCCAGTGCAGGGT- 3'
	Forward：5'-CTCGCTTCGGCAGCACA- 3'
	Reserve：5'-AACGCTTCACGAATTTGCGT- 3'

3.5. Western Blot

Total protein was extracted by using RIPA lysis buffer (Beyotime Biotechnology) on ice, then concentration of total protein was measured by BCA Protein Assay Kit (Beyotime Biotechnology). 30μg of protein was separated by 10% SDS-PAGE membrane electrophoresis, and then transferred to PVDF membrane. After blocked with 5% non-fat milk for 40min, the membrane was probed with primary anti-body at 4°C overnight and incubated with horse-radish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 40min at room temperature. ECL Western Blotting Substrate Reagent was utilized for developing signal.

3.6. Immunohistochemistry

Tissue sections were obtained from cancer tissues and normal tissues before immunohistochemistry, they were hydrated and Dewaxinged then. After blocked with goat serum blocking solution for 30min, the sections were probed with primary anti-body at 4°C overnight and incubated with horse-radish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1h at 37°C. Sections were visualized with DAB working solution, and images were acquired using an inverted microscope.

3.7. CCK-8 Assay

Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies) was applied to analyze cell proliferation. Experimental cell lines

(5×103 cells per well) were plated into a 96-well plate. After 0, 24, 48, 72 and 96h, 10μl of CCK-8 solution was supplemented and incubated 2h at 37°C. The optical density (OD) was measured using a spectrophotometer at 450nm.

3.8. Apoptosis Assay

Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN Bio-TECH) was applied to analyze cell apoptosis. Experimental cell lines were resuspended in the appropriate binding buffer. Then, the cell suspension was stained with 5μl of Annexin V-FITC solution for 5min and subsequently incubated with 5μl of propidium iodide (PI) solution for 15min at room temperature. The rate of apoptotic cell was measured using a flow cytometer.

3.9. Transwell Assay

Transwell chambers (BD Biosciences) was applied to analyze cell migration and invasion. For the migration assay, experimental cell lines (5×104 cells per well) were plated into the upper chamber with 200μl FBS-free medium, while the lower chamber was added with 500μl medium containing 10% FBS. After incubated 24h at 37°C, the migrating cells were fixed by methanol for 10min and subsequently stained by 0.5% crystal violet for 15min. The rate of migrating cell was measured using an inverted microscope. The processes of the invasion assay were basically the same as described for the migration assay, except that the upper chamber was pre-coated with Matrigel (BD Biosciences).

3.10. β-Galactosidase Assay

β-Galactosidase Assay Kit (GENMED Scientifics) was applied to analyze cell apoptosis. Experimental cell lines were fixed by dyeing fixing solution for 15min at room temperature. Then Dyeing working solution was add and incubated at 37°C overnight. The condition of cell staining was observed using an optical microscope.

3.11. Statistical Analysis

GraphPad Prism 8.0 software was used to produce Bar graphs, and SPSS 22.0 software was used for statistical analysis. $p>0.10$ for all variables conformed to a normal distribution by the D-W (Kolmogorov-Smirnov) test or the Shapiro-Wilk test. All quantitative data are described as mean ± standard deviation (±s). Between-group comparisons were completed via Student's t-test, while multi-sample comparisons were calculated using one-way ANOVA (Analysis of variance) followed by Dunnett's test. P value<0.05 was considered statistically significant.

4. Results

4.1. miR-186-5p is Lowly Expressed in Prostate Cancer Tissues and Cells

We analyzed transcriptome miRNA by downloading GEO dataset GSE36802, and filtered with | logFC | > 1, pvalue < 0.05. a total of 58 differentially expressed miRNA were obtained, of which 21 were up-regulated and 37 down-regulated (Figure 1A-B). Then the screening criteria were further adjusted to | logFC | > 2, pvalue < 0.05. finally, 14 miRNAs were obtained. it was found that the expression of miR-186-5p was significantly decreased in prostate cancer (Figure 1C). In order to verify the results of the above bioinformatics analysis, we detected the relative expression of miR-186-5p in tissues and cells by qRT-PCR. The results showed that the expression of miR-186-5p was significantly decreased in prostate cancer tissues and prostate cancer cell lines 22RV1 and DU145, while PC-3 and LNCaP were decreased but not statistically significant, so 22RV1 and DU145 cell lines were selected for follow-up vitro experiments (Figure 1D-E).

