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microRNA-218 inhibits prostate cancer cell growth and promotes apoptosis by repressing TPD52 expression

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ABSTRACT

The tumor protein D52 (TPD52) is an oncogene overexpressed in prostate cancer (PC) due to gene amplification. Although the oncogenic effect of TPD52 is well recognized, how its expression is regulated is still not clear. This study tried to explore the regulative role of miR-218, a tumor suppressing miRNA on TPD52 expression and prostate cancer cell proliferation. We found the expression of miR-218 was significantly lower in PC specimens. Based on gain and loss of function analysis, we found miR-218 significantly inhibit cancer cell proliferation by inducing apoptosis. These results strongly suggest that miR-218 plays a tumor suppressor role in PC cells. In addition, our data firstly demonstrated that miR-218 directly regulates oncogenic TPD52 in PC3 cells and the miR-218-TPD52 axis can regulate growth of this prostate cancer cell line. Knockdown of TPD52 resulted in significantly increased cancer cell apoptosis. Clearly understanding of oncogenic TPD52 pathways regulated by miR-218 might be helpful to reveal new therapeutic targets for PC.

1. Introduction

Prostate cancer (PC) is the most common male malignancy and accounts for about 10% of all male cancer-related deaths across the world [1]. Most of the PC patients are responsive to androgen-deprivation therapy (ADT) in first treatment. However, a large proportion of the cancer cases develop castration-resistance and distant metastasis, which is the leading cause of death [2]. Thus, clear understanding of the molecular mechanisms of PC development and metastasis is beneficial for development of effective therapy.

The tumor protein D52 (TPD52) is an oncogene overexpressed in prostate cancer due to gene amplification [3]. Previous studies found TPD52 transcript levels were significantly higher in high- versus low-grade localized prostate cancers [4]. Its expression was also closely related to development of systemic progression within 5 years [5]. In cell line models, increased TPD52 led to increased proliferation and colony formation and significantly higher expression of phospho Akt (pSer 473) in LnCaP cells [6–8]. Increased TPD52 is also associated with higher LnCaP cell migration [6]. Besides, TPD52 is an upstream mediator of the mitochondrial apoptotic reaction. Transient knockdown of TPD52 increased cell death of both LnCaP and C4-2, two prostate cancer cell lines [6–8]. Therefore, TPD52 plays a quite critical role in survival, proliferation, migration, and invasion of prostate cancer cells.

Although the oncogenic effect of TPD52 is well recognized, how its expression is regulated is still not quite clear. microRNAs (miRNAs) are a set of endogenous small (19–22 bases in length) non-coding RNA. They can regulate the gene expression by inhibiting translation or cleaving RNA transcripts in a sequence specific manner [9]. Approximately 80% of the TPD52 transcript was originally identified as 3′-untranslated region (3′-UTR) [10], which usually contains targeting sequence of miRNA [11]. Previous studies observed that TPD52 is a target of miR-34a [12] in colorectal cancer cells and a target of miR-224 in prostate cancer cell [13]. However, whether other miRNAs are involved in regulating TPD52 in prostate cancer is not clear.

This study firstly reported TPD52 is a downstream target of miR-218, a tumor suppressing miRNA in several cancers [14,15]. Through repressing TPD52 expression, miR-218 could inhibit prostate cancer growth and promote apoptosis.

2. Methods

2.1. Human prostate tissue specimens

All human tissue based studies were proved by the ethics committee of Xinxiang Medical University and all participants were recruited from the first affiliated hospital of Xinxiang Medical University. Men who were suspected of having PC due to elevated

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serum prostate-specific antigen (PSA) levels were possible candidates of this study. A pair of 10 cores of prostate specimens were obtained from each patient at the same region by using transrectal ultrasound guided prostate biopsy. The paired two specimens were used for pathological and molecular analysis respectively. If none of the biopsy cores from a patient were found with cancerous cells in pathological analysis, this patient was confirmed as non-PC and were recruited as normal control in this study. Finally, 46 patients had prostate cancer and 25 cancer free controls were recruited. Informed consent was obtained every patient before tissue collection. 5 mL blood sample was obtained from each participant. The blood samples were centrifuged at 3500 rpm for 10 min after coagulation to isolate serum.

2.2. Patient follow up

Survival estimates were calculated using the Kaplan–Meier method and compared using the log-rank test. Comparison of the overall survival (%) between the high (higher than average) and low (lower than average) miR-218 expression group was performed by using the Student's *T* test.

2.3. Cell culture

HEK 293T cell and human prostate cancer cell line PC-3 were obtained from the American Type Culture Collection and were maintained in DMEM (HEK 293T) and RPMI 1640 (PC-3) supplemented with 10% fetal bovine serum (Gibco, New York, USA), 100 U/mL penicillin and 100 mg/mL streptomycin in humidified air with 5% CO_2 at 37 °C.

2.4. Quantitative real-time PCR

TRIzol and TRIzol LS Reagent (Invitrogen) were used to extract total RNA from prostate tumor tissues, cell samples and serum samples respectively. The purity and concentration of the RNA samples were identified by using a UV–visible spectrophotometer (NanoDrop Technologies). Mature miR-218 expression was quantified by using Taqman miRNA Assays. RNU6B was used as an internal control. $2^{-\Delta\Delta CT}$ method was used to determine the relative quantitation of miR-218 levels.

