miR-129-5p inhibits prostate cancer proliferation via targeting ETV1

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Ge Gao^{1,*}
Dianhui Xiu^{2,*}
Bin Yang³
Daju Sun¹
Xin Wei⁴
Youpeng Ding⁴
Yanan Ma⁴
Zhixin Wang⁴

¹Department of Pathology, China-Japan Union Hospital of Jilin University, Changchun, Jilin 130033, P.R. China; ²Department of Radiology, China-Japan Union Hospital of Jilin University, Changchun, Jilin 130033, P.R. China; ³Department of Breast Surgery, China-Japan Union Hospital of Jilin University, Changchun, Jilin 130033, P.R. China; ⁴Department of Urology, China-Japan Union Hospital of Jilin University, Changchun, Jilin 130033, P.R. China

*These authors contributed equally to this work

Background: Prostate cancer is one of the most commonly diagnosed diseases in males.

Methods: RT-qPCR was used to detect miR-129-5p expression in tumor tissues and adjacent normal tissues from patients with prostate cancer. The cell proliferation assay and colony forming assay were used to study the role of miR-129-5p in mediating prostate cancer cell growth. Bioinformatic analysis and dual luciferase assay were performed to predict and confirm ETV1 as a target gene of miR-129-5p.

Results: We found that miR-129-5p levels were decreased significantly in human prostate cancer tissues compared with matched normal tissues from patients with prostate cancer. Overexpression of miR-129-5p suppressed prostate cancer cell growth while antagonist of miR-129-5p promoted cell proliferation in immortal prostate cell line RWPE-1. In addition, elevation of miR-129-5p decreased ETV1 expression in prostate cancer cells while downregulation of miR-129-5p increased ETV1 expression in RWPE-1. Mechanistically, ETV1 is confirmed a direct target of miR-129-5p in prostate cancer cells. Through repression of ETV1 expression, miR-129-5p could inactivate YAP signaling in prostate cancer cells. In addition, overexpression of ETV1 attenuated miR-129-5p induced cell proliferation in prostate cancer cells. Correlation analysis further revealed that there was a negative correlation between miR-129-5p levels and ETV1 mRNA levels in tumor tissues from patients with prostate cancer.

Conclusion: Our results identified miR-129-5p as a tumor suppressor in prostate cancer via repression of ETV1.

Keywords: microRNA, prostate cancer, ETV1, proliferation

Introduction

Prostate cancer is the most commonly diagnosed malignant cancer types in males and ranks third among cancer-related deaths in men worldwide.¹ Globally, there were estimated 903,500 new prostate cancer cases and 258,400 estimated deaths in 2012.² Currently, prostate-specific antigen is used for early prediction of prostate cancer.³ When patients are diagnosed as early stage prostate cancer, radical prostatectomy that can greatly improve patients' overall survival is performed.⁴ Although many patients with prostate cancer are curable, tumor recurrence, metastasis, and development of castration resistance frequently occur and eventually lead to patient death.⁵⁻⁷ Advances in genetics have deepened our understandings on the carcinogenesis of prostate cancer,⁸ but the precise molecular mechanism of prostate cancer progression is not fully elucidated. As a result, further investigation is still needed to provide valuable targets for prostate cancer treatment.

MicroRNAs (miRNAs) belong to a class of noncoding RNAs with the features of small (19–25 nucleotides), single-strand, and ubiquitously expression in human.⁹ Mechanistically, miRNAs bind to complementary sites on 3'-untranslated region (3'UTR) of messenger RNA (mRNA) and lead to mRNA degradation or translational inhibition, resulting in repression of target gene expression.¹⁰ MiRNAs are important regulators of

Correspondence: Zhixin Wang
Department of Urology, China-Japan Union Hospital of Jilin University, No.126 Xiantai Street, Changchun, Jilin 130033, P.R. China
Tel +86 431 8464 1024
Email wangzhixin1234@aliyun.com