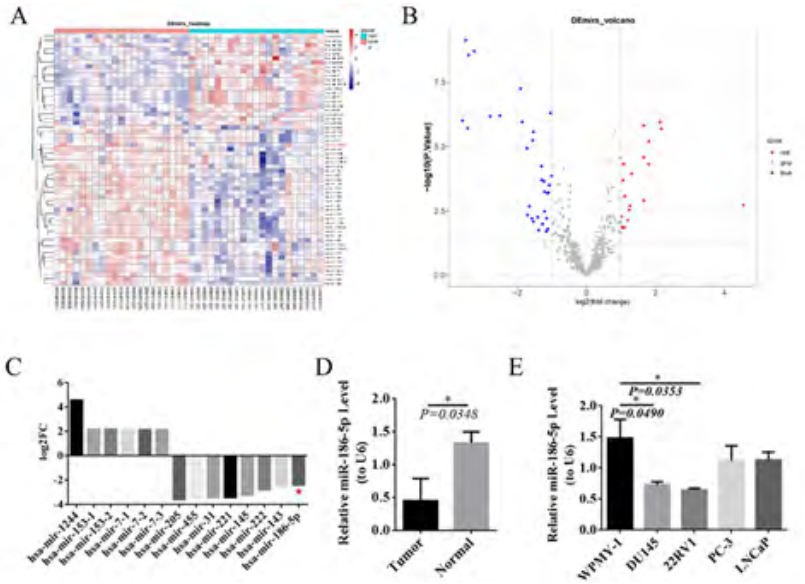

Figure 1: miR-186-5p is down-regulated in PCa. (A) Heat map for differential expressed miRNAs (DEMs) in GEO dataset. (B) Volcano map for DEMs, red dots represented upregulated genes, and blue dots represented downregulated genes. (C) Expression of 14 miRNA in GEO dataset. (D) qRT-PCR was performed to detect the expression levels of miR-186-5p in prostate cancer tissues and normal prostate tissues. (E) qRT-PCR was performed to detect the expression levels of miR-186-5p in prostate cancer cell lines (DU145, 22RV1, PC-3 and LNCaP) and the normal human prostate cell line (WPMY-1).

4.2. miR-186-5p Plays a Tumor Suppressor Role in the Occurrence and Development of Prostate Cancer

miR-186-5p mimics, miR-186-5p inhibitor and NC was transfected into 22RV1 and DU145 cell lines respectively to investigate the role of miR-186-5p in prostate cancer (Figure 2A). The results of CCK-8 indicated that compared with NC group, the ability of cell proliferation was significantly decreased in mimics group, while on the contrary in inhibitor group (Figure 2B). Similarly, transwell assay implied that highly expressed miR-186-5p was markedly weaken migration and invasion abilities (Figure 2D-E). Whatsmore, apoptosis assay unveiled that in comparison with the NC group, the proportion of apoptotic cells was increased in the mimics group, while the proportion was significantly reduced in the inhibitor group (Figure 2C). These results demonstrate that miR-186-5p plays a cancer suppressor role in the occurrence and development of prostate cancer cell.

