miR-195 Inhibits Tumor Progression by Targeting RPS6KB1 in Human Prostate Cancer

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Abstract

Purpose: To investigate the involvement of hsa-miRNA-195-5p (miR-195) in progression and prognosis of human prostate cancer.

Experimental Design: qRT-PCR was performed to detect miR-195 expression in both prostate cancer cell lines and clinical tissue samples. Its clinical significance was statistically analyzed. The roles of miR-195 and its candidate target gene, ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (RPS6KB1) in prostate cancer progression were confirmed on the basis of both in vitro and in vivo systems.

Results: miR-195 downregulation in prostate cancer tissues was significantly associated with high Gleason score (P = 0.001), positive metastasis failure (P < 0.001), and biochemical recurrence (BCR, P < 0.001). Survival analysis identified miR-195 as an independent prognostic factor for BCR-free survival of prostate cancer patients (P = 0.022). Then, we confirmed the tumor suppressive role of miR-195 through prostate cancer cell invasion, migration, and apoptosis assays in vitro, along with tumor xenogriffath, angiogenesis, and invasion in vivo according to both gain-of-function and loss-of-function experiments. In addition, RPS6KB1 was identified as a novel direct target of miR-195 through proteomic expression profiling combined with bioinformatic target prediction and luciferase reporter assay. Moreover, the reexpression and knockdown of RPS6KB1 could respectively rescue and imitate the effects induced by miR-195. Importantly, RPS6KB1 expression was closely correlated with aggressive progression and poor prognosis in prostate cancer patients as opposed to miR-195. Furthermore, we identified MMP-9, VEGF, BAD, and E-cadherin as the downstream effectors of miR-195–RPS6KB1 axis.

Conclusion: The newly identified miR-195–RPS6KB1 axis partially illustrates the molecular mechanism of prostate cancer progression and represents a novel potential therapeutic target for prostate cancer treatment.

Introduction

Clinical behavior of prostate cancer ranges from indolent tumors with no or little clinical significance to aggressive metastatic and lethal diseases (1). Treatment options for prostate cancer depend on clinicopathologic features of the disease, such as TNM stage, serum prostate-specific antigen (PSA), surgical margin status, and Gleason score. However, these factors explain only a moderate proportion of the observed heterogeneity in treatment outcome (2–4). Therefore, it is urgent to better understand the molecular mechanism underlying the progressive process of prostate cancer and to identify more accurate predictors for stratifying patients into indolent and aggressive cases, so that the optimal therapeutic strategies can be decided.

miRNAs represent the best characterized class of small (19–25 nt in length) noncoding RNA transcripts that are endogenously expressed in animal and plant cells (5–7). Because of their important roles in the regulation of genes that are involved in many physiologic processes, including cell development, proliferation, differentiation, and apoptosis, it is not of a surprise that miRNAs are also involved in the initiation and progression of various human diseases such as cancer (8). miRNAs can either serve as a tumor suppressor or as an oncogene, depending on the genes they target (9, 10). According to the Taylor dataset, we noticed that hsa-miR-195-5p (miR-195) has a potential as a marker for biomedical recurrence of prostate cancer patients. It is located from 6881953 bp to 6862065 bp on chromosome 17p13.1 and belongs to the miR-15/16/195/424/497 family (11). It has been reported to be upregulated as an oncogene in malignant melanoma, while downregulated as a strong tumor suppressor in breast cancer, hepatocellular carcinoma, adrenocortical carcinoma, squamous cell carcinoma of tongue, and esophageal cancer (12–17). However, up to now, its roles in prostate cancer are still unclear.
Tumor Suppressive Role of miR-195 in Prostate Cancer

Translational Relevance
Optimal therapeutic strategies for prostate cancer patients are still challenging because of the disease's highly variable natural history. Growing evidence shows that genetic and epigenetic alterations are involved in prostate cancer progression; however, the underlying molecular mechanisms have not been fully elucidated. Here, we identified hsa-miRNA-195-5p (miR-195) as a critical tumor suppressor in prostate cancer progression via reducing the expression of RPS6KB1. More interestingly, both miR-195 and RPS6KB1 axis partially illustrates the molecular mechanism of prostate cancer progression and represents a novel potential therapeutic target for prostate cancer treatment.

Materials and Methods

Patients and tissue samples
The study was approved by the human study ethics committees at Massachusetts General Hospital (Boston, MA) and the Ministry of Public Health of PR China. Written informed consent was obtained from all patients. All specimens were handled and made anonymous according to the ethical and legal standards. Detailed information on patients and tissue samples was summarized in Supplementary File S1.

