Circular RNA Myosin Light Chain Kinase (MYLK) Promotes Prostate Cancer Progression through Modulating Mir-29a Expression

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Background: In developed countries, prostate cancer (PCa) is a frequently diagnosed cancer with the second highest fatality rate. Circular RNAs (circRNAs) are a class of endogenous non-coding RNAs (ncRNAs) stably expressed in cells and involved in a series of carcinomas. However, few research studies have reported on the role of circRNAs in PCa.

Material/Methods: We used qRT-PCR to detect the expression of circMYLK (circRNA ID: hsa_circ_0141940) and miR-29a in PCa tissues and cell lines. MTT, colony formation, and TUNEL assays were performed to analysis the cell viability of PCa cells. Transwell and wound scratch assays were performed to investigate the cell invasion and migration of PCa cells.

Results: In the present study, we confirmed that circMYLK expression level was significantly higher in PCa samples and PCa cells than in normal tissues and normal prostatic cells. The upregulated circRNA-MYLK promoted PCa cells proliferation, invasion, and migration; however, si-circRNA-MYLK significantly accelerated the PCa cell apoptosis. We also observed that the aforementioned function of circRNA-MYLK on PCa cells was affected through targeting miR-29a.

Conclusions: We confirmed circRNA-MYLK was an oncogene in PCa and revealed a novel mechanism underlying circRNA-MYLK in PC progression.

MeSH Keywords: MicroRNAs • Prostatic Neoplasms • RNA, Untranslated

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Prostate cancer (PCa) is considered one of the most life-threatening malignancies, with the highest percentage of localized tumor diagnosed in males [1,2]. In recent years, the molecular mechanisms, especially the genetic changes in the process of PCa, have been studied extensively. A series of non-coding RNAs, such as miRNAs and IncRNAs, were considered to take part in the PCa carcinogenesis. For example, miR-135a and miR-27a were identified as tumor suppressors that mediate the development and progression of PCa, while mir-301a and mir-301b promoted radioresistance of PCa [3–5]. LncRNA HCG11, IncRNA LINC01138, and SUZ12P1 were considered to play important roles in regulating PCa carcinogenesis through promoting proliferation and inhibiting apoptosis of PC cells [6,7]. In addition, many biomarkers including miRNAs have been investigated as diagnostic value in prostate cancer [8–10]. However, few reports have described the role of circular RNAs (circRNAs) in PCa.

The circRNAs are a class of RNAs that stably exist in cells, and characteristically lack 5’-3’ ends and poly A tail [11]. In the past few years, the characteristics of circRNAs have garnered much scientific attention. An increasing number of research studies have confirmed that circRNAs take part in the development and progression of a variety of diseases such as diabetes, heart failure, osteoarthritis, neurological disorders, and especially carcinomas [12–16].

It has been widely identified that circRNAs can play a crucial role in regulating gene expressions by acting as microRNA (miRNA) sponges, RNA-binding protein (RBP) sequestering agents, and transcription regulators [17–20]. And the circRNA-miRNA-mRNA axis is considered to play an important role in cancer-related pathways. In addition, recent studies have suggested that enhanced expression of miR-29a inhibited the process of PCa [21,22].

In our study, we identified that circRNA-MYLK (circRNA ID: hsa_circ_0141940) expression was significantly upregulated in PCa tissues and PCa cells. The upregulated circRNA-MYLK enhanced PCa cells proliferation, colony formation, invasive migration, and the ability of wound healing; whereas si-circRNA-MYLK remarkably inhibited the aforementioned viabilities of PCa cells. On the contrary, upregulation of circRNA-MYLK remarkably decreased the apoptotic of PCa cells; whereas knock-down of circRNA-MYLK increased the apoptotic of PCa cells.

Moreover, we observed that the aforementioned function of circRNA-MYLK on PCa cells was affected through targeting miR-29a. Our data revealed a new mechanism underlying circRNA-MYLK in the progression of PCa and provided strong evidence for circRNA-MYLK as a tool to use to diagnose and treat PCa.