normal biological processes including cell proliferation, cell differentiation, cell motility, and cell death. Accumulating evidences suggested that deregulation of miRNAs was associated with cancer initiation and progression.^{11,12} In prostate cancer, many miRNAs were reported as oncogenes or tumor suppressors via regulation of their numerous target genes.^{13–15} For example, miR-203 was reported as an upregulated miRNA in breast cancer and promoted breast cancer progression via targeting SOCS3.¹⁶ Moreover, several miRNAs were proved to be promising biomarkers for prostate cancer diagnosis.¹⁷ Profiling of miRNAs suggested that four miRNAs (miR-4289, miR-326, miR-152-3p, and miR-98-5p) were biomarkers to identify patients with prostate cancer from healthy people.¹⁷ Using microarray, many cancer-associated miRNAs have been identified in prostate cancer.^{18,19} In 2016, one study involving miRNA profiling of 21 normal prostate tissues and 56 prostate tumor tissues has discovered many deregulated miRNAs in prostate cancer; among them, miR-424 was experimentally validated as an oncogene.²⁰ However, the potential function of other aberrant expressed miRNAs has not been studied yet.

ETS factors are transcription factors that can directly bind to promoters and enhancers to recruit other transcriptional machinery components.²¹ Overexpression of ETS family members such as ETV1, ETV4, and ETV5 are associated with prostate cancer progression.²² In prostate cancer cells, ETV1 cooperates with androgen receptor signaling and contributes to highly aggressive phenotype in mice and human.²³ ETV1 also activated transcription of TAZ to promote prostate cancer development.²⁴ Upregulated by JMJD2A, ETV1 activated transcription of YAP, a TAZ homologue, in prostate cancer.²⁵ Therefore, the regulation of ETV1 in prostate cancer needs to be investigated.

In the current study, we showed that miR-129-5p was down-regulated in prostate cancer tissues and cell lines. Overexpression of miR-129-5p inhibited cell proliferation of PC-3, while antagonizing of miR-129-5p promoted RWPE-1 cell growth. Mechanistic studies indicated that miR-129-5p negatively regulated ETV1 to inactivate YAP signaling in prostate cancer. Forced overexpression of ETV1 reversed miR-129-5p mimics inducing cell growth arrest. Reverse transcription-quantitative PCR (RT-qPCR) suggested a negative correlation between miR-129-5p and ETV1 mRNA expression in tumor tissues from patients with prostate cancer. Our findings revealed miR-129-5p as a tumor suppressor in prostate cancer via repression of ETV1.

Materials and methods

Patient samples and patient tumor data analysis

Prostate tumor tissues and matched normal prostate tissues were collected from 30 patients with prostate cancer receiving

radical prostatectomy during 2015–2017 in China-Japan Union Hospital of Jilin University. Written informed consents were provided by all patients before experiments. All procedures were carried out under the supervision of medical ethics committee of Jilin University. The study was conducted in accordance with the Declaration of Helsinki. For bioinformatic analysis of miRNA profiling in prostate cancer, GSE60117 containing data of 21 normal tissues and 56 prostate tumor tissues were analyzed on Gene Expression Omnibus (GEO) using GEO2R to obtain most significantly deregulated miRNAs.

Cell lines

Human normal epithelial cell line RWPE-1 and human prostate cancer cell lines PC-3, DU145, and LNCaP were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were used within 6 months after receipt. PC-3, DU145, and LNCaP cells were cultured in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (HyClone, Thermo Fisher Scientific) and 1% Penicillin–Streptomycin solution (Invitrogen, Thermo Fisher Scientific). RWPE-1 cells were maintained in Keratinocyte Serum Free Medium (Invitrogen, Thermo Fisher Scientific) supplemented with bovine pituitary extract (Invitrogen, Thermo Fisher Scientific) and human recombinant EGF (Invitrogen, Thermo Fisher Scientific). All cell lines were maintained in a 37°C incubator with 5% CO₂.

MiR-129-5p overexpression and antagonizing

MiR-NC mimics, miR-129-5p mimics, miR-NC antagonist, and miR-129-5p antagonist were bought from RiboBio (Guangzhou, P.R. China). For miR-129-5p overexpression or downregulation, miR-129-5p mimics or miR-129-5p antagonist was mixed with Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific), maintained for 15 minutes, and then added into culture medium of cells. Seventy-two hours after transfection, the cells were harvested and the RNA and/or protein was extracted for the following experiments.

Western blot

Protein lysates were prepared using RIPA lysis buffer (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. Antibodies for ETV1, YAP, CTGF, and CYR61 were bought from Cell Signaling Technology (CST, Danvers, MA, USA). β -actin antibody was purchased from Sigma-Aldrich. Secondary antibodies against rabbit and mouse were products of Proteintech (Chicago, IL, USA). Western blot was performed in a standard

procedure. Briefly, lysates were loaded on SDS gel; proteins were separated and then transferred on a PVDF membrane. The membrane was blocked by 5% nonfat milk and incubated in indicated primary antibodies overnight. On the next day, membrane was incubated in secondary antibodies for 1 hour. The membrane was developed by ECL Western blot substrate (Pierce, Thermo Fisher Scientific).

