MiR-203 over-expression promotes prostate cancer cell apoptosis and reduces ADM resistance

L.-Z. CHEN1,2, Z. DING3, Y. ZHANG2, S.-T. HE2, X.-H. WANG1

1Department of Urology, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, China
2Department of Urology, Baoan Central Hospital of Shenzhen, Shenzhen, Guangdong, China
3Department of Science and Education, Baoan Central Hospital of Shenzhen, Guangdong, China

Lezhong Chen and Zan Ding contributed equally to this work

Abstract. – OBJECTIVE: Extra-cellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK) signaling pathway is widely involved in cell proliferation, apoptosis, and drug resistance. MAPK kinase 1 (MEK1) is the upstream protein kinase of ERK that can activate ERK/MAPK signaling pathway. microRNA 203 (MiR-203) down-regulation is found to be associated with prostate cancer pathogenesis. Bioinformatics analysis showed the complementary targeted relationship between miR-203 and the 3’-UTR of MEK1 mRNA. This study explored the role of miR-203 in regulating prostate cancer cell proliferation, apoptosis, and ADM resistance through affecting MEK1 expression.

MATERIALS AND METHODS: Dual luciferase assay confirmed the targeted relationship between miR-203 and MEK1. MiR-203, MEK1, p-ERK1/2, and B cell lymphoma 2 (Bcl-2) expressions were compared in normal prostate epithelial cells PrEC, prostate cancer cells PC-3M, and drug resistant cells PC-3M/ADM. PC-3M, PC-3M/ADM cell proliferation and apoptosis were detected with flow cytometry under ADM treatment at IC50 concentration of PC-3M cells. PC-3M cells were cultured in vitro and divided into four groups including microRNA-normal control (mir-NC), mir-203 mimic, small interfering RNA (siRNA) control (si-NC), and si-MEK1.

RESULTS: MiR-203 targeted and inhibited MEK1 expression. MiR-203 levels and cell apoptosis were significantly lower, while MEK1, p-ERK1/2, Bcl-2, and cell proliferation were significantly higher in PC-3M/ADM cells compared to the PC-3M cells. MiR-203 mimic and/or si-MEK1 transfection significantly reduced MEK1, p-ERK1/2, and Bcl-2 levels, attenuated cell proliferation, induced cell apoptosis, and decreased drug resistance.

CONCLUSIONS: MiR-203 elevation suppressed prostate cancer PC-3M cell proliferation, promoted apoptosis, and weakened ADM resistance through targeted inhibiting MEK1 expression, alleviating ERK/MAPK signaling pathway, and Bcl-2 expression.

Key Words: miR-203, MEK1, ERK/MAPK, ADM, Prostate cancer, Drug resistance.

Introduction

Prostate cancer (PC) refers to epithelial malignant tumor occurring in prostate. Though the incidence of PC is lower in China than Europe and the USA, it shows an increasing trend that accounts for the 6th in male and 3rd in urinary system1,2. Chemotherapy is one of the most important measures in PC treatment. Chemotherapy resistance often affects the treatment effect and poor prognosis of PC.

Mitogen activated protein kinase (MAPK) signaling pathway is widely expressed in various tissues and cells, regulating multiple biological processes, including cell survival, proliferation, cycle, apoptosis, and drug resistance3,4. Extra-cellular signal regulated kinase (ERK) mediated MAPK signaling pathway is considered as a canonical MAPK signal transduction pathway. ERK/MAPK signaling pathway excessive activation induces cell abnormal proliferation, apoptosis, and differentiation, which are closely related to tumorigenesis5-7. Ras/Raf/ERK is the major mode of ERK/MAPK signaling pathway. MAPK kinase 1 (MEK1) phosphorylates the residue of Tyr/Thr on ERK protein to activate ERK/MAPK signaling pathway. MEK1 up-regulation
MiR-203 over-expression promotes prostate cancer cell apoptosis and reduces ADM resistance

is associated with tumor pathogenesis, progression, metastasis, and drug resistance. MEK1 elevation plays a promoting role in PC occurrence and development. MiRNA is a type of endogenous single stranded noncoding RNA at the length of 20-24 nt. It plays a degrading or inhibiting role on mRNA by binding with the 3’-UTR, thus negatively regulates gene expression at posttranscriptional level. Multiple studies revealed that miR-203 level significantly decreased in PC tissue and cell lines, suggesting that miR-203 plays a tumor suppressor role in PC. Bioinformatics analysis shows the complementary targeted relationship between miR-203 and the 3’-UTR of MEK1 mRNA. We explored the role of miR-203 in regulating MEK1 expression, ERK/MAPK signaling pathway activation, PC pathogenesis, progression, and ADM resistance.

