MicroRNA-194-5p directly targets Rab23 and inhibits tumor progression in prostate cancer

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Abstract: Objectives: The prostate cancer is among the leading causes of tumors in men. Resistance to conventional treatment for advanced prostate cancer has posed serious threat. The microRNAs denote a class of noncoding RNAs and regulate numerous processes. Here we aim to investigate the function of miR-194-5p in prostate cancer. Methods: The expression of miR-194-5p and Rab23 is determined by qRT-PCR. Association between miR-194-5p and clinicopathological features was also evaluated. The role of miR-194-5p in regulating proliferation and invasion was explored in vivo. Results: The expression of miR-194-5p was frequently downregulated in prostate cancer tissues as well as cell lines. Meanwhile, miR-194-5p transfection can significantly impair proliferation and invasion. Furthermore, we identified Rab23 as a target for miR-194-5p and ectopic expression of Rab23 can counteract the effect of miR-194-5p. We also found a significantly inverse correlation between miR-194-5p and Rab23 expression. Conclusions: Our results suggested that decreased miR-194-5p expression may underscore the mechanism of prostate cancer development. The intricate interaction between miR-194-5p and Rab23 may serve as a potential therapeutic target in prostate oncogenesis.

Keywords: MiR-194-5p, prostate cancer, Rab23, survival

Introduction

The prostate cancer (PCa) is among the leading causes of tumors in men and about 1/4 of total cases can be ascribed to prostate cancer [1]. Multiple treatment strategies can be obtained for PCa and the five year survival for PCa is relatively high [2]. The main challenge for advanced PCa is the lack of effective curative treatment and metastasis to multiple organs or tissues is also a critical feature [3]. Meanwhile, resistance to androgen-deprivation therapy and progression to metastatic PCa also posed serious challenge to the survival of men. Therefore, further understanding of the underlying molecular mechanisms of PCa will provide crucial insight into the development of effective therapeutics or treatment options for prostate cancer.

The microRNAs denote a class of small noncoding RNAs about 22 nucleotides and can regulate gene expression by base-pairing with transcripts [4]. Even imperfect binding of microRNAs to the targets can cause degradation and translation inhibition [5]. Differential microRNA expression patterns can be found in different types of tumors and therefore suggest that microRNAs may be used as diagnostic markers or therapeutic targets [6]. Many studies have implied that microRNAs might play pivotal roles in regulating carcinogenesis and may further be taken as biomarkers. Recent study for PCa showed that miR-223 is involved in the progression of PCa as miR-223 is frequently decreased in tumorous tissues compared with normal adjacent tissues [7]. The miR-21 has been also suggested as an oncomir in prostate cancer [8]. BTG2 loss is further identified as a lead cause of miR-21 decrease and transformation of prostate cancer [9]. However, the role of miR-194-5p in PCa is rarely reported.

The Rab23 belongs to a Rab GTPase family member and plays important roles in various types of tumors [10]. For instance, the association between Rab23 GTPase and hepatocellular carcinoma and gastric cancer has been established [11, 12]. These studies imply that...
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Rab23 may be a potential oncogene in human malignancies. However, the association between miR-194-5p and Rab23, as well as the involvement of the interaction in PCa is still elusive.

Here we reported that miR-194-5p is frequently downregulated in prostate cancer tissues as well as cell lines. Introducing miR-194-5p mimics can inhibit proliferation and restrain the invasive capacity in PC-3 and LNCaP cells. Furthermore, we identified that Rab23 might be the direct target of miR-194-5p. Overexpression of Rab23 can counteract the effect of miR-194-5p and restored the malignant phenotypes of PCa. We could also identify a significantly reverse correlation between miR-194-5p expression and Rab23 levels in specimens. Our results collectively suggested a tumor suppressive role of miR-194-5p in PCa and implied that miR-194-5p might be a putative target for targeted therapy and diagnosis.