RNA extraction and real-time RT-PCR

Total RNA from tissues and cells were extracted using TRIzol Reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol. For miR-129-5p expression detection, RNA was reverse transcribed using a stem-loop primer with RevertAid First Strand cDNA kit (Thermo Fisher Scientific). For gene expression, RNA was reverse transcribed into first-stranded cDNA using PrimeScript RT Master Mix (Takara, Kusatsu, Japan). Real-time RT-PCR was carried out using SYBR Premix Ex Taq kit (Takara). U6 and β -actin were served as internal controls for miRNA and mRNA, respectively. The primer sequences were listed as follows: ETV1-forward: 5'-CTGAACCCTGTAACCTCTTCC-3'; ETV1-reverse: 5'-AGACATCTGGCGTTGGTACATA-3'; CTGF-forward: 5'-CAGCATGGACGTTCTGCTG-3'; CTGF-reverse: 5'-AACCACGGTTTGGTCCTTGG-3'; CYR61-forward: 5'-CTCGCCTTAGTCGTCACCC-3'; CYR61-reverse: 5'-CGCCGAAGTTGCATTCCAG-3'; β -actin-forward: 5'-CATGTACGTTGCTATCCAGGC-3'; β -actin-reverse: 5'-CTCCTTAATGTACGCACGAT-3'.

ETV1 overexpression

Full length of ETV1 open reading frame was amplified from RWPE-1 cDNA and ligated into pcDNA3.1 plasmid (OriGene, Rockville, MD, USA). For ETV1 overexpression, pcDNA3.1-ETV1 plasmid was mixed with Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific) in serum-free DMEM for 15 minutes and added into culture medium.

Cell proliferation assay

For analysis of cell growth ability, cells were seeded in 96-well plate. Every 24 hours from day 0 to day 3, 10 μ L CCK-8 solution (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) was added into culture medium in each well for 2 hours. After that, medium containing CCK-8 solution was transferred into a new 96-well plate and the absorbance at 450 nm was detected to reflect cell proliferation ability.

Colony forming assay

For colony forming assay, 500 treated cells were seeded in each well in 6-well plates. After growing for 16 days,

the culture medium was discarded, and each well was slightly washed with PBS. Then, cell colonies were fixed with 4% paraformaldehyde (Sigma-Aldrich) followed by staining with 0.5% crystal violet (Sigma-Aldrich). Images of colony were captured, and colony numbers were counted using ImageJ software.

Dual luciferase reporter assay

ETV1 3'UTR was cloned from cDNA of RWPE-1 and annealed into pGL3-basic (Promega, Fitchburg, WI, USA). The primer sequences were as follows: ETV1 3'UTR-forward: 5'-GCTCTAGATTCTGTAAATGTGAT-3'; ETV1 3'UTR-reverse: 5'-GCTCTAGATGAAATTGGAGTACTT-3'. Two site mutations were introduced into pGL3-ETV1 3'UTR-WT (wild type) to construct pGL3-ETV1 3'UTR-Mut (mutant) using GeneArt™ Site-Directed Mutagenesis PLUS System (Thermo Fisher Scientific). For dual luciferase reporter assay, cells were cotransfected with pGL3-ETV1 3'UTR-WT or pGL3-ETV1 3'UTR-Mut in combination with miR-NC mimics or miR-129-5p mimics and pRL plasmid. After 48 hours, relative luciferase activity was detected using Dual Luciferase Reporter Assay System (Promega) in accordance with the manufacturer's protocol.

Statistical analysis

All data were analyzed using GraphPad Prism 7 and expressed as mean $\pm$ SD. Student's *t*-test was used to compare differences between two groups. One-way ANOVA was carried out to compare differences among three groups, followed by Newman-Keuls test. Correlation analysis between miR-129-5p and ETV1 expression was conducted using Pearson correlation analysis. *P*-values <0.05 were considered to be statistically significant.