Materials and Methods

Main Reagents and Materials

PC-3M cells were purchased from Zeye Biotech Co., Ltd., (Shanghai, China). The human normal prostate epithelial cell line, PrEC, was purchased from Xuran Biotech Co., Ltd., (Shanghai, China). Dulbecco’s Modified Eagle Medium (MEM) and PrEGM medium were obtained from Lonza Inc. (Allendale, NJ, USA). Fetal bovine serum (FBS) was bought from Gibco (Grand Island, NY, USA). Total RNA extraction kit TRIzol Universal was purchased from Tiangen Biotech Co., Ltd., (Beijing, China). Real-time PCR TransScript Green One-Step qRT-PCR SuperMix was provided by Transgen (Beijing, China). The miR-203 mimic, miR-203 inhibitor, and miR-NC were purchased from RiboBio (Guangzhou, China). Transfection kit FuGENE6 was purchased from Roche Diagnostics (Indianapolis, IN, USA). Antibodies against MEK1, p-ERK1, p-ERK2, and horseradish peroxidase (HRP) conjugated secondary antibodies were derived from Abcam Biotech. (Cambridge, MA, USA). Mouse anti-human B cell lymphoma-2 (Bcl-2) and β-catenin antibodies, si-NC, and si-MEK1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Annexin V/PI cell apoptosis detection kit was purchased from MultiSciences (Hangzhou, China). EdU cell proliferation detection kit was purchased from Molecular Probes (Eugene, OR, USA). Dual luciferase activity detection kit Dual-Glo Luciferase Assay System and pGL3 luciferase reporter gene vector were purchased from Promega (Madison, WI, USA). Adriamycin (ADM) was obtained from Wanle Pharmaceutical Industry (Shenzhen, China).

Cell Culture

The PC-3M cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) medium, while PrEC cells were cultured in PrEGM medium at 37°C and 5% CO2. The cells were passaged at 1:4. PC-3M and PC-3M/ADM cells were cultured by ADM at 0, 20, 40, 80, 160, 320, and 640 ng/ml. After 48 h, the cells were added with 10 μl CCK-8 for 4 h and tested at 450 nm to obtain the absorbance value (A450). Inhibitory rate = (1-ADM group A450)/control A450 × 100%. IC50 was calculated by SPSS software. Resistance index (RI) = IC50 of PC-3M/ADM / IC50 of PC-3M.

Flow Cytometry

The cells were added with 10 μM EdU solution at 37°C for 2 h. After the cells were incubated for 48 h, they were digested by trypsin and collected. After fixation and permeabilization, the cells were stained by reaction liquid containing Alexa Fluor 488 at room temperature avoiding light for 30 min and tested on CytoFLEX flow cytometry (Beckman Coulter Inc., Brea, CA, USA).

Dual-Luciferase Reporter Gene Assay

The PCR products containing the full-length of MEK1 gene 3’-UTR or mutant segment were cloned to pGL3. Next, they were transformed to DH5a competent cells and sequenced to select the plasmid with correct sequence. After that, pGL3-MEK1-3’-UTR-wt (or pGL3-MEK1-3’-UTR-mut) was co-transfected to HEK293T cells using FuGENE6 together with miR-203 mimic (or miR-
203 inhibitor, or miR-NC). The luciferase activity was detected according to the Dual-Glo Luciferase Assay System manual after cultured for 48 h.

**Cell Transfection and Grouping**

PC-3M/ADM cells were divided into four groups, including mimic-NC, miR-203 mimic, si-NC, and si-MEK1 groups. MiR-NC or miR-203 mimic at 30 nmol/l, si-NC or si-MEK1 at 10 nmol/l, and FuGENE6 at 10 μl were diluted in serum free DMEM medium at room temperature for 5 min. Then they were added to the cells and incubated for 72 h for the following experiments.