Materials and methods

Cell culture and human samples

The prostate cancer cell lines (PC-3, PC-93, ARCaP, DU-145, ALVA-101, LNCaP, LNCaP-C4 and LAPC4) as well a normal cell line (hFOB) were all purchased from The Shanghai Institute of Cell Biology (Shanghai, China). The 293T cell line was also obtained from The Shanghai Institute of Cell Biology (Shanghai, China). The PCa cells were maintained in RPMI-1640 medium (Sigma, Shanghai, China) supplemented by 10% fetal bovine serum (FBS, Sigma, Shanghai, China) streptomycin (100 μg/ml, Sigma, Shanghai, China) and penicillin (100 U/ml, Sigma, Shanghai, China) in 5% CO₂ at 20°C. Matched fresh tumor tissue specimens and adjacent nontumorous tissues were collected before any anticancer treatment such as chemotherapy and radiotherapy, and immediately stored at -80°C. The surgically resected specimens were acquired from West China Hospital from February 2012 to July 2015. All patients provided written informed consents. The research related to human specimens were reviewed and approved by Ethics Committee of West China Hospital (NO. 2012L0003).

Quantitative real-time RT-PCR (qRT-PCR)

Total RNAs were isolated from both PCA cell lines (PC-3 and LNCaP) and human specimens with Trizol reagent (Invitrogen, Carlsbad, CA, USA). 5 μg total DNA in a final volume of 10 μl containing 10 mM dNTP Mix (Sigma, Shanghai, China) was used to generate complementary DNA (cDNA). The mixture was maintained in 70°C for 5 min and then a mix containing 5 × RT buffer, 100 U/μl reverse transcriptase, 50 U/μl RNase inhibitor was appended (Sigma, Shanghai, China). GAPDH was used as the control. Reactions were performed by the ABI PRISM® 7000 Sequence Detection System (Applied Biosystem, Foster City, USA) according to the manufacturer’s protocols. The expression of miR-194-5p was calculated by the 2⁻ΔΔCt method. At least triplicates were performed. The primer sequences were: miR-194-5p: sense: 5’-GTGCTATGACTG-3’; anti-sense: 5’-CTTGATTGCTGCTT-3’; GAPDH: sense: 5′-GATGCAATTGCTGCATTGT-3’; anti-sense: 5′-AGGTACCGTTAGCTAGT-3’.

Transfection of plasmid

The miR-194-5p mimics, miR-194-5p inhibitor and negative controls were all synthesized and cloned in pcDNA3.1 vector (TIANGEN, Shanghai, China). The negative control, scramble and pcDNA-miR-194-5p was then transfected into PC-3 and LNCaP cells in 12-well plates using Lipofectamine 2000 system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. After incubation for 24 h, the culture was replaced with fresh medium. The expression of miR-194-5p was determined by qRT-PCR.

Western blot

Cells were harvested 48 h post transfection for preparing cell lysates (20 μg each). 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to extract proteins. The proteins were then transferred to polyvinylidene difluoride membranes (Sigma, Shanghai, China) and then probed using the rabbit antibodies against Rab23 and GAPDH (1:1000 dilution, Cell Signaling Technology, Boston, MA, USA). The peroxidase-conjugated secondary antibody was used (Cell Signaling Technology, Boston, MA, USA). Chemiluminescence was used to visualize the results (GE, Fairfield, CT, USA).

Proliferation assay

We used the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) to measure the
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Figure 1. The miR-194-5p was downregulated in PCa cells and specimens. A: qRT-PCR results for 161 PCa samples as well as normal tissues. B: Expression of miR-194-5p in normal prostate cell line PZ-HPV-7 and tumor cell lines. **: P<0.01.