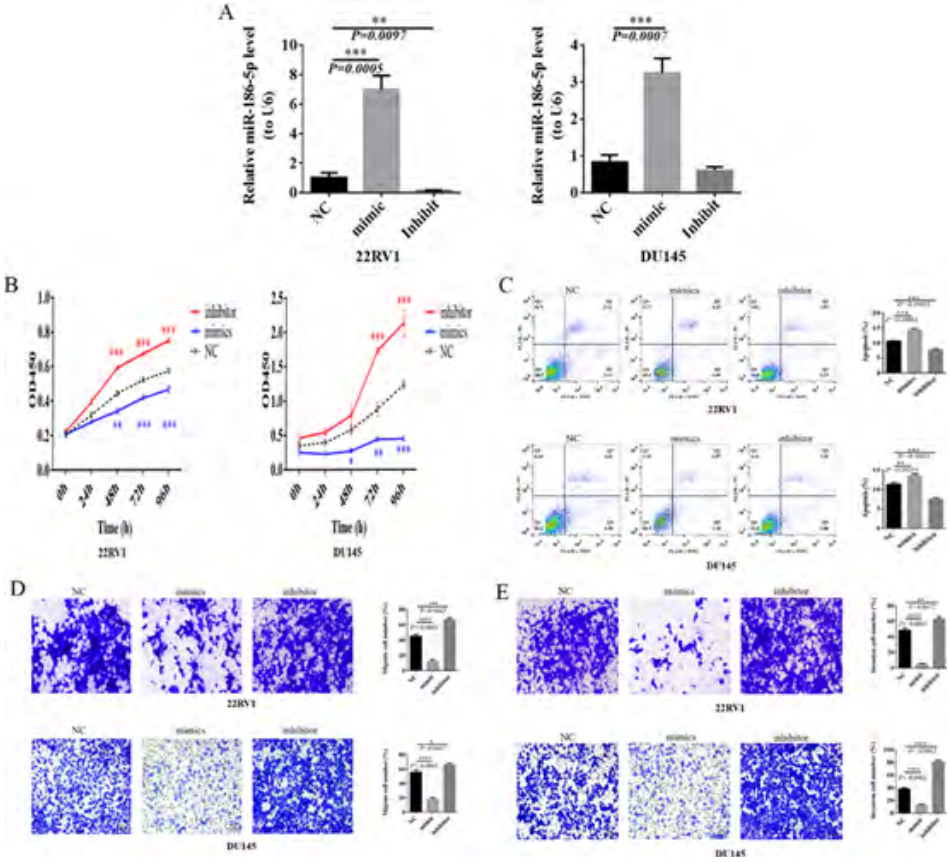

Figure 2: miR-186-5p inhibits proliferation, migration and invasion, and promote apoptosis in prostate cancer cells. (A) Transfection efficiency examined by qRT-PCR. (B) Cell proliferation rate in DU145 and 22RV1 cell lines was measured by CCK-8 assay. (C) Cell apoptosis rate in DU145 and 22RV1 cell lines was determined by apoptosis assay. (D) Cell migration ability in DU145 and 22RV1 cell lines was measured by transwell assay. (E) Cell invasion ability in DU145 and 22RV1 cell lines was measured by transwell assay.

4.3. CALB1 is a Target of miR-186-5p

In order to further clarify the mechanism of miR-186-5p in prostate cancer, we downloaded data from GEPIA database and TargetScan8.0 database to analyze the target genes related to miR-186-5p, and identified 519, 953 target genes respectively. Later on, the Venn diagram was used to screen for common target genes of both, eventually a total of 16 target genes were obtained, of which only CALB1 was highly expressed (Figure 3A, 3C). In addition, we also found that miR-186-5p had binding sites with CALB1 through TargetScan8.0 database (Figure 3B). So CALB1was finally selected as the downstream target gene of miR-186-5p. Then, the levels of CALB1 in tissues were analyzed using qRT-PCR and immunohistochemistry. As shown in Figure 3D and E, compared to normal tissues, the level of CALB1 was significantly higher in prostate cancer tissues. these demonstrate that CALB1 is a target gene of miR-186-5p.

