miR-128 modulates chemosensitivity and invasion of prostate cancer cells through targeting ZEB1

Xianglun Sun1,†, Youkong Li2,†, Jie Yu2, Hong Pei2, Pengcheng Luo3, and Jie Zhang1,3,*

1Department of Urology, Renmin Hospital of Wuhan University, Wuhan, Hubei, 2Jingzhou Central Hospital, Huazhong University of Science and Technology, Jingzhou, Hubei, and 3Huangshi Central Hospital, Hubei Polytechnic University, Huangshi, Hubei, PR China

*For reprints and all correspondence: Jie Zhang, Department of Urology, Renmin Hospital of Wuhan University, Zhangzhidong Road 99#, 430060 Wuhan, Hubei, PR China. E-mail: zhangjie1231994@126.com

†Xianglun Sun and Youkong Li contributed equally to this work.

Received 14 November 2014; Accepted 7 February 2015

Abstract

Objective: Recent reports strongly suggest the profound role of miRNAs in cancer therapeutic response and progression, including invasion and metastasis. The sensitivity to therapy and invasion is the major obstacle for successful treatment in prostate cancer. We aimed to investigate the regulative effect of miR-128/zinc-finger E-box-binding homeobox 1 axis on prostate cancer cell chemosensitivity and invasion.

Methods: The miR-128 expression pattern of prostate cancer cell lines and tissues was detected by real-time reverse transcriptase-polymerase chain reaction, while the mRNA and protein expression levels of zinc-finger E-box-binding homeobox 1 were measured by real-time reverse transcriptase-polymerase chain reaction and western blot assay, respectively. Dual-luciferase reporter gene assay was used to find the direct target of miR-128. Furthermore, prostate cancer cells were treated with miR-128 mimic or zinc-finger E-box-binding homeobox 1-siRNA, and then the cells’ chemosensitivity and invasion were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and transwell assay, respectively.

Results: We found miR-128 expression obviously decreased in prostate cancer tissues compared with paired normal tissues. Restored miR-128 expression sensitized prostate cancer cells to cisplatin and inhibited the invasion. Furthermore, there was an inverse expression pattern between miR-128 and zinc-finger E-box-binding homeobox 1 in prostate cancer cells and tissues, and zinc-finger E-box-binding homeobox 1 was identified as a direct target of miR-128 in prostate cancer. Knockdown of zinc-finger E-box-binding homeobox 1 expression efficiently sensitized prostate cancer cells to cisplatin and inhibited the invasion. However, ectopic zinc-finger E-box-binding homeobox 1 expression impaired the effects of miR-128 on chemosensitivity and invasion in prostate cancer cells.

Conclusions: miR-128 functions as a potential cancer suppressor in prostate cancer progression and rational therapeutic strategies for prostate cancer would be developed based on miR-128/zinc-finger E-box-binding homeobox 1 axis.

Key words: miR-128, ZEB1, chemosensitivity, invasion
Introduction
Prostate cancer (PCa) is one of the most common malignant cancers among males all over the world. It was estimated that 238,590 new cases and 29,720 death cases of PCa occurred in the USA in 2013 (1). Chemoresistance and invasion remain the main causes of treatment failure and mortality in PCa patients. Although several advances have been made in the control of PCAs with some newly developed drugs, there is still an urgent need to investigate the mechanisms of PCa chemoresistance and invasion, identify useful therapeutic targets, develop novel treatment approaches, improve current therapeutic modalities and increase patients’ survival.

MicroRNAs (miRNAs) are a class of endogenous, small, non-coding single-stranded RNAs that post-translationally regulate gene expression through binding to the 3’-untranslated region (UTR) of target mRNAs, causing translational repression and/or degradation of target mRNAs (2,3). Emerging evidences suggested that miRNAs were abnormally expressed in many kinds of cancers, functioning as oncogenes and tumor suppressors (4). Several reports point to the fact that some miRNAs played an important role in PCa chemosensitivity and invasion. For example, miR-130b exerts a suppressive effect on PCa metastasis by downregulating MMP2 (5), miR-34a inhibits PCa regeneration and metastasis by directly repressing CD44 (6). Paclitaxel and cyclophosphamide combination-based therapy upregulates miR-200c and miR-34a. While, miR-200c increased sensitivity of PCa cells to paclitaxel, and miR-34a decreased the spread of PCa (7). On the other hand, miR-125b promotes growth of prostate xenograft tumors by downregulating three key pro-apoptotic genes, and PCa cells could be sensitized to chemotherapy through the inhibition of miR-125b (8,9). miR-616 induces androgen-independent growth of PCa cells by suppressing expression of tissue factor pathway inhibitor TFPI-2 (10).