Table 1. Association of miR-194-5p expression with clinicopathological features of prostate cancer

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>No.</th>
<th>miR-194-5p expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High (n, %)</td>
<td>Low (n, %)</td>
</tr>
<tr>
<td>Age &lt;65</td>
<td>78</td>
<td>42 (53.8%)</td>
<td>36 (46.2%)</td>
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<tr>
<td>Age ≥65</td>
<td>83</td>
<td>39 (47.0%)</td>
<td>44 (53.0%)</td>
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<tr>
<td>Gleason score &lt;8</td>
<td>73</td>
<td>45 (61.6%)</td>
<td>28 (38.4%)</td>
</tr>
<tr>
<td>Gleason score ≥8</td>
<td>88</td>
<td>36 (40.9%)</td>
<td>52 (59.1%)</td>
</tr>
<tr>
<td>Clinical stage &lt;T2A</td>
<td>92</td>
<td>53 (57.6%)</td>
<td>39 (42.4%)</td>
</tr>
<tr>
<td>Clinical stage ≥T2A</td>
<td>69</td>
<td>28 (40.6%)</td>
<td>41 (59.4%)</td>
</tr>
<tr>
<td>Serum PSA levels (ng/mL) &lt;4</td>
<td>98</td>
<td>59 (60.2%)</td>
<td>39 (39.8%)</td>
</tr>
<tr>
<td>Serum PSA levels (ng/mL) ≥4</td>
<td>63</td>
<td>22 (34.9%)</td>
<td>41 (65.1%)</td>
</tr>
<tr>
<td>Metastasis Absent</td>
<td>102</td>
<td>60 (58.8%)</td>
<td>42 (41.2%)</td>
</tr>
<tr>
<td>Metastasis Present</td>
<td>59</td>
<td>21 (35.6%)</td>
<td>38 (64.4%)</td>
</tr>
</tbody>
</table>

Transwell invasion assay

We used transwell chambers to evaluate the invasion (8 μm size, Sigma). The upper chamber was coated with Matrigel (Invitrogen, Shanghai, China) overnight. 10⁵ cells transfected with pcDNA-miR-194-5p, miR-194-5p inhibitor, scramble or negative controls were seeded into the upper chambers. The lower chambers were covered with RPMI-1640 medium plus additional 10% FBS as chemottractants. After 36 h, the cells remaining on the top were removed using cotton swabs. Migrating cells into the lower chamber were fixed with 5% PFA and stained by 0.05% crystal violet. Leica microscope fluorescent microscope (DM-IRB, Leica, Germany) was used to visualize the results.

Luciferase reporter

The 3' UTR of Rab23 with predicted miR-194-5p binding sites were amplified with PCR and inserted into pRL-TK luciferase reporter vectors (Sigma, Shanghai, China) to obtain the Rab23 3' UTR WT. The Rab23 3' UTR MUT was obtained using the primers containing the mutant sequences. The recombinant plasmids were transfected into the 293T cells by lipofectamine 2000 system (Invitrogen, Carlsbad, CA, USA). The pRL-TK plasmid containing Renilla lucifer-
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Figure 2. MiR-194-5p transfection inhibited PCa cell proliferation and invasion. (A) PC-3 and LNCaP cells were left untreated or transfected with control, miR-194-5p mimics or miR-194-5p inhibitor. The expression was quantified using qTR-PCR. A five-day proliferation assay for (B) PC-3 and (C) LNCaP cells untreated or transfected with control, miR-194-5p mimics or miR-194-5p inhibitor. Transwell invasion assays for PC-3 (D) and LNCaP (E) cells. Representative images were shown on the left. The quantification was shown on the right. **: P<0.01.
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A

5’ ...CCAGCUGUCAUGUA----UGUUACAA... 3’ Rab23 WT
3’ AGGUGUACCUCACGACAAGUG hsa-miR-194-5p
5’ ...CCAGCUGUCAUGUA----UCAAUGUA 3’ Rab23 MUT

Figure 3. Rab23 was the direct target of miR-194-5p. (A) The alignment between miR-194-5p and Rab23. The mutant Rab23 plasmid was shown at bottom. (B) Relative luciferase activities of 293T cells transfected with either WT or mutant Rab23 plasmids. Western blot to measure Rab23 protein expression in (C) PC-3 and (D) LNCaP cells transfected with control or miR-194-5p mimics. Expression of Rab23 in PC-3 (E) and LNCaP (F) cells transfected with empty control, pcDNA-Rab23 or pcDNA-Rab23 + miR-194-5p mimics. Invasion assays for PC-3 (G) and LNCaP (H) cells. The PCA cells were either transfected with empty control, pcDNA-Rab23 or pcDNA-Rab23 + miR-1945p mimics.

ase was used as the control. Luciferase activities were quantified using Dual Luciferase Assay (Promega, Shanghai, China) following the manufacturer’s protocols.