Results

Expression of miR-129-5p was downregulated in prostate cancer tissues and cell lines

To explore deregulated miRNAs in prostate cancer, we analyzed miRNA profiling of 21 normal prostate tissues and 56 prostate cancer tissues from data set GSE60117 in GEO database. As shown in Figure 1A, miR-129-5p was significantly downregulated in prostate cancer tissues compared with normal prostate tissues ($P<0.001$). To confirm this result, RT-qPCR was performed to detect miR-129-5p expression in 30 pairs of prostate cancer tissues and matched normal tissues collected. Similarly, a decrease of miR-129-5p expression was observed in prostate cancer tissues compared with normal tissues (Figure 1B, $P<0.001$). Next, association

Figure 1 Downregulation of miR-129-5p in prostate cancer tissues and cell lines.

Notes: (A) Through analysis of miRNA profiling in GSE60117, miR-129-5p was one of the most significantly downregulated miRNAs in 56 prostate tumor tissues compared with 21 normal prostate tissues. (B) Reverse transcription quantitative PCR indicated that miR-129-5p was decreased in 30 prostate cancer tissues in comparison with 30 matched normal prostate tissues. (C) In comparison with normal epithelial prostate cell line RWPE-1, expression of miR-129-5p was reduced in prostate cancer cell lines (PC-3, DU145, and LNCaP). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

between miR-129-5p expression and clinicopathological factors in 30 patients with prostate cancer was analyzed. No significant association was observed between miR-129-5p expression with age, Gleason score, or distant metastasis,

but high expression of miR-129-5p was associated with early pathologic stage ($P < 0.01$) (Table 1). Additionally, compared with normal epithelial prostate cell RWPE-1, expression of miR-129-5p was decreased in prostate cancer cell lines

Table 1 Association of miR-129-5p with clinicopathological features of 30 prostate cancer patients

Clinicopathological variables	No. of cases	miR-129-5p expression		P-value
		Low expression (n=15)	High expression (n=15)	
Ages (years)				1.000
≤65	17	10	7	
>65	13	5	8	
Gleason score				0.128
≤7	19	12	7	
>7	11	3	8	
Distant metastasis				0.245
No	20	12	8	
Yes	10	3	7	
Pathologic stage				0.009
≤T2	14	3	11	
>T2	16	12	4	

(PC-3, DU145, and LNCaP) (Figure 1C, $P<0.001$, $P<0.05$, and $P<0.01$, respectively).

MiR-129-5p regulated prostate cancer cell proliferation

To investigate the potential role of miR-129-5p in prostate cancer, miR-129-5p mimics was transfected into PC-3 cells. Overexpression of miR-129-5p by transfection of miR-129-5p mimics significantly inhibited cell growth of PC-3 cells (Figure 2A and B, $P<0.001$). Additionally, in the colony forming assay, miR-129-5p mimics reduced colony number formed by PC-3 cells (Figure 2C and D, $P<0.01$). We next downregulated miR-129-5p expression in RWPE-1 cells by transfection of miR-129-5p antagonist (Figure 2E). In contrast, miR-129-5p downregulation promoted cell proliferation of RWPE-1 cells (Figure 2F, $P<0.05$). The colony forming assay showed that miR-129-5p antagonist increased colony number formed by RWPE-1 cells (Figure 2G and H, $P<0.001$).

Thus, these data suggested that miR-129-5p could suppress prostate cancer cell proliferation.

MiR-129-5p repressed ETV1 expression by directly binding to its 3'UTR

MiRNA exerts its function via binding to 3'UTR on its target gene mRNA.¹⁰ ETV1 functions as an oncogene and is overexpressed in prostate cancer.²³ Using RT-qPCR, we found that ETV1 was overexpressed in all prostate cancer cell lines examined including PC-3 ($P<0.01$), DU145 ($P<0.05$), and LNCaP ($P<0.01$) compared with normal epithelial prostate cell RWPE-1 (Figure 3A). In PC-3, overexpression of miR-129-5p decreased ETV1 mRNA levels (Figure 3B, $P<0.001$). Additionally, Western blot showed that miR-129-5p mimics induced reduction of ETV1 protein expression (Figure 3C) and quantification of protein levels suggested that ETV1 protein expression was significantly downregulated toward miR-129-5p overexpression (Figure 3D, $P<0.01$). Moreover, in RWPE-1 cells, downregulation of miR-129-5p elevated ETV1 mRNA levels (Figure 3E). Western blot showed that miR-129-5p antagonist induced elevation of ETV1 protein expression (Figure 3F) and quantification of protein levels suggested that ETV1 was significantly upregulated toward miR-129-5p overexpression (Figure 3G, $P<0.01$). Using miRanda, our bioinformatic analysis showed that there was a complementary site between miR-129-5p sequence and ETV1 mRNA 3'UTR sequence (Figure 4A). To validate ETV1 as a target gene of miR-129-5p, we carried out the Dual Luciferase Reporter Assay in PC-3 cells. As expected,