Material and Methods

PCa tissues and cell culture

Seventeen paired PCa and matched non-tumor normal tissues were collected from XiangYa Hospital, Central South University. This study was approved by the ethics committee of XiangYa Hospital, Central South University and written informed consent was obtained from all patients. The four PCa cell lines (DU145, LNCaP, PC-3, and PC-3MIE8 cells) and normal prostate cell line (WPMY-1 cell) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Sigma Chemical Co., St. Louis, MO, USA) and 1% penicillin-streptomycin G (Invitrogen Life Technologies, Carlsbad, CA, USA). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

RNA transfection

For RNA transfection, 2×10⁵ PC-3 or PC-3MIE8 cells were cultured in six-well plates with antibiotic-free complete medium. Then the PC-3 cells were transfected with circMYLK or mock vector, and PC-3MIE8 cells were transfected with si-circMYLK or mock vector. Transfection was performed by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Then circMYLK and si-circMYLK sequences were synthesized by GenePharma (Shanghai, China). For miRNA transfection, the miR-29a mimics and miRNA controls (miR-NC) were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). PC-3 cells were seeded in six-well plates (1×10⁵ cells/well) and were transfected with miR-29a mimics and miR-NC using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer’s protocol. After 48 hours, the cells were harvested for further experiments.

RNA extraction and quantitative RT-PCR (qRT-PCR)

RNA was extracted from all tissues and cells using TRIzol reagent (Life Technologies, CA, USA) and reversely transcribed to cDNA by real-time (RT)-PCR assay. The manual of NovoScript® Reverse Transcriptase was used for reverse transcription and the SYBR-Green PCR Master Mix kit (Takara) was used for quantitative RT-PCR. The reactions were performed on ABI 7500 system (Applied Biosystems, Carlsbad, CA, USA). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.
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Results

CircMYLK expression was upregulated in PCa

The qRT-PCR assay was used to detect circMYLK expression levels in tumor samples and PCa cell lines. We randomly selected 17 paired PCa tissues and adjacent normal tissues, and detected the mRNA expression of circMYLK. We found that the relative expression of circMYLK was significantly upregulated in tumor samples compared with the matched normal prostate tissues (Figure 1A, 1B). mRNA expression levels of circMYLK in noncancerous prostatic cell line WPMY-1 and four PCa cell lines including DU145, LNCaP, PC-3, and PC-3MIE8 were detected by RT-PCR. The result showed the expression of circMYLK was significantly higher in four PCa cell lines compared with the WPMY-1 cells (Figure 1C). In addition, we found that the increase of circMYLK levels was minimal in PC3 cells and maximal in PC-3MIE8 cells. Consequently, we chose these two cell lines for further study. Relative circMYLK expression was significantly upregulated in the PC-3 cells transfected with circMYLK overexpression vector. However, MYLK expression was remarkably downregulated in the PC-3MIE8 cells transfected with si-circMYLK vector (Figure 1D).

The effects of circMYLK on PCa cell proliferation and apoptosis

We further studied the effects of circMYLK on PCa cell proliferation and apoptosis. The transfection efficiency was determined by qRT-PCR. The result showed that circMYLK overexpression significantly enhanced PCa cells proliferation and colony formation, whereas si-circMYLK remarkably inhibited cell viability (Figure 2A–2D). On the contrary, upregulation of circMYLK significantly decreased the apoptotic cells number, whereas knockdown of circMYLK remarkably increased the apoptotic cells number (Figure 2E).

The effects of circMYLK on PCa cell invasion and migration

Then we further researched the effects of circMYLK on PCa cell invasion and migration. We found that upregulation of circMYLK noticeably increased the invasive and migratory number of PC cells, however, downregulation of circMYLK significantly decreased the invasive and migratory
number of PCa cells (Figures 3A–5D). Moreover, overexpression of circRNA-MYLK remarkably enhanced wound healing ability of PC cells, whereas si-circRNA-MYLK noticeably inhibited the PC cell motility (Figure 5E, 5F).

CircRNA-MYLK promoted PCa cells proliferation and invasion through targeting miR-29a

According to miRBase prediction, circRNA-MYLK possessed a complementary sequence to miR-29a seed region (Figure 4A up-panel). Our data revealed that miR-29a expression was significantly decreased in PCa cells transfected with circRNA-MYLK overexpression vector, whereas si-circRNA-MYLK played an opposite role in PCa cells (Figure 4A, 4B). We used miR-29a mimics to analyze if circRNA-MYLK affected the proliferation, invasion and migration of PC cells through inhibiting miR-29a. PC-3 cells were transfected with circRNA-MYLK overexpression vector and miR-29a mimics or miR-NC, and we found that miR-29a mimics reversed the promotive effect of circRNA-MYLK mediated PC-3 cell proliferation, invasion, and migration (Figure 4C–4E). Consequently, we predict circRNA-MYLK promoted PCa cell proliferation, invasion and migration by downregulating the expression of miR-29a.