Immunohistochemistry

The Ki67 immunostaining was carried out using primary anti-Ki67 antibody (TIANGEN, Shanghai, China) to measure the cell proliferation of prostate cancer cells. The Ki-67-labeled nuclei were quantified by monitoring the number of Ki-67 positive cells in five fields with 20 × magnification.

Statistical analysis

Results were presented as mean ± SD. Statistical significance was determined by Student’s t-test (SPSS, version 16.0, Inc., Chicago, IL, USA). Spearman correlation was used to measure the association between miR-194-5p and Rab23 expression. Fisher exact test was used to evaluate the correlation between miR-194-5p and clinicopathological characteristics.
All experiments were performed with at least triplicates.

Results

The miR-194-5p was downregulated in human samples and prostate cancer cell lines

To quantify the intrinsic levels of miR-194-5p, we performed qTR-PCR analysis. We found that the miR-194-5p was significantly decreased in 161 prostate cancer samples compared with normal adjacent tissues (Figure 1A). Meanwhile, in well-established cell lines, miR-194-5p was also downregulated compared with that in normal cells (Figure 1B). Notably, reduced expression of miR-194-5p was significantly associated with high Gleason score, clinical stages, high serum prostate specific antigens (PSA) levels and metastasis (Table 1). These results suggested that the level of miR-194-5p was lowered in PCa cell lines as well as specimens and may contribute to PCa malignancy. Since PC-3 and LNCaP cells were among the most significantly downregulated cells regarding miR-194-5p expression, we selected these two cell lines for further analysis.

The miR-194-5p inhibits prostate cancer cell proliferation and invasion

We then identified whether miR-194-5p may affect the malignant phenotypes of PCa cells. PC-3 and LNCaP cells were either left untreated or transfected with miR-194-5p mimics or inhibitors. The miR-194-5p mimics transfection significantly upregulated intrinsic levels of miR-194-5p in PC-3 and LNCaP cells (Figure 2A). We then found that miR-194-5p transfection can substantially decrease the proliferation of PC-3 cells compared with the control group (Figure 2B). Qualitatively similar results were also observed in LNCaP cells (Figure 2C). Meanwhile, we also noticed that transfection with miR-194-5p inhibitor can reverse the effect and greatly promote the proliferation (Figure 2B and 2C). Consistently, miR-194-5p transfection can also inhibit invasion of PC-3 cells (Figure 2D). Qualitatively similar results can also be observed in LNCaP cells (Figure 2E). These data suggested that miR-194-5p can inhibit PCa cells through decreasing proliferation and invasion.

The miR-194-5p targets Rab23 in prostate cancer

To identify possible targets of miR-194-5p, we used TARGETSCAN-VERT (http://www.targetscan.org) for prediction. We noticed that Rab23 might be a potential target. The base pairing between hsa-miR-194-5p and Rab23 was shown (Figure 3A). The paired regions were underlined. We next used luciferase reporter assay and confirmed that the luciferase intensi-
ties were significantly downregulated with wild type Rab23 (Figure 3B). Instead, mutations in the paired region of Rab23 showed little difference compared with control groups (Figure 3B). Consistently, the expression of Rab23 proteins was downregulated with miR-194-5p transfection (Figure 3C and 3D). These data suggested that Rab23 may be a direct target of miR-194-5p.

Rab23 overexpression can counteract miR-194-5p transfection in prostate cancer cells

To further validate the direct target of Rab23 by miR-194-5p, we performed rescue studies. We used pcDNA3.1 to overexpress Rab23 in both PC-3 and LNCaP cells. The results showed that pcDNA-Rab23 transfection can significantly increase intrinsic Rab23 levels compared with controls (Figure 3E and 3F). We noticed that overexpressing Rab23 greatly enhance the invasive capacity of PC-3 and LNCaP cells (Figure 3G and 3H). Cotransfection of miR-194-5p mimics and pcDNA-Rab23 consistently reduced the invasion of PC-3 and LNCaP cells compared with that in pcDNA-Rab23 transfection group (Figure 3G and 3H). These results further confirmed that the miR-194-5p can mediate its tumor suppressive effect by targeting Rab23.