Cell culture
Normal human prostate epithelial cells (PrEC) were purchased from Lonza Company in 2012 and were cultured in PrECGM Bullet kit (Lonza) with antibiotics. Human prostate cancer cell lines, PC-3, LNCaP, and DU145 were purchased from the American Type Culture Collection (Manassas, VA) in 2012 and were cultured in RPMI1640 medium (Hyclone), 2 mmol/LL-glutamine, and antibiotics. The companies RPMI1640 medium (Hyclone) supplemented with 10% FBS (Gibco), 2 mmol/LL-glutamine, and antibiotics. The companies maintained at 37°C in a humidified chamber supplemented with 5% CO₂.

Animals
Animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research at Guangzhou Medical University (Guangzhou, PR China). Twenty BALB/c nude mice (4- to 5-week-old males) were purchased from Guangdong Medical Laboratory Animal Center and were housed five per cage in wire-top cages with sawdust bedding in an isolated, clean, air-conditioned room at a temperature of 25–26°C and a relative humidity of approximately 50%, lit 12 hours/day.

Cell line construction and transfection
The miR-195 coding sequence was cloned into the pMIRNA1 Lentivectors (Human pre-microRNA Expression Construct Lentivector miR-195 MI0000489, Cat No: PMIRH195PA-1, SBI) for expressing the miR-195 precursor (miR-195). Precursor Scramble control hairpin in pCDH-CMV-Scramble hairpin-EF1-copGFP (CD511B-1) was purchased from the same vendor (cat. no: PMIRHO000PA-1, SBI) for negative controls (miR-NC). The miR-Zip anti-miRNA Constructs (miRZip-195 anti-miR-195 miRNA construct, cat. no: MZIP195-PA-1) and miRZip control vectors (pGreenPuro Scramble Hairpin Control-Construct, cat. no: MZIP000-PA-1) were designed and cloned by SBI. To package the construct, 293TN cells were transfected with miR-195/miR-NC or anti-195 anti-NC by pPACKH1 Packaging Plasmid Mix (cat. no: LV500A-1, SBI), and then after 3 days, the virus particles were collected according to the packaging protocol of SBI with the Lenti-Concentrin Virus Precipitation Solution (cat. no: LV810A-1, SBI). DU145 and LNCaP cells were infected with TransDux virus transduction reagent (Cat No: LV850A-1, SBI). The infected cells were isolated with a flow cytometer and cultured in 96-well plates.

Oligonucleotide and plasmid transfection
The miRNA mimics (cat. no: miR0000461-1-5) and miRNA mimics negative control (cat. no: miR01201-1-5) that were used for transient transfection were designed and synthesized by Ribobio. The RPS6KB1 coding sequence (without 3’-UTR) was cloned into pCDNA3.1(−)Vector (cat no: V790-20, Invitrogen). The blank vector was used as negative control. siRNA against RPS6KB1 (si-RPS6KB1) and negative control (si-NC) with nonspecific sequences was synthesized by SigmaUSA. The sequence of siRNA targeting RPS6KB1 was as follows: 5’-CCACAAUICGUGICGUG-GAUIdTdT-3’ (sense), 5’-AUCCCACACCGAICUIGUGdGdTdT-3’ (antisense). The sequence of siRNA negative control was as follow: 5’-IUCUICCCAGCAUGGCAGCGUUTT-3’ (sense), 5’-AGCGUGAGACGUCGAAAAT-3’ (antisense). Cells were transfected with miRNA mimic, siRNA, and pCDNA3.1(−)RPS6KB1 using Lipofectamine 2000 Reagent (cat. no: 11668019, Invitrogen) according to the manufacturer’s protocol. For migration, invasion, apoptosis, proliferation, RNA extraction, and Western blot assays, cells were used 48 hours after transfection.

Proteomics expression profiling by mass spectrometric analysis using the isobaric tagging reagent iTRAQ
Identification of novel targets for miR-195 by proteomic expression profiling with iTRAQ tagging was performed according to the protocol as described in Supplementary File S2.

Bioinformatic miRNA target prediction
Three online programs Target-Scan (release 6.2; ref. 18), miRWalk (last update: March 29, 2011; ref. 19), and miRanda (August 2010 Release, last update: November 01, 2010; ref. 20) were used to predict potential target genes for miR-195.

qRT-PCR
Expression levels of miR-195, SMAP2, DCUN1D1, SMAD4, IPO9, RPS6KB1, CAPZA2, and CPNE1 mRNA in prostate cancer
cell lines, xenograft tumors, and clinical prostate cancer tissues were detected by qRT-PCR analysis according to the protocol of our previous studies (21, 22). The sequences of all the primers used in this study were shown in Supplementary Table S2.