The zinc-finger E-box-binding homeobox 1 (ZEB1) gene encodes a zinc-finger transcription factor that plays a role in normal embryonic development. Mutations in this gene have been associated with posterior poly morphous corneal dystrophy-3 and late-onset Fuchs endothelial corneal dystrophy (11). Data also indicated that it emerged as a key player in cancer progression. Aberrant expression of ZEB1 in endometrial cancers, pancreatic head cancer and hepatocellular carcinoma had been associated with aggressive disease, poor differentiation, development of metastasis and poor clinical prognosis (12–15). Previous study had showed that ZEB1 was regulated by miR-200 family miRNAs in several tumors, yet expression of ZEB1 by miRNAs in PCa was rarely reported (16–18).

In this study, we showed that miR-128 is downregulated in PCa and inversely correlated with ZEB1 levels. Our collective data indicated that miR-128 directly targets 3’UTR of ZEB1 to suppress its expression, therefore enhances chemosensitivity of PCa cells to cisplatin (cDDP) and inhibits invasion of PCa cells.

Patients and methods

Clinical specimens
Eight prostate tissues and normal tissues were obtained from PCa patients at Renmin Hospital of Wuhan University. Tissue samples were cut into two parts and immediately snap-frozen in liquid nitrogen. One section was used for mRNA and miRNA extraction, and the other section was used for protein extraction. The study was approved by the ethics committee of Renmin Hospital of Wuhan University. Written informed consent was obtained from all patients.

Cell culture
Human PCa cell lines DU-145 and LNCaP, HEK-293T cells were obtained from Cell Bank of Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences. Cells were cultured in RPMI-1640 (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) in a humidified cell incubator with an atmosphere of 5% CO2 at 37°C.

RNA preparation and real-time RT-PCR
Total RNA was purified by TRIzol Reagent (Life Technologies), and then cDNA was synthesized by ReverTra Aid First Strand cDNA Synthesis Kit (Fermentas Life Science). Real-time RT-PCR for ZEB1 detection was performed by SYBR Select Master Mix (Life Technologies) according to the manufacturer’s instructions. GAPDH was used as an internal control. The primers for GAPDH were: forward primer 5’-ATTCATGGCACCGTCAAGGCTGA-3’, reverse primer 5’-TTTTCCATGTTGTAAGAGCCCA-3’; for ZEB1: forward primer 5’-CAGCTGTGATACCTGTGAATGGG-3’, reverse primer 5’-CAGCTGTGATACCTGTGAATGGG-3’.

miRNAs were isolated and purified by miRNA isolation system (OMEGA Bio-Tek). cDNA was generated with the miScript II RT Kit (QIAGEN) and the real-time RT-PCR was done by miScript SYBR Green PCR Kit (QIAGEN) following the manufacturer’s instructions. Real-time RT-PCR primers for miR-128 and endogenous control RNU6 were purchased from QIAGEN. The gene expression threshold cycle values of miRNAs were calculated by normalizing with internal control RNU6 and relative quantization values were calculated.

Plasmid construction
Two single strands of the wild-type miR-128 binding site (represented by underscores) at 3’UTR of ZEB1 were synthesized as following: (sense): 5’-ctagTGUUAUAAAUAAACUUAAUUUGAAAUUCACUGUGUCAGCUUAAUAAUGCAUCUGCACGCAGCUUUAUA-3’; antisense: 5’-agctTTAATAAACTGCGAGCAGCATGCTTTATATGAGAACAC-3’. Two single strands of the mutant one (represented by underscores) were synthesized as following: (sense): 5’-ctagTGUUAUAAAUAAACUUAAUUUGAAACGCGUCGCUAUAAGCAUCGUCGUCAGCUUUAUAUA-3’; antisense: 5’-agctTTAATAAACTGCGAGCAGCATGCTTTATATGAGAACAC-3’.

miRNAs were isolated and purified by miRNA isolation system (OMEGA Bio-Tek). cDNA was generated with the miScript II RT Kit (QIAGEN) and the real-time RT-PCR was done by miScript SYBR Green PCR Kit (QIAGEN) following the manufacturer’s instructions. Real-time RT-PCR primers for miR-128 and endogenous control RNU6 were purchased from QIAGEN. The gene expression threshold cycle values of miRNAs were calculated by normalizing with internal control RNU6 and relative quantization values were calculated.

Dual-luciferase reporter gene assay
HEK-293T cells were seeded into 96-well plates at a density of 10,000 cells per well and co-transfected with miR-128 mimic, pMir-Report plasmid, then constructs were verified by sequencing, and was designated as pMir-ZEB1-wt and pMir-ZEB1-mut, respectively.