Lower miR-194-5p expression correlates with higher Rab23 and proliferation in specimens

To further confirm the role of miR-194-5p, we performed pair wise analysis on the expression of miR-194-5p and Rab23. We found that the expression of miR-194-5p was inversely correlated with Rab23 in specimens (Figure 4A). In specimens with relatively lower miR-194-5p expression (miR-194-5p -), we found elevated Ki-67 staining which indicated that reduced miR-194-5p expression may dictate higher proliferation. The characteristics were less significant in samples with higher miR-194-5p expression (Figure 4B). These results further suggested that downregulation of miR-194-5p correlated with higher Rab23 expression and proliferation.

Discussion

Deregulated microRNA expression has been documented in many tumors and to date, various microRNAs are critically involved in the malignancy of cancer. The effect of microRNA is primarily ascribed to the dynamic regulation of oncogenes and/or tumor suppressors [13]. The microRNAs can play diversely roles possibly in a tumor type specific manner. Therefore, identifying key microRNAs in tumorigenesis is important for unraveling the molecular mechanisms of tumorigenesis.

In current work, we showed that miR-194-5p is critically involved in prostate cancer. The miR-194-5p is significantly downregulated in prostate cancer specimens compared with normal tissues. Similar results were also observed in prostate cancer cell lines. Meanwhile, miR-194-5p can inhibit malignant phenotypes of prostate cancer such as proliferation and invasion. By using online database, we uncovered Rab23 as a potential target of miR-194-5p. Overexpressing Rab23 can counteract the effect of miR-194-5p. More importantly, we found a reverse correlation between miR-194-5p expression and Rab23 levels, further consolidating the direct targeting effect. Our data support that miR-194-5p can behave like a tumor suppressor by targeting Rab23.

The role of miR-194 in the progression of cancer has been reported. Meng et al. showed that miR-194 can suppress the metastasis of hepatocellular carcinoma and may serve as a marker [14]. The miR-194 is also downregulated together with other tumor suppressor microRNAs in renal childhood neoplasms [15]. Meanwhile, another report also showed that miR-194 can target BMP1 and p27 to suppress metastasis of non-small cell lung cancer (NSCLC) [16]. However, several other reports also suggest that miR-194 may be involved in promoting carcinogenesis. For example, miR-194 has been shown to increase metastasis of colon cancer cells as a p53-responsive gene product [17]. The miR-194 can also promote the tumorigenesis of ovarian carcinoma by directly targeting PTPN12 [18]. Therefore, the role of miR-194 in tumor cells remains controversial. Here, we for the first time reported a function of miR-194-5p in prostate cancer and established an association between miR-194-5p and Rab23.

Rab23 is functionally an oncogene in hepatocellular carcinoma and astrocytoma [19]. Aberrant expression of Rab23 has also been found in gastric cancer via gene amplification [11]. Rab23 is critically implicated in migration of cells and overexpressing Rab23 can significantly increase migratory capacity [11]. However, the tumorigenic effect of Rab23 and
especially its association with miR-194-5p remains unknown. Here we reported that miR-194-5p can directly target Rab23 and suppress prostate cancer development. Therefore, Rab23 may also play a pivotal role in prostate cancer via deregulated miR-194-5p expression.

In summary, the current work indicates that the miR-194-5p is a potent tumor suppressor in prostate cancer. MiR-194-5p can directly targets Rab23 for tumor suppression. As Rab23 is critically involved in prostate cancer development, manipulating the intricate interaction between miR-194-5p and Rab23 might be an effective and potential rationale for diagnosis.

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Disclosure of conflict of interest

None.

Authors’ contribution

WW and LL conceived the study; WW, GJZ, CL performed the experiments; CL, JHG analyzed the data; WW and LL wrote the paper. All authors have read and approved the final version of the paper.

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