Biochemical and Biophysical Research Communications 456 (2015) 804–809

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Guangye Han *, Maochuan Fan, Xinjun Zhang

Urology Department, The First Affiliated Hospital of Xinxiang Medical University, Henan 453100, China

ARTICLE INFO

Article history:
Received 2 December 2014
Available online 13 December 2014

Keywords:
miR-218
TPD52
Prostate cancer
Apoptosis

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

* Corresponding author at: The First Affiliated Hospital of Xinxiang Medical University, Healthy Road No. 88, Weihui, Henan 453100, China.

E-mail addresses: guangyehan@126.com (G. Han), maochunfan@outlook.com (M. Fan), xinjunzhang11@163.com (X. Zhang).

http://dx.doi.org/10.1016/j.bbrc.2014.12.026
0006-291X/

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

with 5% nonfat milk, the membrane was incubated with anti-TPD52 (1:2000, ab181260, Abcam) and anti-GAPHD (loading control) (1:2000, ab37168, Abcam) at 4 °C overnight, following by incubation with the secondary HRP conjugated anti-rabbit IgG (1:10,000, ab97064, Abcam) for 1 h at room temperature. The proteins were visualized using ECL reagents (Beyotime, China).

2.11. Statistical analysis

Experimental data are presented as mean ± SD with at least three repeats. Between group comparisons were performed by using paired or non-paired Student's *t* test, according to the detail assessment required. *p* value of <0.05 was considered significant and <0.01 was considered very significant.

3. Results

3.1. miR-218 expression is downregulated in prostate cancer

miR-218 expression in 46 prostate cancer patients (cancer tissue, adjacent normal tissue and serum) and in 25 normal controls (normal tissue and serum) were quantified by qRT-TPCR analysis. Generally, in prostate cancer patients, miR-218 expression was significantly lower in cancer tissues than in adjacent normal tissue (Fig. 1A). In addition, compared with normal controls, cancer cases also had significantly lower miR-218 expression in both cancer tissue ($p < 0.001$) and serum ($p < 0.01$) (Fig. 1B and C).

3.2. miR-218 inhibits prostate tumor cells proliferation partially through promoting apoptosis

Considering the lowered expression of miR-218 in prostate cancer patients, we further explored its function in prostate cancer cells. PC-3 cells with miR-218 overexpression or downregultion were generated (Fig. 2A). The growth rate of PC-3 cells with miR-218 overexpression was significantly lower than the normal control, while growth of miR-218 knockdown cells was significantly higher than the control (Fig. 2B). This trend was further confirmed in colony formation assay. The clonogenicity rate (%) of miR-218 overexpression, normal and downregulation groups were 10.5 ± 3.5, 20.7 ± 3.5 and 30.4 ± 4.2 respectively (Fig. 2C). In addition, miR-218 overexpression promoted apoptosis of PC3 cells, while its downregulation inhibited apoptosis (Fig. 2D). These data suggest that miR-218 could negatively regulate the growth of prostate cancer cells, which was partially through promoting apoptosis.

3.3. miR-218 targets TPD52 by binding to its 3′-UTR

To further explore the mechanism of miR-218's regulative role in prostate cancer, we screened its downstream targets in microRNA database TargetScan 5.1 (http://www.targetscan.org/). Bioinformatics prediction showed there were two putative binding sites between miR-218 and 3′-UTR of TPD52 (Fig. 3A). Overexpression of miR-218 in HEK 293T cells were confirmed by qRT-PCR (Fig. 3B). To confirm the binding, we constructed both a wide type and three mutants of firefly luciferase reporters containing the 3′-untranslated region (UTR) of TPD52. The reporters were cotransfected with either miR-218 mimic or NC oligos to HEK293T cells. The reporter assay showed that miR-218 could significantly repress luciferase expression of Luc-TPD52-Wt1+Wt2. Mutation at any of the two sites could partly abrogate the effect. Mutation at the two sites led to complete abrogation of the suppressive effect (Fig. 3C). In addition, we also observed that ectopic expression of miR-218 could affect the expression of TPD52 protein in PC3 cells (Fig. 3D). To further explore the association between miR-218 and TPD52 in prostate cancer patients, their expressions in serum sample of the patients were quantified through qRT-PCR. It was observed TPD52 protein level in the cancer tissues was inversely correlated with miR-218 expression in 46 PC patients ($R = -0.512$, $p < 0.001$) (Fig. 3E).