miR-129-5p mimics reduced relative luciferase activity in PC-3 cells transfected with ETV1 3'UTR-WT not ETV1 3'UTR-Mut (Figure 4B). Similarly, in RWPE-1 cells, overexpression of miR-129-5p repressed luciferase activity of ETV1 3'UTR-WT (Figure 4C, $P<0.01$). These data suggested that miR-129-5p might inhibit prostate cancer progression via regulation of ETV1.

MiR-129-5p repressed YAP and its target gene expression

ETV1 is a transcription factor and accelerates prostate cancer cell growth via transcriptional activation of YAP.²⁵ Consistent with ETV1 downregulation, miR-129-5p mimics also triggered the decrease of YAP mRNA expression (Figure 5A). Also, the protein levels of YAP were decreased upon miR-129-5p overexpression (Figure 5B and C, $P<0.001$). As a transcription coactivator, YAP stimulated transcription of CTGF and CYR61.²⁶ RT-qPCR results indicated that CTGF and CYR61 expression was decreased (Figure 5D) and Western blot showed that CTGF and CYR61 protein expression was decreased (Figure 5E and F, $P<0.01$ and $P<0.001$, respectively) toward miR-129-5p overexpression, suggesting that miR-129-5p suppressed YAP target gene expression. In contrast, downregulation of miR-129-5p significantly elevated YAP mRNA and protein ($P<0.01$) expression in RWPE-1 cells (Figure 6A–C). Additionally, miR-129-5p antagonist also stimulated transcription of CTGF and CYR61 (Figure 6D, $P<0.001$), leading to enhanced protein expression of CTGF and CYR61 in RWPE-1 cells (Figure 6E and F, $P<0.05$ and $P<0.01$, respectively). The results demonstrated that miR-129-5p could repress YAP signaling via targeting ETV1 in prostate cancer.

ETV1 overexpression reversed miR-129-5p mimics-induced cell proliferation inhibition in PC-3 cells

To examine whether ETV1 was pivotal for miR-129-5p function in prostate cancer cells, we constructed plasmid containing recombinant ETV1. Western blot showed that miR-129-5p mimics reduced ETV1 protein ($P<0.001$) expression, which could be reversed ($P<0.01$) by pcDNA3.1-ETV1 transfection in PC-3 cells (Figure 7A and B, $P<0.001$ and $P<0.01$, respectively). In the cell proliferation assay, forced expression of ETV1 reversed cell growth arrest induced by miR-129-5p mimics in PC-3 (Figure 7C, $P<0.001$ and $P<0.05$, respectively). Moreover, the colony forming assay showed that reduced colony number toward miR-129-5p mimics was recovered after ETV1 overexpression

Figure 2 MiR-129-5p regulated cell proliferation in PC-3 and RWPE-1 cells.

Notes: (A) Transfection of miR-129-5p mimics elevated miR-129-5p expression by more than three-fold in PC-3 cells. (B) MiR-129-5p mimics inhibited cell proliferation of PC-3 cells in the CCK-8 assay. (C) In the colony forming assay, overexpression of miR-129-5p decreased colony number formed by PC-3 cells. (D) Quantitative analysis of colony number in (C). (E) Transfection of miR-129-5p antagonist decreased miR-129-5p expression in RWPE-1 cells. (F) Cell growth ability was elevated toward miR-129-5p downregulation in RWPE-1 cells. (G) MiR-129-5p antagonist increased colony number formed by RWPE-1 cells. (H) Quantitative analysis of colony number in (G). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Abbreviation: CCK-8, Cell Counting Kit-8.

Figure 3 ETV1 was negatively regulated by miR-129-5p.