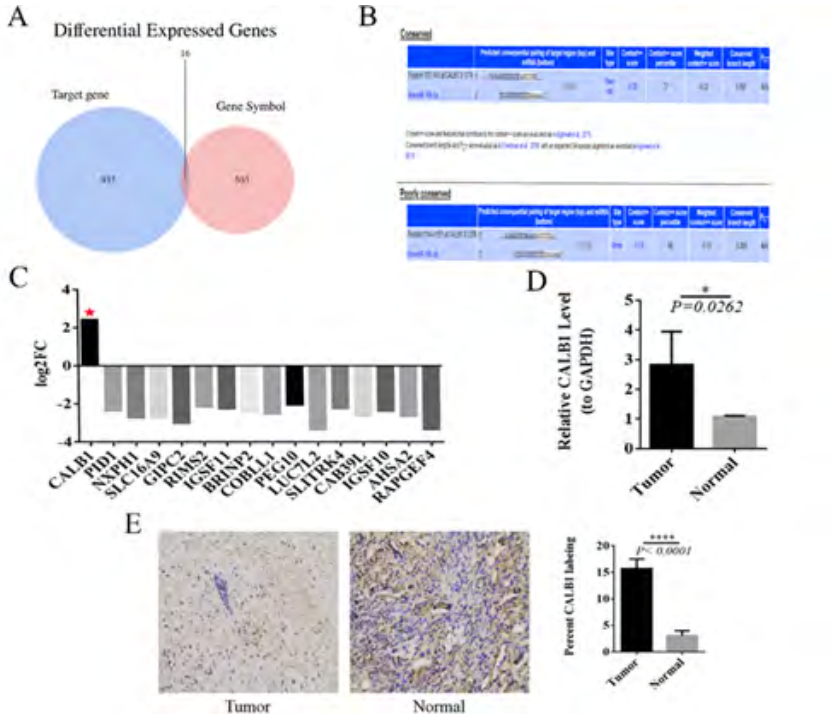

Figure 3: CALB1 is a target of miR-186-5p in prostate cancer cell. (A) The common targets of miR-186-5p identified using GEPIA and TargetScan. (B) Binding sites between miR-186-5p and CALB1. (C) Expression of 16 differential expressed genes (DEGs) in database. (D) qRT-PCR was performed to detect the expression levels of CALB1 in prostate cancer tissues and normal prostate tissues. (E) immunohistochemistry was performed to detect the expression levels of CALB1 in prostate cancer tissues and normal prostate tissues.

4.4. CALB1 Silencing Reverses the Promoting Effects of miR-186-5p Inhibitor in Prostate Cancer

To find out how CALB1 plays a role in in miR-802 medicating prostate cancer cell aggressiveness, we performed several rescue experiments. First, 3 siCALB1 was constructed and transfected into 22RV1 to silence the CALB1 expression. qRT-PCR and western blot results showed that only siCALB1-1 had the significant interference effect, so it was chosen for the subsequent experiments (Figure 4A-B). Then, si-CALB1 and miR-186-5p inhibitor were co-transfected into 22RV1 and DU145 cell lines and the CALB1 expression was opposite, which indicated that CALB1 expression was affected by miR-186-5p (Figure 4C-D). The CCK-8 and transwell assay suggested that in both 22RV1 and DU145 cell lines, the cell proliferation, migration and invasion abilities were repressed after transfecting si-CALB1, whereas these were reversed by miR-186-5p down-expression (Figure 4E, 4G-H). Moreover, flow cytometry unveiled that down expression of CALB1 reversed miR-186-5p inhibitor-induced cell apoptotic (Figure 4F). All these findings suggest CALB1 silencing reverses the promoting effects of miR-186-5p inhibitor on prostate cancer.

4.5. CALB1 is Closely Related to Apoptosis

To further understand the mechanism of CALB1 in prostate cancer, qRT-PCR was applied to detect apoptosis and senescence-related genes. The result showed that compared with the NC group, the expression of p21 and p27 in 22RV1 cells in the si-CALB1 group was significantly higher, and the expression of TH and BCL-2 was significantly lower, while there was no difference in p16 and p53; the expression of p21, p27 and p53 in DU145 cells was significantly higher, and the expression of TH and BCL-2 was significantly lower, while there was no difference in p16 (Figure 5A). Similar to qRT-PCR, β-galactosidase assay showed that compared to NC group, the apoptosis rate of 22RV1 and DU145 cells in si-CALB1 group was higher (Figure 5B). Collectively, the above results uncovered that CALB1 is closely related to apoptosis.