MiR-29a expression is downregulated in PCa

The qRT-PCR assay revealed that the relative expression levels of miR-29a in noncancerous prostatic cell line (WPMY-1 cells) was significantly higher compared to the four PCa cell lines (DU145, LNCaP, PC-3, and PC-3MIE8 cells) (Figure 5A). Moreover, miR-29a expression was significantly lower in tumor samples compared with the match normal prostate tissues (Figure 5B, 5C). What’s more, we identified that miR-29a expression was negatively correlated with the expression level of circRNA-MYLK (Figure 5D).
Figure 2. The effects of circRNA-MYLK on prostate cancer cell proliferation and apoptosis. **(A)** The growth curves were measured by CCK-8 assays. The growth curves of PC-3 cells transfected with circRNA-MYLK or mock vector. **(B)** The growth curves of PC-3MIE8 cells transfected with si-circRNA-MYLK or mock vector. **(C, D)** Colony formation assays to test the proliferation of circRNA-MYLK or mock vector transfected PC-3 cells and si-circRNA-MYLK or mock vector transfected PC-3MIE8 cells. **(E)** The percent of FITC-labeled TUNEL positive circRNA-MYLK or mock vector transfected PC-3 cells and si-circRNA-MYLK or mock vector transfected PC-3MIE8 cells. The error bars indicate the mean ±SD. Experiments were performed in triplicate; *p<0.05 compared with the control group.
Paired PCa tissue and adjacent normal tissues were examined and compared by qRT-PCR. The result suggested that the relative fold change of circRNA-MYLK in PC tissues/normal tissues was almost greater than one, however, the relative fold change of miR-29a in PC tissues/normal tissues was almost smaller than one (Figure 5E). In this study, we confirmed that in prostate normal low-expression of circRNA-MYLK suppressed PC cell growth, invasion, and migration by upregulated miR-29a. On the contrary, in prostate tumor high-expression of circRNA-MYLK promoted PC cell growth, invasion, and migration by downregulated miR-29a (Figure 5E). In conclusion, we predict circRNA-MYLK promoted PC cell proliferation, colony formation, invasion, and migration by downregulating the expression of miR-29a.

**Figure 3.** The effects of circRNA-MYLK on prostate cancer cell invasion and migration. (A, C) Cell invasion and migration assays were performed using Transwell chamber. The invasion and migration of PC-3 cells after transfection with circRNA-MYLK or mock vector. (B, D) The invasion and migration of PC-3MIE8 cells after transfection with circRNA-MYLK or mock vector. (E) The relative migration rate of cells was examined by wound healing assay. The relative migration rate of PC-3 cells after transfection with circRNA-MYLK or mock vector. (F) The relative migration rate of PC-3MIE8 cells after transfection with circRNA-MYLK or mock vector. *p<0.05 compared with the control group.
Figure 4. CircRNA-MYLK promoted PC cells proliferation and invasion through targeting miR-29a. (A) Schematic of putative binding site between circRNA-MYLK and miR-29a predicted by miRBase (up-panel). The relative miR-29a expression was analyzed using qRT-PCR in PC-3 cells and PC-3MIE8 cells transfected with circRNA-MYLK or mock vector. (B) The relative miR-29a expressions in PC-3 cells and PC-3MIE8 cells transfected with si-circRNA-MYLK or mock vector. (C) The growth curves of PC-3 cells were measured after transfection with circRNA-MYLK with/without miR-29a overexpression plasmid using CCK-8 assays. (D, F) Cell invasion and migration assays were performed in PC-3 cells transfected with circRNA-MYLK with/without miR-29a overexpression plasmid using Transwell chamber, respectively. * p<0.05 compared with the miR-24-3p group.
Figure 5. MiR-29a expression is downregulated in prostate cancer. (A) The relative expression level of miR-29a was measured in normal prostate epithelial cell line WPMY-1 and four prostate cancer cell lines, DU145, LNCaP, PC-3, and PC-3MIE8 by qRT-PCR. (B, C) qRT-PCR was performed to evaluate the expression levels of miR-29a from 17 prostate cancer tumor tissues compared with their corresponding adjacent prostate normal tissues. (D) The negative correlation between miR-29a and circRNA-MYLK in clinical samples was determined by Pearson’s correlation coefficient ($R^2=0.629$, $p<0.01$). (E) Data represents the relative fold change of circRNA-MYLK and miR-29a mRNA expression in prostate cancer tissues/normal tissues. (F) Schematic representation of the possible pathogenetic mechanism of prostate cancer. * $p<0.05$ compared with the control group.
Discussion