Notes: (A) In comparison with normal epithelial prostate cell line RWPE-1, expression of ETV1 mRNA was reduced in prostate cancer cell lines (PC-3, DU145, and LNCaP). (B) MiR-129-5p mimics decreased ETV1 mRNA expression in PC-3 cells. (C) MiR-129-5p mimics decreased ETV1 protein expression in PC-3 cells. (D) Quantitative analysis of ETV1 protein expression in (C). (E) MiR-129-5p antagonist increased ETV1 mRNA expression in RWPE-1 cells. (F) MiR-129-5p antagonist increased ETV1 protein expression in RWPE-1 cells. (G) Quantitative analysis of ETV1 protein expression in (F). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4 ETV1 was a direct target of miR-129-5p.

Notes: (A) Sequence alignment of ETV1 mRNA 3'UTR-WT (wild type), 3'UTR-Mut (mutant), and miR-129-5p sequence. (B) Dual Luciferase Reporter Assay showed that miR-129-5p mimics reduced luciferase activity of PC-3 cells transfected with pGL3-ETV1 3'UTR-WT not mutant ETV1 3'UTR. (C) Dual Luciferase Reporter Assay showed that miR-129-5p mimics reduced luciferase activity of RWPE-1 cells transfected with pGL3-ETV1 3'UTR-WT not mutant ETV1 3'UTR. ** $P < 0.01$.

(Figure 7D and E, $P < 0.001$ and $P < 0.01$, respectively). Collectively, these results manifested that miR-129-5p relied on regulation of ETV1 to inhibit prostate cancer cell proliferation.

Expression of miR-129-5p was negatively associated with ETV1 mRNA levels in prostate cancer tissues

Next, we sought to investigate the relationship between miR-129-5p expression and ETV1 expression levels in prostate cancer tissues. Consistent with previous study, ETV1 protein ($P < 0.001$) and mRNA levels were elevated in prostate cancer tissues compared with matched normal prostate tissues (Figure 8A–C, $P < 0.001$ and $P < 0.05$, respectively). Furthermore, Pearson correlation assay showed that miR-129-5p expression was inversely correlated with ETV1 mRNA levels in prostate cancer tissues (Figure 8D, $P = 0.0018$). Our data suggested an miR-129-5p/ETV1/Hippo-YAP axis in regulating proliferation of prostate cancer cells (Figure 8E).

Discussion

It is well characterized that aberrant expression of miRNAs is one of hallmarks of carcinogenesis.²⁷ Recent years, many researches carried out miRNA profiling of prostate cancer tissues and discovered several miRNAs with oncogene or tumor

suppressor potential.^{20,28,29} Although studies have identified numerous differentially expressed miRNAs in prostate cancer tissues compared with normal tissues, little was known about the function and underlying molecular mechanism of specific miRNA in prostate cancer. In the current study, we found that miR-129-5p was downregulated in prostate cancer tissues and functioned as a tumor suppressor via regulation of ETV1.

MiR-129-5p has been reported as a tumor suppressor in many cancer types including glioblastoma, papillary thyroid cancer, colorectal cancer, and breast cancer.^{30–33} Several studies provided evidences that miR-129-5p inhibited cancer progression via repression of HMGB1.^{34,35} In prostate cancer, miR-129-5p was found as one of the most significantly downregulated miRNA in prostate cancer cell and was involved in the regulation of metabolism and cell proliferation via repression of key proteins in carnitine cycle.³⁶ Most recently, miR-129 was discovered to inhibit cell proliferation, migration, and invasiveness in prostate cancer cells.³⁷ Here, through screen of differentially expressed miRNAs in GSE60117, we also observed the decreased expression of miR-129-5p in prostate tumor tissues and further validated its downregulation in 30 pairs of prostate tumor and normal tissues we collected. Our cell functional assays validated tumor suppressor role of miR-129-5p in PC-3 and RWPE-1 cells; these results were consistent with previous reports.

Figure 5 MiR-129-5p mimics repressed YAP and its target gene expression in PC-3 cells.

Notes: (A) MiR-129-5p overexpression decreased YAP mRNA level in PC-3 cells. (B) Western blot showed that YAP protein level was reduced toward miR-129-5p overexpression. (C) Quantitative analysis of YAP protein level in (B). (D) MiR-129-5p overexpression decreased CTGF and CYR61 mRNA levels in PC-3 cells. (E) Western blot showed that CTGF and CYR61 protein levels were reduced toward miR-129-5p overexpression. (F) Quantitative analysis of CTGF and CYR61 protein levels in (E). ** $p < 0.01$, *** $p < 0.001$.