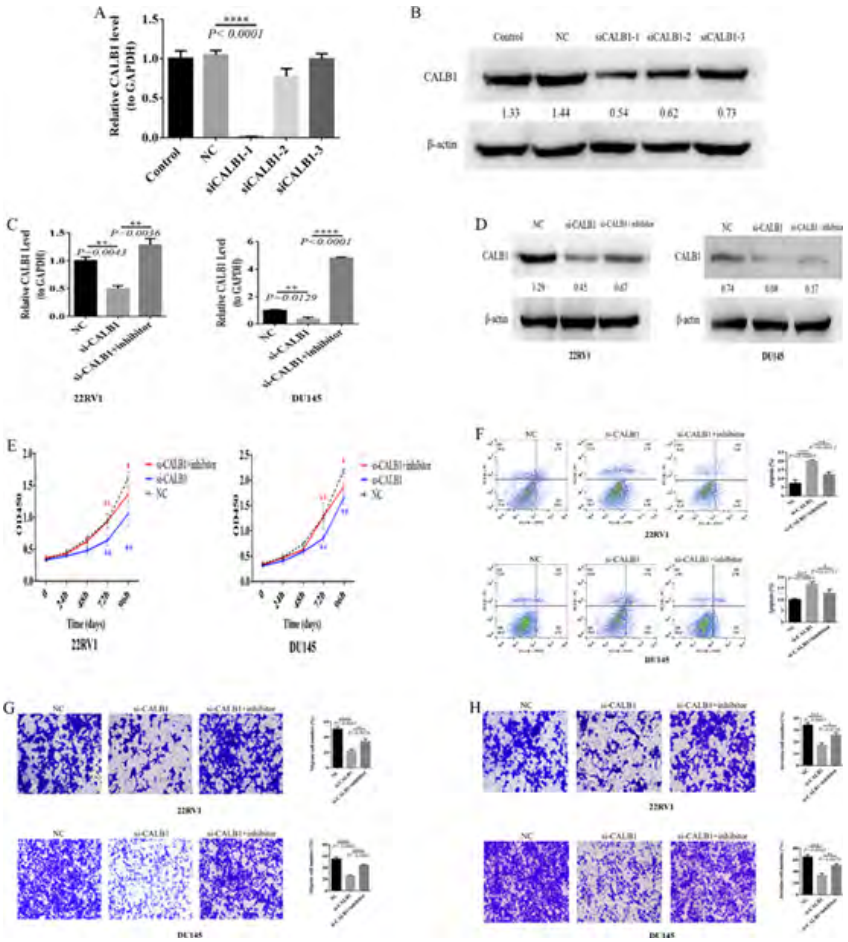

Figure 4: CALB1 silencing reverses the inhibitory effects of miR-186-5p in prostate cancer. (A) Interference efficiency examined by qRT-PCR. (B) Interference efficiency examined by western blot. (C) qRT-PCR was applied to detect CALB1 expression in 22RV1 and DU145 cell lines that co-transfected with si-CALB1 and miR-186-5p inhibitor. (D) Western blot was applied to quantitate CALB1 protein levels in 22RV1 and DU145 cell lines that co-transfected with si-CALB1 and miR-186-5p inhibitor. (E) Cell proliferation rate in DU145 and 22RV1 cell lines that co-transfected with si-CALB1 and miR-186-5p inhibitor was measured by CCK-8 assay. (F)Cell apoptosis rate in DU145 and 22RV1 cell lines that co-transfected with si-CALB1 and miR-186-5p inhibitor was determined by apoptosis assay. (G) Cell migration ability in DU145 and 22RV1 cell lines that co-transfected with si-CALB1 and miR-186-5p inhibitor was measured by transwell assay. (H) Cell invasion ability in DU145 and 22RV1 cell lines that co-transfected with si-CALB1 and miR-186-5p inhibitor was measured by transwell assay.

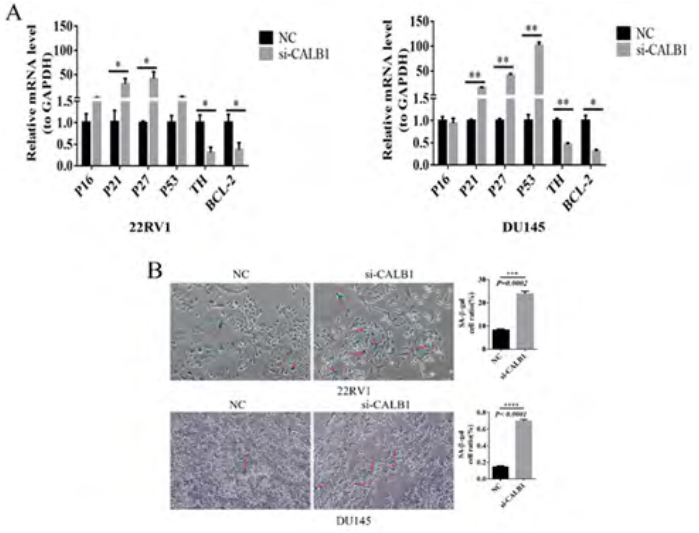

Figure 5: CALB1 is closely related to apoptosis. (A) qRT-PCR was applied to detect the levels of apoptosis and senescence-related genes in 22RV1 and DU145 cells that transfected with si-CALB1. (B) β-galactosidase assay was performed to assess the apoptosis rate of 22RV1 and DU145 cells that transfected with si-CALB1.

5. Discussion

High incidence rate and poor prognosis make PCa a great threat to the survival and life of men. We need to explore the mechanism of the occurrence and development of prostate cancer and explore new promising clinical diagnosis and treatment directions, such as miRNA. miRNA-186-5p was derived from miRNA-186, which was localized at human 1q31.1. miRNA-186-5p is down-regulated and plays a tumor-suppressor specific role in multiple cancers, such as bladder cancer [7], neuroblastoma [8], non-small colorectal cancer [9] and breast cancer [10]. However, the role of miRNA-186-5p in talignant phenotypes of PCa, is not clear and needs to be further explored.