**Western blot analysis**

Expression levels of RPS6KB1, MMP-9, VEGF, BAD, and E-cadherin proteins in prostate cancer cell lines, xenograft tumors and clinical prostate cancer tissues were detected by Western blot analysis according to the protocol of our previous studies (21, 22). The antibodies used in this study were shown in Supplementary Table S3.

**Immunohistochemistry**

Expression pattern and subcellular localization of RPS6KB1 protein in clinical prostate cancer tissues, and those of CD31 and VIMENTIN proteins in subcutaneous tumor xenografts of nude mice were detected by immunohistochemistry and the immunoreactivity scores (IRS) of VIMENTIN were calculated according to the protocol of our previous studies (21, 22). Vasculature density in tumor xenografts was determined by the number of CD31-positive vessels. For Immunohistochemistry evaluation of RPS6KB1 in clinical prostate cancer tissues, tumor specimens were scored as positive if greater than 10% of the tumor cells exhibited immunoreactivity. The
antibodies used in this study were shown in Supplementary Table S3.

Generation of the in vivo xenograft model
For the in vivo tumor formation assays, DU145 or LNCaP cells transfected with the miR-195, miR-NC, anti-195, or anti-NC lentivectors were trypsinized and suspended in PBS. Then, the cells were subcutaneously injected into the flanks of each nude mouse (5 per group). DU145 cells were subcutaneously injected at a concentration of 1 × 10⁶ cells. LNCaP cells were subcutaneously injected as a mixture of 2 × 10⁶ cells and an equal volume of Matrigel (cat. no: 356234, BD Biosciences), reaching a total concentration of 10 mg/mL. The tumor sizes were measured at 4-day intervals as soon as the tumors were measurable and the tumor volumes were calculated: V(mm³) = width²(mm²) × length (mm)/2. On day 44 for LNCaP and day 36 for DU145 groups, the mice were sacrificed. The mice were manipulated and housed according to protocols approved by the Institute for Laboratory Animal Research at Guangzhou Medical University (Guangzhou, China).

Luciferase reporter assay
The expression of miR-195–targeted gene was evaluated by using a luciferase reporter assay in LNCaP cells. The putative miR-195 complementary site in the 3′-UTR of RPS6KB1 mRNA (NM_001272060; 3′-UTR: 1624-1630) or its mutant sequence was cloned into the psiCHECK-2 luciferase reporter vector (Promega). LNCaP cells were cotransfected with 50 nmol/L miR-195 mimic (cat. no: miR10000461-1-5, RiboBio) or the negative control (cat. no: miR01201-1-5, RiboBio) and 0.5 μg of psiCHECK-2-RPS6KB1-3′UTR-WT or psiCHECK-2-RPS6KB1-3′UTR-MUT. Cells were collected 48 hours after transfection and analyzed with the Dual-Luciferase Reporter Assay System (Promega). The firefly and Renilla luciferase signals were generated by the GloMax fluorescence reader (Promega), the Renilla luciferase signal normalized to the firefly luciferase signal.

Cell invasion and migration assays
Cell invasion and migration were respectively detected by the Transwell and the scratch wound-healing motility assay according to the protocol of our previous studies (21, 22).

Apoptosis assay
Cell apoptosis was detected using APC-conjugated Annexin V (Annexin V-APC) Kit (cat. no: 550474, BD Biosciences) and 7-aminoactinomycin D (7-AAD; cat. no: AP104-60-AAD, Multi-science) according to the protocol of our previous studies (21, 22).

Statistical analysis
The version 13.0 SPSS for Windows (SPSS Inc.) and SAS 9.1 (SAS Institute) softwares were used for statistical analysis. Continuous variables were expressed as X ± s. Statistical analyses of qRT-PCR and Western blot were conducted using Wilcoxon signed-rank test. Statistical analysis was performed independently by two biostatisticians with the Fisher exact test for any 2 × 2 tables and Pearson χ² test for non-2 × 2 tables. Kolmogorov–Smirnov test was used to test for normality of the distribution of miR-195 expression level. The Mann–Whitney U and Kruskal–Wallis H tests were performed to examine the associations between miR-195 expression and clinicopathologic characters of prostate cancer patients in Taylor dataset. The Kaplan–Meier method was used for the survival analysis and Cox regression analysis was used for the univariate and multivariate analysis. The Spearman correlation was calculated between the expression levels of miR-195 and RPS6KB1 in prostate cancer tissues. Differences were considered statistically significant when the P value was less than 0.05.