Several oncogenes, such as PAK5 and RET, have been validated as target genes of miR-129-5p in cancer cells.^{38,39} Study in mice and human showed that ETV1 cooperated with androgen receptor signaling to promote highly aggressive nature of advanced prostate cancer.²³ ETV1 was proved to be regulated by several miRNAs such as miR-17-5p and

miR-34b.^{40,41} However, whether and how ETV1 was regulated by miR-129-5p was not known. In PC-3, overexpression of miR-129-5p decreased ETV1 at both mRNA and protein levels. In contrast, downregulation of miR-129-5p increased ETV1 mRNA and protein expression in RWPE-1 cells. Bioinformatic analysis of miR-129-5p and ETV1 3'UTR sequences indicated

Figure 6 MiR-129-5p downregulation elevated YAP and its target gene expression in RWPE-1 cells.

Notes: (A) MiR-129-5p downregulation increased YAP mRNA level in RWPE-1 cells. (B) Western blot showed that YAP protein level was elevated toward miR-129-5p downregulation. (C) Quantitative analysis of YAP protein level in (B). (D) MiR-129-5p downregulation increased CTGF and CYR61 mRNA levels in RWPE-1 cells. (E) Western blot showed that CTGF and CYR61 protein levels were elevated toward miR-129-5p downregulation. (F) Quantitative analysis of CTGF and CYR61 protein levels in (E). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

a complementary site between them. The Dual Luciferase Reporter Assay confirmed ETV1 as a target gene of miR-129-5p in PC-3 and RWPE-1 cells. Similar to ETV1, YAP was overexpressed in prostate cancer and mediated prostate cancer metastasis, stemness, cell growth, and development of castration resistance.^{42,43} A recent study declared that ETV1

stimulated prostate cancer development through activation of YAP and its target gene expression.²⁵ In PC-3 and RWPE-1 cells, miR-129-5p suppressed YAP and its target gene CTGF and CYR61 expression at both mRNA and protein levels, suggesting that miR-129-5p might suppress YAP expression via repression of ETV1. RT-qPCR further showed a strong

Figure 7 Forced elevation of ETV1 expression rescued cell growth arrest and inhibition of colony forming ability induced by miR-129-5p mimics in PC-3 cells.

Notes: (A) MiR-129-5p mimics decreased ETV1 protein level, which could be reversed by transfection of pcDNA3.1-ETV1. (B) Quantitative analysis of ETV1 protein level in (A). (C) Overexpression of ETV1 attenuated the suppression effect of miR-129-5p mimics on cell growth. (D) In a colony forming assay, ETV1 overexpression attenuated the decrease of colony number induced by miR-129-5p mimics. (E) Quantitative analysis of colony number in (D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Abbreviation: CCK-8, Cell Counting Kit-8.

negative correlation between miR-129-5p expression and ETV1 mRNA levels in prostate cancer tissues, implying a regulatory relationship between ETV1 and miR-129-5p in prostate cancer. In a recent study, overexpression of miR-129 was shown to inactivate PI3K/AKT signaling in the PC-3.³⁷ Since the cross talk between PI3K/AKT and YAP signaling existed in

cancer cells,⁴⁴ miR-129-5p might play a central role in prostate cancer via regulating a complicated signaling network.

Conclusion

Our study provided evidences confirming that miR-129-5p was a tumor suppressor in prostate cancer and was

Figure 8 Expression of miR-129-5p was inversely associated with ETV1 mRNA levels in prostate cancer tissues.

Notes: (A) Representative Western blot of ETV1 expression in three pairs of prostate tumor tissues and matched normal tissues. (B) Quantification of ETV1 protein levels in prostate tumor tissues and matched normal tissues in (A). (C) Reverse transcription-quantitative PCR showed that ETV1 mRNA levels were significantly elevated in 30 prostate tumor tissues compared with adjacent normal prostate tissues. (D) Analysis of miR-129-5p expression and ETV1 mRNA levels showed a negative correlation between them. (E) Diagram of an miR-129-5p/ETV1/Hippo-YAP axis in prostate cancer cells. * $P < 0.05$, *** $P < 0.001$.

downregulated in tissues of prostate cancer. Mechanistic study discovered an miR-129-5p/ETV1/YAP axis in prostate cancer cells. Thus, miR-129-5p might be a promising target for the treatment of patients with prostate cancer.

Data sharing statement

Data are available on request.

Disclosure

The authors report no conflicts of interest in this work.

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