PLEX-MS was kept that in our laboratory was a control plasmid for overexpression genes in eukaryotic cells. We cloned ZEB1 open reading frame (ORF) without 3’UTR into pLEX-MS. Certainly, the construct pLEX-ZEB1 is not targeted by miR-128 mimic. We synthesized one pair of primers for ZEB1 ORF: sense: 5’-GGGCTGTGGATACCTGTGAATGGG-3’, antisense: 5’-GGGCTGTGGATACCTGTGAATGGG-3’. Lowercase letters located at both ends of the oligonucleotides were restriction sites for SpeI and HindIII. The corresponding sense and antisense strands were annealed and subsequently cloned into pMIR-Report plasmid, then constructs were verified by sequencing, and was designated as pMIR-ZEB1-wt and pMIR-ZEB1-mut, respectively.
miR-128 directly targets ZEB1 in PCa

Potential targets of miR-128 were predicted using public database miRNA.org (http://www.microrna.org/microrna/home.do), and ZEB1 with critically conserved binding site was selected for further expression and function confirmation (Fig. 2A). We analyzed not only ZEB1 expression in each pair of normal and PCa tissues, but also its average expression in eight patients of normal and PCa tissues by real-time RT-PCR. Both suggested that ZEB1 expression was higher in PCa than in normal tissues (Fig. 2B). Similar results were got by western blot assay (Fig. 2C). Meanwhile, it is showed that ZEB1 expression level decreased 74% in DU-145 cells with miR-128 mimic and 63% in LNCaP with miR-128 mimic, when cells were compared with scramble. Western Blot assay also suggested that ZEB1 declined in PCa cells with miR-128 mimic, compared with control-transfected cells (Fig. 2D). To assess whether ZEB1 is a direct target of miR-128, the luciferase reporter vectors with the putative ZEB1 3’-UTR target site for miR-128 (pMir-ZEB1-wt, set as wild-type) and mutant version with a 9 bp change in the seed region (pMir-ZEB1-mut, set as mutant type) were constructed. It is confirmed that miR-128 significantly reduced luciferase activity in HEK-293T cells with pMir-ZEB1-wt plasmid but not pMir-ZEB1-mut plasmid (Fig. 2E). These results demonstrated that miR-128 targets to the 3’UTR of ZEB1 and then suppresses its expression.

ZEB1 knockdown sensitizes PCa cells to cDDP and inhibits the invasion

As shown above, ZEB1 was upregulated in PCa tissues. To investigate the role of ZEB1 in PCa cells, we first used siRNAs to silence ZEB1. According to the results of RNAi effection test, siRNA-ZEB1-#1 was used in the following study, and siRNA-ZEB1-#2 can be used as a negative control (Fig. 3A). As shown in Fig. 3B, knocked-down ZEB1 with siRNA-ZEB1-#1 enhanced the sensitivity of PCa cells to cDDP, when compared with the negative control and cells with siRNA-ZEB1-#2 (Fig. 3B). Moreover, we found that the invasion of DU-145 and LNCaP cells were decreased upon ZEB1 knocked-down (Fig. 3C). All these data suggested that ZEB1 results in enhancing chemoresistance and invasion of PCa cells.

ZEB1 reverses the effects of miR-128 on chemosensitivity and invasion in PCa cells

To determine whether ZEB1 could reverse the effects of miR-128-induced in PCa cells, we transfected pLEX-ZEB1 plasmid into PCa cells with miR-128 mimic, and measured the expression levels of
miR-128 downregulates in prostate cancer (PCa) and functions on chemosensitivity and invasion of PCa cells. (A) Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was used to determine miR-128 expression patterns in each pair of PCa and normal tissues; the average expression level of miR-128 in PCa and normal tissues among eight patients was also analyzed. (B) miR-128 mimic upregulates miR-128 in PCa cells, when compared with the control group. MTT assays (C) and transwell assays (D) of PCa cells transfected with miR-128 mimic or corresponding control. *P < 0.01.
Figure 2. miR-128 targets ZEB1 by binding to its 3’ UTR in PCa. (A) Schematic of the putative binding sites of miR-128 in 3’-UTR of ZEB1 is presented. (B) mRNA expression levels of ZEB1 were detected in each pair of PCa and normal tissues by real-time RT-PCR; Meanwhile, the average ZEB1 expression level in PCa and normal tissues among eight patients was analyzed. (C) Western blot assay was used to detect ZEB1 protein expression levels in each pair of PCa and normal tissues. (D) The expression levels of ZEB1 were measured in PCa cells with miR-128 mimic or scramble, through real-time RT-PCR and western blot assay. (E) Diagram of constructing plasmids pMir-ZEB1-wt and pMir-ZEB1-mut was showed first, and then luciferase reporter assay was performed in PCa cells co-transfected with miR-128 mimic, plasmids pMir-ZEB1-wt or pMir-ZEB1-mut, and a renilla luciferase reporter for normalization. The mean of the results from cells transfected with pMir-ZEB1-wt and scramble was set as 1. **P<0.01, *P<0.05.
**ZEB1** in these cells via real-time RT-PCR, also detected chemosensitivity and invasion of these cells. It is showed that **ZEB1** upregulated 3.87-fold in DU-145 cells with miR-128 mimic, when compared with that of the control group; **ZEB1** increased 4.81-fold in LNCaP cells with miR-128 mimic when compared with that of the control. Western blot assay got similar results (Fig. 4A). We found that **ZEB1** could significantly abolished miR-128 mimic inducing chemosensitivity of PCa cells (Fig. 4B). As shown in Fig. 4C, **ZEB1** remarkably enhanced the invasion of PCa cells with miR-128 mimic. Our data strongly suggested that **ZEB1** could reverse miR-128 inducing chemosensitivity and inhibit invasion of PCa cells.