Prior to the experiment, we first gained preliminary insight into the low expression of miR-186-5p in prostate cancer by bioinformatics analysis, and we hypothesized that miR-186-5p might inhibit the development of prostate cancer. In order to verify our conjecture, we used qRT-PCR experiment in both tissues and cells to confirm the down-regulated expression of miR-186-5p in prostate cancer. On this basis, we overexpressed and knocked down miR-186-5p, respectively, and performed a series of experiments in vitro. We proved that the expression of miR-186-5p suppressed the proliferation, migration and invasion of prostate cancer cells and promoted the apoptosis, which was consistent with Jin's study [11]. They found that circZNF609 is highly expressed in prostate cancer cells and negatively correlated with miR-186-5p. When circZNF609 is silenced, it can inhibit the growth, colony formation, migration and invasion of prostate cancer cells by up-regulating miR-186-5p. However, research results of Jones et al [12] were diametrically opposed to ours. their findings suggested that miR-186-5p was upregulated and played an oncogenic role in PCa. This may be due to different types of control cell lines used for comparison and different methods for miRNA isolation and detection, but the deeper reasons need to be explored.

According to its influence on the occurrence and development of cancer, miRNA can be divided into onco-miRNA and suppressor-miRNA. However, the identity is not immutable, as one miRNA is a onco-miRNA in one cancer and a suppressor-miRNA in another [13]. The characteristics of its function mainly depend on the properties of the downstream target genes regulated by it [14]. For example, miR-20b-5p inhibits cancer by targeting cyclinD1 in colon cancer [15], while down-regulates the expression of CPEB3 in hepatocellular carcinoma to promote proliferation, migration and invasion [16].

In the prebiotic bioinformatics analysis, we identified CALB1 as a potential target gene for miR-186-5p. CALB1, also known as CaBP28k, is a Ca2+ binding protein with high affinity, widely expressed in kidney, brain and male and female reproductive organs. It is mainly responsible for maintaining low intracellular calcium levels and has anti-apoptosis properties [17, 18]. Numerous studies have shown that CALB1 plays a tumor-promoting role in a variety of cancers such as osteosarcoma [19], ovarian cancer [20] and non-small cell lung cancer [21] and so on. For example, Huang et al [19] found that after knocking down the expression of CALB1 in osteosarcoma U2OS cells, the proliferation and colony formation ability of the cells received significant inhibition, and CALB1 helped protect osteosarcoma cells from apoptosis. In our work, we first examined the relative expression of CALB1 by qRT-PCR and immunohistochemistry in prostate cancer and paracancerous tissues, and confirmed that CALB1 was highly expressed in prostate cancer, which verified the results of our bioinformatics analysis. Furthermore, our study has shown that CALB1 plays a cancer-promoting role in prostate cancer cells, and by negatively regulating CALB1 expression, miR-186-5p can inhibit the proliferation, migration, and invasion of prostate cancer cells, and promoting apoptosis.

In addition, several studies have shown that CALB1 has a close relationship with apoptosis and senescence. For example, CALB1 inhibits the upregulation of apoptosis-related genes and protects human lens epithelial cells from apoptosis[22]; Cao et al[20] found that CALB1 can inhibit senescence of ovarian cancer cells and promote proliferation. In order to further understand the mechanism of CALB1 in prostate cancer, we used qRT-PCR to detect apoptosis and aging related genes. p21, p27 and p53 are apoptosis and senescence related genes, p53 is an important oncogene that encodes a protein that blocks the cell cycle and promotes apoptosis. Mutations in the p53 gene are present in about 50% of tumor tissues, indicating its close association with tumorigenesis. p21 and p27 are the downstream factors of p53, which can reduce the replication and accumulation of damaged DNA, thus delaying cell aging [23, 24]. Th and bcl-2 proteins are important anti-apoptotic proteins in humans, with bcl-2 can inhibit p53-mediated apoptosis [25]. After interfering with the expression of CALB1, the expression of p21 and p27 were significantly increased in both 22RV1 and DU145 prostate cancer cell lines, while p53 was significantly increased only in DU145, and P16 was not significantly increased; th and bcl-2 were significantly decreased with the decrease of CALB1 expression, indicating that CALB1 can inhibit apoptosis and senescence of prostate cancer cells by affecting the expression of the above apoptotic or anti-apoptotic genes.

6. Conclusion

Our study revealed that there is a negative correlation between miR-186-5p and CALB1, and miR-186-5p can significantly inhibit the proliferation, migration and invasion of prostate cancer cells and promote the apoptosis of prostate cancer cells by targeting the expression of CALB1.

7. Funding

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