Results
Decreased expression of miR-195 in human prostate cancer cells and tissues
Expression levels of miR-195 in three human prostate cancer cell lines (LNCaP, DU145, and PC-3) and prostate cancer tissues were respectively lower than those in a nonmalignant prostate epithelial cell line (PrEC) and adjacent noncancerous prostate tissues (Supplementary Table S1A and S1B). Among the three prostate cancer cell lines, DU145 cells had the lowest miR-195 expression. LNCaP cells are androgen-sensitive human prostate cancer cells, while DU145 cells are not hormone sensitive. Thus, DU145 and LNCaP cells were chosen for further studies.

Decreased expression of miR-195 is associated with the aggressive progression of human prostate cancer
To investigate whether the expression of miR-195 was associated with clinicopathologic features of prostate cancer patients, a publicly available dataset (Taylor dataset) consisting of miRNAs expression profiles for 113 primary prostate cancer tissues was used (23). The data shown in Supplementary Table S4 revealed that miR-195 downregulation was frequently found in prostate cancer tissues with high Gleason score (P = 0.001), positive metastasis failure (P < 0.001), and biochemical recurrence (P < 0.001).

Decreased expression of miR-195 predicts poor prognosis of human prostate cancer
To evaluate the prognostic value of miR-195 expression in prostate cancer, the Kaplan–Meier method was performed to analyze the correlations between miR-195 expression with BCR-free survival, overall survival, nonmetastatic BCR-free survival and nonmetastatic survival of prostate cancer patients in Taylor dataset. Pairwise comparisons showed a significant difference in the BCR-free survival (Supplementary Fig. S2A and S2B) and nonmetastatic BCR-free survival (Supplementary Fig. S2B) between patients with high and low miR-195 expression. However, the data revealed that there were no significant differences in overall survival and nonmetastatic survival between high and low miR-195 expression group (Supplementary Fig. S2C and S2D). Multivariate analysis revealed that downregulation of miR-195 had the potential to serve as an independent predictor for shorter BCR-free survival (Supplementary Table S5).

miR-195 suppresses invasion and migration but promotes apoptosis of prostate cancer cells in vitro
To determine the suppressive role of miR-195 in prostate cancer in vitro, we first constructed a lentiviral vector expressing miR-195 and established stable cell lines LNCaP and DU145 expressing miR-195 after lentivectors transduction. qRT-PCR analysis confirmed that the cell lines were successfully established (Fig. 2A). Transwell assays clearly revealed that enforced expression of
Figure 2.
The reexpression of miR-195 inhibits invasion and migration but promotes apoptosis of LNCaP and DU145 cells in vitro. A, miR-195 was reexpressed in the miR-195 stably transfected cell lines LNCaP and DU145. miR-195 levels were determined by qRT-PCR after transfection. miR-195 expression (miR-195/U6) was calculated as the fold change relative to the negative control (NC). B, Transwell analysis showed that miR-195 overexpression suppressed invasive abilities of LNCaP and DU145 cells. Statistical analysis was performed with three independent experiments. C, wound-healing assays indicated that miR-195 upregulation inhibited migration of LNCaP and DU145 cells. Statistical analysis was performed with three independent experiments. D, enforced expression of miR-195 promoted the cell apoptosis of LNCaP and DU145 cells. Statistical analysis was performed with three independent experiments. Data, mean ± SD. *, P < 0.05; **, P < 0.01 compared with negative control.
miR-195 significantly reduced the invasive activities of both DU145 and LNCaP cells compared with these cells (Fig. 2B). Wound-healing assays demonstrated that miR-195 upregulation markedly weakened the migratory abilities of both DU145 and LNCaP cells (Fig. 2C). In contrast, the apoptotic rates of miR-195–transfected DU145 and LNCaP cells were significantly higher than those of control cells (Fig. 2D). Moreover, we also stably suppressed the miR-195 expression in LNCaP and DU145 cell lines via lentivectors transduction (Supplementary Fig. S3A). Intriguingly, the knockdown of miR-195 expression with lentivectors in LNCaP and DU145 cell lines would dramatically enhance the abilities of cellular invasion, motility, and reduce cellular apoptosis (Supplementary Fig. S3B–S3D).