3.4. miR-218 suppresses prostate cancer cell growth through inhibiting TPD52 expression

TPD52 is oncogene regulating cell migration and invasion. Elevated TPD52 expression was observed in prostate cancer. Considering the confirmed binding between miR-218 and TPD52, we hypothesized that the tumor suppressing effect of miR-218 in prostate cancer cells might be mediated by modulating TPD52 expression. PC3 cells with TPD52 overexpression and downregulation was generated (Fig. 4A). TPD52 overexpression was associated with significantly higher growth rate of PC3 cells, while interfere of its expression had the opposite effect (Fig. 4B). Growth suppression effect of miR-218 could be rescued by transfection of TPD52 (Fig. 4C). Cytoflowmetry analysis showed that there were similar apoptosis-promoting role between TPD52 knockdown and miR-218 overexpression. However, transfecting TPD52 vector with mutated 3′UTR specific for miR-218 could significantly attenuated the apoptosis induced by miR-218, suggesting the growth suppressing effect of miR-218 is mainly mediated by TPD52 (Fig. 4D). Based on follow up data of 76 prostate cancer patients, the overall 3 year survival rate of 30 patients with high miR-218 expression and 46 patients with low miR-218 expression were 39.1% and 63.3% respectively, suggesting the level of miR-218 expression could affect tumor progression and overall survival.

4. Discussion

In the current study, we found the expression of miR-218 was significantly lower in PC specimens. Based on gain and loss of

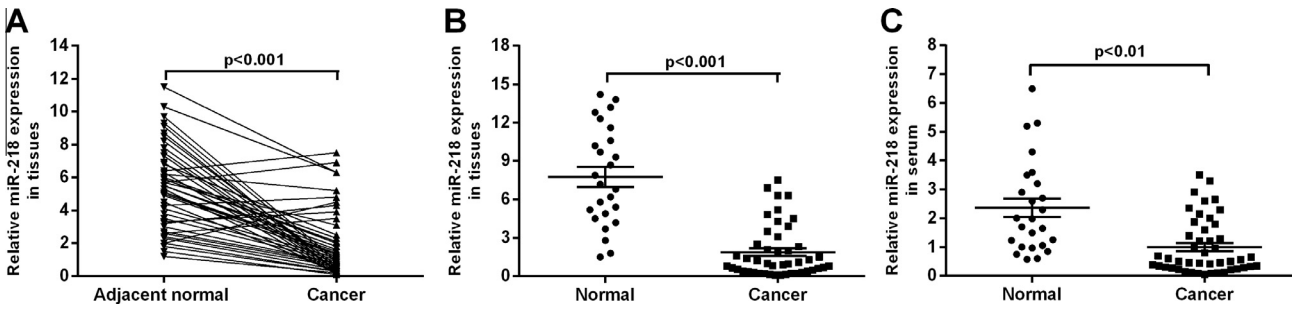

Fig. 1. miR-218 was downregulated in patients with PC. (A) Real-time PCR analysis of expression of miR-218 between PC and matched adjacent normal tissues from PC patients ($n = 46$). (B and C) Real-time PCR analysis of expression of miR-218 in the prostate tissue (B) and serum (C) of PC patients ($n = 46$) and healthy controls ($n = 25$).