In the past few years, there has been an increasing number of research studies that have focused on the role of circRNAs in carcinomas. In the present study, we found that circRNA-MYLK was significantly upregulated in human PCa tissues and PCa cells. Functionally, our result revealed that the upregulation level of circRNA-MYLK could enhance PCa cells proliferation, colony formation, invasion, migration, and the ability of wound healing; whereas it inhibited the apoptosis in vitro studies. However, knockdown of the circRNA-MYLK by siRNA exerted a contrary role. Moreover, we confirmed that the promotive function of circRNA-MYLK on PCa cells was affected through downregulating the miR-29a expression. Thus, our results suggested that circRNA-MYLK played an important role in the development and progression of PCa.

Recently, mounting evidence has demonstrated that circRNAs play important roles in cancer cell proliferation, apoptosis, invasion, and migration, including gastric cancer, colon cancer, human hepatocellular carcinoma, and PCa. Chen et al. suggested that circPVT1 acted as a carcinogenic gene in gastric cancer through promoting gastric cancer cells proliferation [24]. Hsiao et al. confirmed that circCCDC66 promoted colon cancer cell proliferation, invasion, and migration and acted as an oncogene [25]. Yao et al. considered that circZKSCAN1 repressed cell proliferation, promoted apoptosis, and acted as a tumor suppressor gene in human hepatocellular carcinoma [26]. Kong et al. found that circ-SMARCAS5 promoted cell cycle, inhibited cell apoptosis, and acted as an oncogene in PCa [27].

Reports have shown that the circRNA-miRNA-mRNA axis plays a crucial role in cancer-related pathways. For instance, Chen et al. considered that CircRNA_100290 acted as a carcinogenic gene in human oral squamous cell carcinoma (OSCC) by regulating the activity of miR-29b [28]. Zhong et al. confirmed that circRNA TCF25 bound to miR-103a-3p/miR-107 and promoted expression of targets genes related to cell proliferation, migration, and invasion in bladder carcinoma [29]. Wan et al. found that cir-ITCH inhibited lung cancer cell proliferation and function as a tumor suppressor gene by regulating the activities of miR-7 and miR-214 [30]. Wang et al. identified that circHIAT1 could inhibit clear cell renal cell carcinoma cell migration and invasion through binding to miR-195-5p/29a-3p/29c-3p to upregulate CDC42 expression [31].

Currently, miR-29a has been shown to be involved in different kinds of cancers, such as chronic lymphocytic leukemia [32], acute myeloid leukemia [33], and some solid tumors [22,34]. And there are a lot of reports about the function of miR-29a on prostate cancer. Nishikawa et al. showed that miR-29s functioned as tumor suppressors in PCa and inhibited PCa cells migration and invasion through regulation of LAMC1 [35]. Ahmed et al. demonstrated that miR-29a acted as a tumor suppressor gene in metastatic prostate cancer by regulating the TRAF4 expression [21]. In the present study, our results showed that miR-29a was downregulated in PCa cells and tumor samples compared with the match normal prostate tissues. In addition, we found that miR-29a expression was negatively correlated with the expression level of circRNA-MYLK. In conclusion, we suggested that circRNA-MYLK promoted PCa cell proliferation, invasion, and migration; whereas it inhibited PCa cell apoptosis by downregulating the expression of miR-29a.

Conclusions

In conclusion, we confirmed that circRNA-MYLK was an oncogene in PCa cells and revealed a novel mechanism underlying circRNA-MYLK in PC progression.

Conflict of interests

None.

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