**miR-195 suppresses tumor growth, angiogenesis, and invasion in vivo**

To better evaluate the biologic functions of miR-195 in vivo, LNCaP and DU145 prostate cancer cell lines stably expressing miR-195 or vector control via lentivectors transduction were used. The miR-195–overexpressed cell lines (miR-195) were subcutaneously injected into the flank of each male nude mice, simultaneously, the vector control prostate cancer cell lines (miR-NC) were subcutaneously injected into the other flank of the same mice. The LNCaP and DU145 cells stably expressing miR-195 formed significantly smaller tumor nodules (Fig. 3A) and remarkably slowed tumor xenografts growth compared with the controls (Fig. 3B). On the other hand, we further found that the prostate cancer cells that permanently suppressed the expression of miR-195 with lentivectors could enhance tumor growth compared with the controls (Supplementary Fig. S4A and S4B). Next, immunohistochemical analysis using pan-endothelial marker CD31 and VIMENTIN antibody, which is a marker of mesenchymal derived cells or cells undergoing an epithelial-to-mesenchymal transition (EMT; ref. 24), were employed to evaluate the angiogenesis and invasive tendency of the tumor xenografts. The results indicated that the expression level of CD31 and VIMENTIN protein in the tumor xenografts established by LNCaP or DU145 cells stably expressing miR-195 was remarkably lower than that in the xenografts established by cells transfected with control vectors (Fig. 3C and D). Moreover, the tumor xenografts established by LNCaP or DU145 cells with low miR-195 expression presented significantly more CD31 and VIMENTIN protein than the control xenografts (Supplementary Fig. S4C and S4D). These results strongly demonstrated that miR-195 could significantly inhibit tumor growth, angiogenesis, and invasion in vivo.

**Proteomics analysis of miR-195–induced changes in protein synthesis**

To identify direct targets of miR-195 in prostate cancer, we performed differential tagging with iTRAQ followed by nanoreversed phase liquid chromatography (nano-LC) and tandem mass spectrometry (MS-MS) analysis on LNCaP cells (miR-195 vs. miR-NC) to detect miR-195–induced changes in protein synthesis. As a result, a total of 3,194 proteins were identified, when the endogenous miR-195–encoded downregulated proteins were set as a requirement for unambiguous identification. All proteins were reliably quantified in at least two experiments. Of note, ectopic expression of miR-195 caused only moderate changes in overall protein synthesis, with the majority of proteins having miR-195/miR-NC ratios between −1.5 and 1.5. Among the 3,194 proteins quantified in at least two experiments, only 78 (2.44%) were differentially regulated with fold changes <−1.5 or >1.5 (miR-195 vs. miR-NC), including 50 downregulated proteins and 28 upregulated proteins (Supplementary Table S6). Consistent with these findings, recent studies have indicated that ectopic miRNA expression often exerts overall moderate effects on global protein synthesis (25).

As miRNAs are noncoding RNA transcripts and they can only execute their biologic functions through their direct and indirect targets that are coding genes, we then investigated the molecular role of all the differentially expressed proteins induced by miR-195. Pathway and biologic functional enrichment analyses, respectively, based on Ingenuity Pathways Analysis and GO annotation system gave us a global clue of their functional roles in prostate cancer progression. Among the pathways enriched by candidate targets of miR-195, we concentrated on tumor-related pathways, such as mTOR signaling, IL-8 signaling, AMPK, and IGF-1 signaling pathways (Supplementary Fig. S5 and Supplementary Table S7), which all have broad effects on cell behavior. On the other hand, GO annotation system uses a controlled and hierarchical vocabulary to assign function to genes or gene products in any organism. The changed proteins induced by miR-195 significantly controlled many biologic processes directly relevant to cancers, such as cell cycle, cell morphology, cell assembly and organization, and cellular movement (Supplementary Table S8), which are involved in multiple steps of cell viability, and evidence had shown that dysregulations of the cell viability of those ingredients might lead to tumor formation. These results suggested that the candidate targets of miR-195 may control broad biologic functions associated with prostate cancer.

**RP56KB1 is the direct target of miR-195**

We also used three miRNA target predicting programs (Target-Scan, miRWalk, and miRanda) to identify the candidate targets of miR-195. As a result, the three programs all predicted RP56KB1, SMAP2, DCUN1D1, SMAD4, IPO9, CAPZA2, and CPE11 as candidate targets of miR-195. Moreover, they also encoded the corresponding downregulated proteins according to the results of proteomics analysis of miR-195–induced changes in protein synthesis (Fig. 4A). To verify this prediction, qRT-PCR analysis was performed and the results showed that the endogenous SMAP2, RP56KB1, IPO9, and DCUN1D1 expression in cells and established tumors associated with LNCaP cells stably expressing miR-195 were all significantly reduced at mRNA levels (Fig. 4A). Among them, RP56KB1 was the most significantly downregulated gene. Moreover importantly, RP56KB1 is an important component in the mTOR signaling pathway, which was one of the significantly enriched pathways involved by miR-195–induced differentially regulated proteins (Supplementary Fig. S5A; Supplementary Table S7). Therefore, we would like to focus on the biologic role of miR-195-RP56KB1 axis during prostate cancer progression in further experiments.