**qRT-PCR**

Total RNA was extracted using TRIzol Universal RNA Extraction Kit and adopted for PCR reaction by TransScript Green One-Step qRT-PCR SuperMix. The reaction system contained 1 μg template RNA, 0.3 μM primers, 10 μl 2 × TransStart Tip Green qPCR SuperMix, 0.4 μl One-Step RT Enzyme Mix, 0.4 μl Passive Reference Dye II, and RNase-free water. The PCR reaction was composed of 45°C for 5 min and 94°C for 30 s, followed by 40 cycles of 94°C for 5 s and 60°C for 30 s. Real-time PCR was performed on Bio-Rad (Hercules, CA, USA) CFX96 to test the relative expression.

**Western Blot**

Total protein was extracted using RIPA from cells. A total of 40 μg proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. Next, the membrane was blocked by 5% skim milk and incubated in primary antibody at 4°C overnight (MEK1, ERK1/2, p-ERK1/2, Bcl-2, and β-actin at 1:3000, 1:1000, 1:1000, 1:2000, and 1:10000, respectively). The membrane was incubated in horseradish peroxidase (HRP) labeled secondary antibody at room temperature for 1 h after washed by phosphate-buffered saline and tween 20 (PBST) for three times. At last, the protein expression was detected by Beyo ECL Plus.

**Cell Apoptosis Detection**

The cells were digested by trypsin and resuspended in 100 μl Annexin V binding buffer. Next, the cells were incubated in 5 μl Annexin V-FITC and 5 μl PI. At last, the cells were tested on Beckman Coulter CytoFLEX flow cytometry to evaluate cell apoptosis.

**Statistical Analysis**

All data analyses were performed on SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation (SD). The Student’s t-test was used to compare the differences between two groups. Tukey’s post-hoc test was used to validate ANOVA for comparing measurement data between groups. $p < 0.05$ was considered as statistical significance.

**Results**

**MiR-203 Targeted Inhibited MEK1 Expression**

Bioinformatics analysis showed the targeted binding site between miR-203 and 3'-UTR of MEK1 mRNA (Figure 1A). Dual luciferase assay revealed that miR-203 mimic transfection significantly declined, while miR-203 inhibitor transfection significantly elevated the relative luciferase activity of HEK293T cells, indicating the regulatory relationship between miR-203 and MEK1 mRNA (Figure 1B).

![Figure 1. MiR-203 targeted inhibited MEK1 expression. (A) The binding site between miR-203 the 3'-UTR of MEK1 mRNA. (B) Dual luciferase assay. *p < 0.05, compared with mimic NC.](image)
MiR-203 Down-Regulated, while DJ-1 Overexpressed in PC-3M/ADM Cells

The IC₅₀ of PC-3M cells was 32.63 ng/ml, while it was 412.77 ng/ml in PC-3M/ADM cells. The RI was 12.65. qRT-PCR showed that miR-203 was significantly lower in PC-3M/ADM cells compared with PC-3M cells, while it was significantly lower in PC-3M cells compared with PrEC cells. MEK1 and Bcl-2 mRNA levels were markedly higher in PC-3M/ADM cells compared with PC-3M cells (Figure 2A). Western blot revealed that MEK1, p-ERK1/2, and Bcl-2 protein levels were apparently higher in PC-3M/ADM cells compared with PC-3M cells and PrEC cells (Figure 2B). Flow cytometry demonstrated that the cell apoptotic rate was significantly lower (Figure 2C), while cell proliferation (Figure 2D) was significantly stronger in PC-3M/ADM cells than that in PC-3M cells treated by 32.63 ng/mL ADM.

MiR-203 Overexpression or MEK1 Down-Regulation Attenuated ADM Resistance in PC-3M/ADM Cells

Under the 40 ng/ml ADM was used to observe the impact of miR-203 on the ADM sensitivity of PC-3M/ADM cells. MiR-203 mimics and/or si-MEK1 transfection markedly reduced MEK1, p-ERK1/2, and Bcl-2 expressions (Figure 3A and B), attenuated cell proliferation (Figure 3C), enhanced cell apoptosis (Figure 3D). The results showed that ADM resistance.

Discussion

PC is one of the most common malignant tumors in males in Western countries. Its incidence accounts over 15% among malignant tumor with a rising trend linked to the age. Though the incidence of PC in China is lower than West-
Chemotherapy is one of the most important measures in PC treatment. Chemotherapy resistance often affects the treatment effect and poor prognosis of PC. It is of great significance to investigate the mechanism of PC chemotherapy resistance for improving therapeutic effect and prognosis.