**Discussion**

It is well known that PCa is an epithelial malignant cancer characterized by frequent metastasis and chemoresistance (19). So far, functions of miRNAs in PCa occurrence and development are inumerably reported, among which miRNAs regulating invasion and
chemoresistance still highlights in recent several years, such as miR-21, miR-221, miR-181, miR-34a and miR-124 (20–25). And miRNAs possessed invasion and chemoresistance functions were thought to be a promising target for PCa management. In the present study, we initially examined miR-128 expression in normal as well as tumor tissues from PCa patients and found miR-128 was dramatically downregulated in PCa. In addition, we found miR-128 modulated PCa cells chemosensitivity and invasion via directly targeting ZEB1, suggesting their involvement in PCa carcinogenesis and progression.

miR-128 was a kind of brain-enriched miRNA, and played important roles in the development of the nervous system and the maintenance of its normal physiological functions (26). Recent studies demonstrated that miR-128 was involved in proliferation, differentiation, apoptosis, invasion and metastasis of various tumor cells. For example, miR-128 inhibited glioma cells proliferation by

Figure 4. miR-128 modulates chemosensitivity and invasion of PCa cells via downregulating ZEB1. Transfected ZEB1 overexpression plasmid or control plasmid into PCa cells with miR-128 mimic, then real-time RT-PCR and western blot assay were used to determine ZEB1 expression in these cells (A), also MTT assay was utilized to measure these cells viability (B), meanwhile transwell assay was made to detect these cells invasion (C). *P < 0.01.
targeting transcription factor E2F3a; miR-128 down-regulated Bax and induced apoptosis in human embryonic kidney cells; miR-128 repressed growth and mediated differentiation of glioma-initiating neural stem cells by targeting oncogenic receptor tyrosine kinases epithelial growth factor receptor and platelet-derived growth factor receptor-α, and repressed gliomagenesis (27–29). Moreover, proteomic alterations in a cohort of 15 prostate-derived tissues that included five each from adjacent benign prostate, clinically localized PCa, and metastatic disease from distant sites revealed a role for miR-128 in PCa (30). In our study, we found that miR-128 decreased in PCa, and overexpression of miR-128 remarkably sensitized chemotherapy and inhibited invasion of PCa, which suggested that it involved in PCa suppression.

Increasing evidences indicated that ZEB1 closely related with the biological behaviors of tumors, such as invasion and metastasis, stemness of cancer stem cells, tumor angiogenesis and chemosensitivity, which largely contributes to tumors initiation and progression (12,13,31–33). Several miRNAs also had been showed to take part in ZEB1 regulation, and therefore adjusted of its function in occurrence and development of tumors. For example, miR-200c suppressed TGF-β signaling and counteracted trastuzumab resistance and metastasis by targeting ZNF217 and ZEB1 in breast cancer; in ovarian carcinoma, miR-101 suppressed the epithelial-to-mesenchymal (EMT) transition by targeting ZEB1 and ZEB2; and miR-1236–3p repressed the cell migration and invasion abilities by targeting ZEB1 in high-grade serous ovarian carcinoma (16,34,35). In addition, it had been reported that NVP-LDE-225/Erisimodegib (smoothened inhibitor) unregulated miR-128 and inhibited ZEB1 in PCa, which revealed a potential relationship between miR-128 and ZEB1 (36). In this article, we found that miR-128 bound to the 3′UTR of ZEB1 and inhibited its expression. And ZEB1 enhanced PCa chemoresistance and invasion, while miR-128 could reverse that by down-regulated ZEB1. These indicated that miR-128-mediated sensitizing chemoresistance and inhibiting invasion of PCa cells by directly targeting ZEB1.

In conclusion, we demonstrated for the first time that downregulation of miR-128 accompanied with ZEB1 upregulation is a common event in PCa. Furthermore, miR-128 functions as a tumor suppressor by directly targeting ZEB1 in PCa. These findings collectively implicate miR-128-ZEB1 axis as a promising prognostic and therapeutic target for future PCa therapy.

Conflict of interest statement
None declared.

References