To confirm RP56KB1 being targeted by miR-195, the luciferase reporter containing the complimentary seed sequence of miR-195 at the 3′–UTR region of RP56KB1 mRNA was constructed (Fig. 4B). Luciferase activity assay showed that the expression of the reporter of RP56KB1 was significantly reduced by cotransfection with hsa-miR-195 mimics. In contrast, the expression of the reporter of
RPS6KB1 containing the mutated sequence of the same fragment was not affected by cotransfection with hsa-miR-195 mimics (Fig. 4B). The result indicated that the fragment at the 3'UTRs of the RPS6KB1 mRNA was the complementary site for the miR-195 seed region, suggesting that RPS6KB1 was the direct target of miR-195.

We performed Western blot analysis to detect the expression levels of RPS6KB1 protein in LNCaP and DU145 cells transfected by lentivectors and in the tumor xenografts established by these prostate cancer cells. RPS6KB1 protein levels were remarkably downregulated in LNCaP or DU145 cells stably overexpressing miR-195 (Fig. 4C). The similar findings were observed in the corresponding tumor xenografts established by cell lines overexpressing miR-195. In line with the above gain-of-function experiments, we also knocked down the expression of miR-195 in LNCaP or DU145 cells using anti-miR-195 lentivectors and found that the RPS6KB1 protein levels in cell lines and corresponding tumor xenografts were all significantly upregulated (Fig. 4D). Overall, miR-195 negatively regulated RPS6KB1 expression in vitro and in vivo.
RPS6KB1 is a critical downstream mediator of miR-195 suppressive effects in prostate cancer progression

To clarify whether the role of miR-195 in prostate cancer was mediated through suppressing RPS6KB1 expression, pCDNA3.1(+) Vectors expressing RPS6KB1 without its 3'-UTR were conducted. As shown in the Fig. 5A, the endogenous RPS6KB1 expression levels detected by Western blot in LNCaP and DU145 cells transfected with the miR-195 mimics or vector control for 48 hours. Migration (Fig. 5B and Supplementary Fig. S7A), invasion (Fig. 5C and Supplementary Fig. S7B), and apoptosis (Fig. 5D and Supplementary Fig. S7C) assays all indicated that restoration of RPS6KB1 expression dramatically attenuated the effects induced by miR-195. Furthermore, to verify whether inhibition of RPS6KB1 expression could generate similar in vitro phenotypes induced by miR-195 overexpression, we knocked down the endogenous RPS6KB1 protein expression in LNCaP and DU145 cells with RPS6KB1 siRNA (Supplementary Fig. S6A). In vitro results showed that the knockdown of endogenous RPS6KB1 expression could imitate the effects associated with
miR-195 overexpression in prostate cancer cells, including inhibiting the abilities of migration (Supplementary Figs. S6B and S7A) and invasion (Supplementary Figs. S6C and S7B), as well as promoting cellular apoptosis (Supplementary Figs. S6D and S7C). Collectively, these findings indicated that RPS6KB1 was a critical mediator in the tumor suppressive roles of miR-195 in prostate cancer.

Reverse correlation between miR-195 and RPS6KB1 expression in human prostate cancer tissues

We have observed that the expression of miR-195 was downregulated in prostate cancer tissues (Supplementary Fig. S1B). In contrast to miR-195, Western blot and qRT-PCR analyses revealed that RPS6KB1 expression at protein and mRNA levels were both upregulated in the same human prostate cancer tissues compared with adjacent noncancerous prostate tissues (Supplementary Fig. S8A–S8C). Importantly, the Spearman Correlation analysis clearly presented a negative correlation between RPS6KB1 mRNA and miR-195 expression in prostate cancer tissues (Supplementary Fig. S8D).

RPS6KB1-positive expression associates with the aggressive progression and poor prognosis of human prostate cancer

To investigate whether RPS6KB1 expression could be linked to the clinicopathologic features of human prostate cancer, the immunohistochemical staining using the antibody that specifically recognizes RPS6KB1 was employed to detect the expression pattern and subcellular localization of RPS6KB1 expression in 225 prostate cancer and 25 adjacent noncancerous prostate tissues. In general, we found that this antibody stained the cytoplasm and cellular membrane of prostate cancer cells and gave evenly distributed staining pattern with various intensities (Fig. 6A). The positive expression rate of RPS6KB1 protein in prostate cancer clinical samples [174/225 (77.3%)] was significantly higher than that in adjacent benign tissues [10/25 (40%), (P < 0.001)].