2.5. In vitro knockdown and overexpression of miR-218 and TPD52

Human miR-218 and TPD-52 expression vector with a pCMV-MIR backbone was purchased from Origene. The mutant TPD52 expression vector (with mutation at the two putative miR-218 binding site) was generated with the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). Chemically synthesized oligonucleotides miR-218a inhibitor and the negative control was purchased from Ambion. TPD52 siRNA and siRNA control were purchased from Life Technologies. PC-3 cells were seeded on 24 well plate before transfection. miR-218 and TPD-52 expression vector alone or in combination were transiently transfected into PC-3 cells by using MegaTran 1.0 Transfection Reagent (Origene) according to the protocol. miR-218a inhibitor (25nM) and TPD52 siRNA (50 nM) were transfected to target cells by using Oligofectamine (Invitrogen). Cells were lysed 48 h post-transfection to confirm the success of knockdown and overexpression.

2.6. Cell viability assay

After transfection, PC-3 cells were plated at 5×10^3 cells/well in 96-well plates. Cells were cultured for 1, 2 and 3 days and cell viability was measured at 12, 24, 48, 60 and 72 h. On the indicated time, MTT reagent (AMRESCO) was added and incubated for 3 h at 37 °C. Then, the supernatant was removed and replaced with dimethyl sulfoxide to dissolve the formazan product. Absorbance was measured at an optical density of 490 nm in a spectrophotometric plate reader. Each test was performed with five repeats.

2.7. Cell clonogenicity assay

PC-3 cells after transfection were plated in six-well plate at 500 cell/well and further cultured in complete medium for 2 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet. Cell cluster with over 50 cells was considered as a colony. The rate of clonogenicity was calculated with the following equation: clonogenicity rate = (number of colonies/number of seeded cells) × 100%. Each test was performed with five repeats.

2.8. Flow cytometric analysis

PC-3 cells transfected with miR-218, siTPD52, miR-218+TPD52 mut and the controls were plated in six-well plates at 4×10^5 cells/well. 72-h after transfection, cell apoptosis was detected by using Annexin V-fluorescein-5-isothiocyanate Apoptosis Detection Kit (Biovision) and the apoptosis rates were measured by using a flow cytometer (FACSCalibur, BD Biosciences).

2.9. Luciferase reporter assay

The possible binding site between TPD52 and miR-218 was searched in TargetScan (http://www.targetscan.org). Since there were two putative binding sites between miR-218 and TPD52, four pairs of double-stranded oligonucleotides of wildtype (Wt) and mutant (Mut) 3′-UTR region of TPD52 corresponding to the miR-218 binding sites were synthesized according to the following sequence: Wt-1 and Wt-2: F: cAGATGCTGCAAGCGAGGTCCAAGCACATCTTGTCAACATGCATTGCCCATTTAGCACTATTTAAAATAAGCACACCAAGTTATATGACTAATATg; R: **tcgac**ATATTAGTCATATAACTTGGTGTGCTTATTTTAAATAGTGCTAAATGGGCAATGCATGTTGACAAGATGTGCTTGGACCTCGCTTGCAGCATCT**gagct**; Mut-1+Wt-2: F: cAGATGCTGCAAGCGAGGTCCAACGTGATCTTGTCAACATGCATTGCCCATTTAGCACTATTTAAAATAAGCACACCAAGTTATATGACTAATATg; R: **tcgac**ATATTAGTCATATAACTTGGTGTGCTTATTTTAAATAGTGCTAAATGGGCAATGCATGTTGACAAGATCACGTTGGACCTCGCTTGCAGCATCT**gagct**. Wt-1+Mut-2: F: cAGATGCTGCAAGCGAGGTCCAAGCACATCTTGTCAACATGCATTGCCCATTTAGCACTATTTAAAATAACGTGACCAAGTTATATGACTAATATg; R: **tcgac**ATATTAGTCATATAACTTGGTCACGTTATTTTAAATAGTGCTAAATGGGCAATGCATGTTGACAAGATGTGCTTGGACCTCGCTTGCAGCATCT**gagct**. Mut-1+Mut-2: F: cAGATGCTGCAAGCGAGGTCCAACGTGATCTTGTCAACATGCATTGCCCATTTAGCACTATTTAAAATAACGTGACCAAGTTATATGACTAATATg. R: **tcgac**ATATTAGTCATATAACTTGGTCACGTTATTTTAAATAGTGCTAAATGGGCAATGCATGTTGACAAGATCACGTTGGACCTCGCTTGCAGCATCT**gagct**. The oligonucleotide pairs were annealed and then inserted into the site between SacI and SalI of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) respectively. This expression vectors constructed were designated as Luc-TPD52-Wt-1+Wt-2, Luc-TPD52-Mut-1+Wt-2, Luc-TPD52-Wt-1+Mut-2 and Luc-TPD52-Mut-1+Mut-2. The insertion was verified by sequencing. 293T cells were co-transfected with either 25 nM miR-218a mimics or NC oligos and 200 ng plasmid. The relative firefly luciferase activity normalized with Renilla luciferase was measured 48 h after transfection by using the Dual-Light luminescent reporter gene assay (Applied Biosystems).

2.10. Western-blot analysis

Protein samples were separated in 12% SDS–PAGE gel and then transferred to nitrocellulose membrane (Bio-Rad). After blocked