MAPK signaling pathway is an important signal transduction system that widely exists in eukaryotes. It regulates various target genes expression and activation mediated by intracellular receptor tyrosine kinase, G-protein coupled receptor, and cytokine receptor under the effect of cytokines, growth factors, neurotransmitter, and G-protein coupled receptor, thus affecting cell survival, proliferation, migration, apoptosis, angiogenesis, and immune response. ERK/MAPK signaling pathway excessive activation leads to cell abnormal proliferation, apoptosis, and differentiation, and promote malignant transformation. It is closely associated with various tumors pathogenesis, apoptosis escape, and differentiation capacity, such as gall bladder cancer, breast cancer, and thyroid cancer. ERK/MAPK signaling pathway conforms to

Figure 3. MiR-203 over-expression or MEK1 down-regulation attenuated ADM resistance in PC-3M/ADM cells. (A) qRT-PCR detection of gene expression. (B) Western blot detection of protein expression. (C) Flow cytometry detection of cell apoptosis. (D) EdU staining detection of cell proliferation. *p < 0.05, compared with miR-NC. b p < 0.05, compared with si-NC.

Counterintuitively, it has gradually increased following aging of population and the change of lifestyle. Chemotherapy is one of the most important measures in PC treatment. It is of great significance to investigate the mechanism of PC chemotherapy resistance for improving therapeutic effect and prognosis.

MAPK signaling pathway is an important signal transduction system that widely exists in eukaryotes. It regulates various target genes expression and activation mediated by intracellular receptor tyrosine kinase, G-protein coupled receptor, and cytokine receptor under the effect of cytokines, growth factors, neurotransmitter, and G-protein coupled receptor, thus affecting cell survival, proliferation, migration, apoptosis, angiogenesis, and immune response. ERK/MAPK signaling pathway excessive activation leads to cell abnormal proliferation, apoptosis, and differentiation, and promote malignant transformation. It is closely associated with various tumors pathogenesis, apoptosis escape, and differentiation capacity, such as gall bladder cancer, breast cancer, and thyroid cancer. ERK/MAPK signaling pathway conforms to
MiR-203 over-expression promotes prostate cancer cell apoptosis and reduces ADM resistance

the classic three-step enzymatic cascade, and presents the same activation mode under different stimuli. Ras/Raf/MEK/ERK is the major mode of ERK/MAPK signaling pathway. MEK1 is a type of kinase with dual specific protease that phosphorylates the Tyr/Thr residue of ERK protein, thus activating ERK/MAPK signaling pathway. MEK1 expression and functional activity are related to a variety of cancers pathogenesis, progression, metastasis, and drug resistance, including hepatic cancer, ovarian cancer, and pancreatic cancer. It was showed that MEK1 over-expression is related to PC pathogenesis and progression. Scholars revealed that miR-203 expression reduced in PC tissue and cell line, suggesting that miR-203 may play a tumor suppressor role in the occurrence and development of PC. Bioinformatics analysis shows the complementary targeted relationship between miR-203 and the 3'-UTR of MEK1 mRNA. This investigation explores the role of miR-203 in regulating MEK1 expression, ERK/MAPK signaling pathway activation, and PC cell proliferation, apoptosis, and ADM resistance.