Then, we analyzed 225 radical prostatectomy specimens represented in tissue microarray from prostate cancer patients. The data shown in Supplementary Table S4 revealed that RPS6KB1-positive expression was significantly associated with advanced pathologic stage (P = 0.036), positive surgical margin status (P = 0.02), positive BCR (P = 0.017), and shorter overall survival (P = 0.02) of prostate cancer patients. Kaplan–Meier analysis was conducted to assess the prognostic value of RPS6KB1 expression in human prostate cancer. We found that there were significant differences in the BCR-free survival (P = 0.011, Fig. 6B) and overall survival (P = 0.022, Fig. 6D), but not in metastasis-free survival (P = 0.058, Fig. 6C), between patients with positive and negative RPS6KB1 expression.

Univariate analysis (a, Supplementary Table S9) revealed that RPS6KB1 expression (P = 0.014 and 0.03, respectively) and Gleason score (P < 0.001 and P = 0.001, respectively) were significant prognostic factors for BCR-free survival and overall survival in patients with prostate cancer. Our analysis also demonstrated that pathologic tumor stage (P = 0.002) and surgical margin (P = 0.001) were significant prognostic factors for BCR-free survival but did not show any correlative relationship with overall survival in patients with prostate cancer (a, Supplementary Table S9). Besides, PSA failure (P = 0.006) was a significant prognostic factor for overall survival. We then used the Cox proportional hazards multivariate model to examine...
the association of clinicopathologic factors and RPS6KB1 staining status with BCR-free survival and overall survival. The results indicated that only RPS6KB1 expression and Gleason score were independent predictors of BCR-free survival and overall survival in prostate cancer patients (b, Supplementary Table S9).

MMP-9, VEGF, BAD, and E-cadherin function as the downstream effectors of miR-195–RPS6KB1 axis

Growing evidence suggests that RPS6KB1 may be required for tumor cell survival, tumor invasion, and angiogenesis through inactivating the proapoptotic molecule BAD and the epithelial marker E-cadherin, inducing expression of MMP-9 and VEGF, respectively (26–29), which prompted us to determine whether MMP-9, VEGF, BAD, and E-cadherin were downstream effectors of miR-195–RPS6KB1 axis in human prostate cancer. As shown in Supplementary Fig. S9A, we found that enforced expression of miR-195 and knockdown of RPS6KB1 protein in two prostate cancer cell lines LNCaP and DU145 both significantly reduced the protein expression levels of MMP-9 and VEGF, but increased those of E-cadherin and BAD, implying the four proteins might function as the

Figure 6.
Immunohistochemical staining of RPS6KB1 in prostate cancer and adjacent benign prostate tissues and its prognostic implications. A, positive RPS6KB1 staining with weak, moderate, and strong expression levels in prostate cancer was found in cell membrane and cytoplasm. Besides, negative RPS6KB1 staining was presented in benign prostate tissues. Left, magnification ×50; right: magnification ×200. B–D, RPS6KB1 protein expression showed a prognostic value in BCR-free and overall survival but not in metastasis-free survival, as indicated by Kaplan-Meier analysis.
downstream effectors of miR-195–RPS6KB1 axis in human prostate cancer and be implicated in various pathologic events during prostate cancer progression (Supplementary Fig. S9B).

Discussion

Extensive evidence shows that the PSA screening worldwide has brought an overdiagnosis of prostate cancer, which subsequently leading to an overtreatment of patients with indolent disease (4). Therefore, it is extremely necessary to identify novel and more efficient biomarkers that can discriminate between indolent and aggressive prostate cancer, so that patients with low risk of progression may better benefit from avoiding unnecessary treatments. miRNAs have been recognized to be promising biomarkers for cancer patients due to their deregulated expression in a variety of human cancer types as well as their tissue-specific expression patterns in cancer and high stabilities in blood (30). In our study, miR-195 was identified as a novel tumor suppressor of prostate cancer. We further determined that miR-195 could directly target RPS6KB1 by binding to its 3′-UTR and be implicated into the progression of prostate cancer via regulating RPS6KB1 signaling. More interestingly, our clinical evidence revealed that both miR-195 and RPS6KB1 could be used to predict prognosis of prostate cancer. These findings provided us with abundant information to further decode the functional implications of miR-195–RPS6KB1 axis in the progression of human prostate cancer.

Altered expression of the miR-15/16/195/424/497 family has been reported to exert diverse effects in tumor cells. Similar with other members of this family, miR-195 expression is downregulated frequently in multiple cancer types and implicates its fundamental role in cell-cycle regulation and carcinogenesis (31). In the current study, we found that the reduced expression of miR-195 was a characteristic molecular change in both prostate cancer cell lines and clinical tissue samples. miR-195 is localized to the segment of chromosome 17p13.1, which has been reported to be frequently deleted in cancer cells (32), implying that miR-195 downregulation in prostate cancer cells and tissues might be related to gene copy number reduction. Besides, the methylation of CpG islands upstream of the miR-195 might be another cause contributing to the downregulation of miR-195 (13). In the current study, we analyzed the CGH array information in the Taylor dataset, but failed to find any copy number variations (CNV) of miR-195, suggesting the CNVs is not the reason for the downregulation of miR-195 here. Then, we also confirmed the significant associations of miR-195 downregulation with aggressive clinicopathologic characteristics, shorter BCR-free and nonmetastatic BCR-free survivals of prostate cancer patients, which prompted us to determine the roles of miR-195 in malignant phenotypes of prostate cancer in vitro systems and in vivo models. Our data confirmed the tumor suppressive role of miR-195 through prostate cancer cells invasion, migration, and apoptosis assays in vitro, along with tumor xenografts growth, angiogenesis, and invasion in vivo according to both gain-of-function and loss-of-function experiments.

To further disclose the molecular mechanisms underlying the involvement of miR-195 in prostate cancer, we combined iTRAQ-based nano- LC/MS-MS and three miRNA target prediction algorithms to identify the potential targets of miR-195. iTRAQ-based nano-LC/MS-MS provides a potential platform for simultaneously identifying and quantifying target proteins of miRNAs (33). Compared with the conventional proteomics methods, such as two-dimensional gel electrophoresis and the peptide mass fingerprint method via matrix-assisted laser desorption/ionization—time-of-flight MS, the iTRAQ-based nano-LC-MS/MS methods can undertake high-throughput tasks and identify proteins more accurately. Then, we also integrated the prediction results of bioinformatic methods. As a result, RPS6KB1, SMAP2, DCUN1D1, SMAD4, IPO9, CAPZA2, and CPNE1 were predicted as candidate targets of miR-195. Numerous genes have been identified as the direct targets of miR-195. For example, miR-195 targeted the TNFα/NFκB pathway by downregulating IκB Kinase Alpha and TAB3 in hepatocellular carcinoma (14). Cell-cycle checkpoint kinase WEE1 was targeted by miR-195 in malignant melanoma (12). Here, we showed great interest in RPS6KB1 biologic role as a downstream target of miR-195, because it is a crucial component of the mTOR signaling pathway which was one of the significantly enriched pathways involved by miR-195–induced differentially expressed proteins and plays an important role in prostate cancer, as reported many times (34, 35). Consequently, RPS6KB1 was identified as a novel direct target of miR-195 confirmed by luciferase report assay. RPS6KB1 is an evolutionarily conserved serine/threonine kinase and has been recognized as an integrator of nutrient and growth factor signals for fundamental cellular processes, such as cell growth and proliferation (36). It plays an important role in protein synthesis, via interacting with or activating other transcriptional factors (37, 38), as well as being a transcriptional factor (28). Growing evidence suggests that the alteration of RPS6KB1 may be a crucial driver of tumor initiation and progression (39, 40), and that the inactivation of RPS6KB1 may be therapeutically effective in many cancers (41, 42). Our data here suggested that the enforced expression and the loss of miR-195 could respectively cause the reduced and the increased expression of RPS6KB1 at both mRNA and protein levels in prostate cancer cells and tumor xenografts dramatically. qRT-PCR analysis in human prostate cancer tissues also showed an adverse relationship between miR-195 and RPS6KB1 expression. Of note, the reexpression and knockdown of RPS6KB1 could respectively rescue and imitate the effects associated with miR-195. Moreover, the prognostic impact of RPS6KB1 expression in prostate cancer was also determined based on a cohort of 225 prostate cancer patients. We further identified the downstream effectors of miR-195–RPS6KB1 axis. Our data showed that the decreased expression of miR-195 might upregulate RPS6KB1, leading to the increasing expression of MMP-9 and VEGF proteins and the decreasing expression of E-cadherin and BAD proteins, which together trigger a series of tumor reactions in prostate cancer, including tumor invasion, angiogenesis, EMT, and survival (Supplementary Fig. S9B).

In conclusion, our data offered the convincing evidence that miR-195 may function as a tumor suppressor with prognostic impact in human prostate cancer. miR-195 deregulation may confer proliferative advantage and promote prostate cancer migration and invasion by regulating RPS6KB1 signaling. The newly identified miR-195–RPS6KB1 axis partially illustrates the molecular mechanism of prostate cancer progression and
represents a novel potential therapeutic target for prostate cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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