Dual luciferase assay revealed that miR-203 mimic transfection significantly declined miR-203 expression was significantly lower in PC-3M/ADM cells compared with PC-3 cells. MiR-203 over-expression significantly arrested PC cell cycle, indicating the regulatory relationship between miR-203 and MEK1 mRNA. MEK1 can activate anti-apoptotic factor Bcl-2 expression to suppress cell proliferation and inducing cell apoptosis. Siu et al demonstrated that miR-203 expression was more reduced in PC tissue and cell lines LNCaP, DU145, and PC3 than that in benign prostate tissue and normal prostate epithelial cell line RWPE-1. Siu et al demonstrated that miR-203 level apparently decreased in PC tissue compared with normal prostate tissue, while it was significantly lower in metastatic PC. In this study, miR-203 expression significantly reduced in PC cells, indicating that its downregulation may be involved in PC pathogenesis, which was similar with Boll et al., Saini et al., and Siu et al. It was showed that MEK1 enhancement played a crucial role in promoting PC cell EMT and metastasis, revealing the tumor-promoting action of MEK1 in PC, which is in accordance with our results. Siu et al. demonstrated that miR-203 down-regulation in PC cells is resistant to tyrosine kinase inhibitors (TKIs). Siu et al. revealed that miR-203 level declined in hormone resistant PC cells compared with LNCaP cells. This study showed that miR-203 significantly reduced in drug resistant PC cells compared with parent drug sensitive cells, suggesting that miR-203 down-regulation may play a role in PC cell resistance, which was in accordance with Siu et al. and Sun et al. MiR-203 mimics and/or si-MEK1 transfection markedly reduced p-ERK1/2, and Bcl-2 expressions, attenuated cell proliferation, enhanced cell apoptosis, and weakened ADM resistance. Fragini et al. reported that the effect of zoledronic acid on suppressing PC cell proliferation and inducing cell apoptosis was related to miR-203 enhancement and survivin decrease, confirming the tumor suppressor role of miR-203 on PC. Boll et al. demonstrated that miR-203 over-expression significantly arrested PC cell cycle. Saini et al. exhibited that miR-203 up-regulation significantly attenuated ZEB2, RUNX2, DLX5, SmMAD4, and Survivin expressions, inhibited EMT process, migration, and invasion, alleviated cell proliferation, and induced cell apoptosis in PC-3 cells. Siu et al. revealed that the inhibition of miR-203 enhanced PC drug resistance to TKIs. In this study, miR-203 over-expression reduced PC cell drug resistance, which verified the result of Siu et al. This work revealed the role of miR-203 in targeting MEK1, regulating ERK/MAPK signaling pathway, and in affecting ADM resistance. We only explored the impact of miR-203 on MEK1, ERK/MAPK signaling pathway, and PC cell proliferation, apoptosis, and ADM resistance. However, we did not test the corresponding mechanism in clinical samples.
Conclusions

We showed that down-regulation of miR-203 was associated with ADM resistance in PC-3M cells. MiR-203 elevation suppressed prostate cancer PC-3M cell proliferation, promoted apoptosis, and weakened ADM resistance through targeted inhibited MEK1 expression to alleviate ERK/MAPK signaling pathway and Bcl-2 expression.

Conflict of Interest
The Authors declare that they have no conflict of interests.

References
4) LIANG T, WEN D, MA B, HU JQ, QIU N, SHI RL, LI X, GUAN Q, LI DS, JI QH. Yes-associated protein 1 promotes papillary thyroid cancer cell proliferation by activating the ERK/MAPK signaling pathway. Oncotarget 2016; 8: 11719-11728.
MiR-203 over-expression promotes prostate cancer cell apoptosis and reduces ADM resistance

23) Boll, K; Reiche, K; Kasack, K; Morit, N; Kreutzschmar, AK; Tomm, JM; Verhaegh, G; Schalken, J; von Bergen, M; Horn, F; Hackermüller, J. MiR-130a, miR-203 and miR-205 jointly repress key oncogenic pathways and are downregulated in prostate carcinoma. Oncogene 2013; 32: 277-285.

24) Hailer, A; Grunewald, TG; Orth, M; Reiss, C; Kneitz, B; Spahn, M; Butt, E. Loss of tumor suppressor mir-203 mediates overexpression of LIM and SH3 Protein 1 (LASP1) in high-risk prostate cancer thereby increasing cell proliferation and migration. Oncotarget 2014; 5: 4144-4153.

25) Siu, MK; Abu-Kheir, W; Yin, JJ; Chang, YS; Barrett, B; Suau, F; Case, O; Chen, WY; Fang, L; Hynes, P; Hsieh, YY; Liu, YN; Huang, J; Kelly, K. Loss of EGFR signaling regulated miR-203 promotes prostate cancer bone metastasis and tyrosine kinase inhibitors resistance. Oncotarget 2014; 5: 3770-3784.

26) Sun, T; Yang, M; Chen, S; Bäck, S; Pomerantz, M; Hsien, CL; Brown, M; Lee, GS; Kantom, PW. The altered expression of MiR-221/-222 and MiR-23b/-27b is associated with the development of human castration resistant prostate cancer. Prostate 2012; 72: 1093-1103.

27) Fragni, M; Bonini, SA; Stabile, A; Bonin, D; Forti, L; Simeone, C; Zani, D; Spano, R; Pezzella, F; Mo, M; Sigala, S. Inhibition of survivin is associated with zoledronic acid-induced apoptosis of prostate cancer cells. Anticancer Res 2016; 36: 913-920.