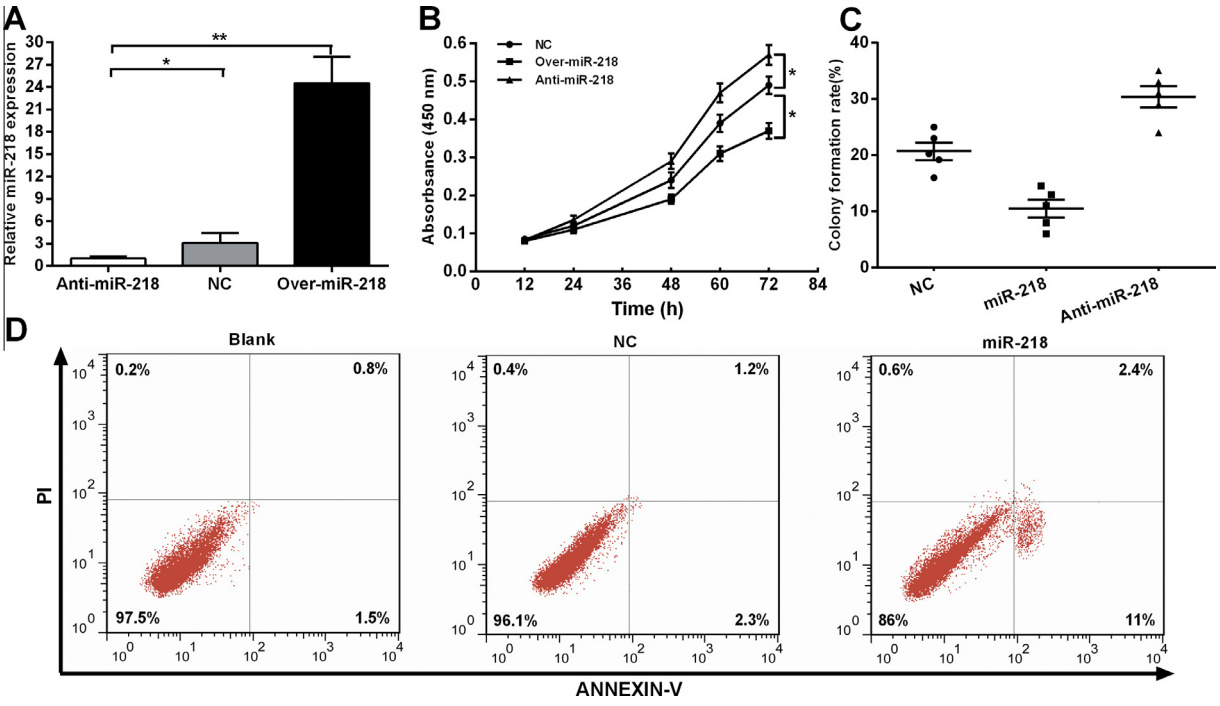

Fig. 2. miR-218 inhibited prostate tumor cells proliferation partially through promoting apoptosis. (A) Confirmation of overexpression and downregulation of miR-218 in PC3 cells using qRT-PCR. (B) miR-218 inhibited the growth of the bladder cancer cells using MTT assay. (C) miR-218 resulted in reduction of colony formation ability. (D) miR-218 promoted apoptosis of PC3 cells by flowcytometry method. Figure is representative of 3 independent experiments. Data are expressed as mean ± S.D. ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$.

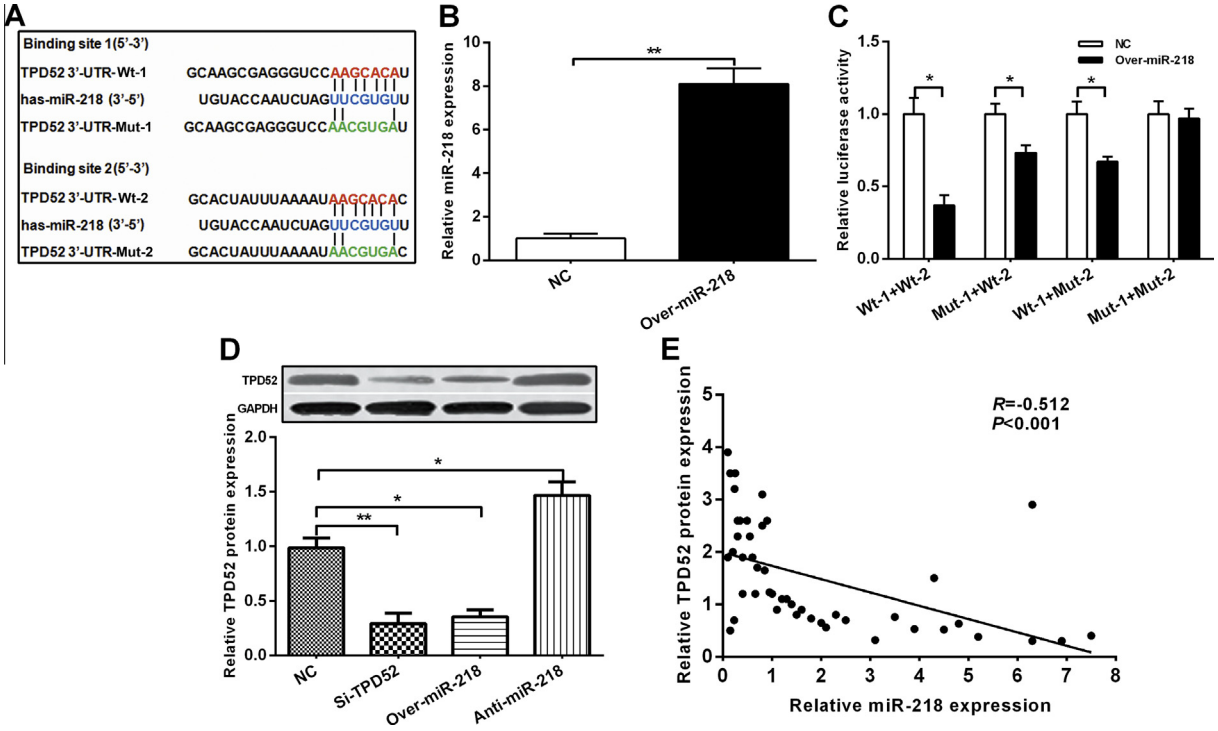

Fig. 3. miR-218 targeted TPD52 by binding to its 3′-UTR. (A) Bioinformatics prediction between miR-218 and 3′-UTR of TPD52. A predicted seed region in red (3′-UTR-Wt-1 and 3′-UTR-Wt-2) in the 3′-UTR of TPD52 was shown. The mutated sequence (mut-1 and mut-2) used was highlighted in blue (bottom). (B) Over-expression of miR-218 was tested by quantitative RT-PCR in HEK-293 cells transfected with 25nM miR-218 mimics. (C) HEK-293 cells were co-transfected with either 25nM miR-218 mimics or NC oligos and 200 ng plasmid carrying either Wt or Mut 3′-UTR of TPD52. The relative firefly luciferase activity normalized with Renilla luciferase was measured 48 h after transfection. (D) Western blot analysis showed that ectopic overexpression of miR-218 resulted in decreased TPD52 protein expression in PC3 cells. (E) miR-218 expression and TPD52 protein level in PC tissues showed an inverse correlated trend from 46 PC patients. Figure is representative of 3 independent experiments. Data are showed as mean ± S.D. ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$.

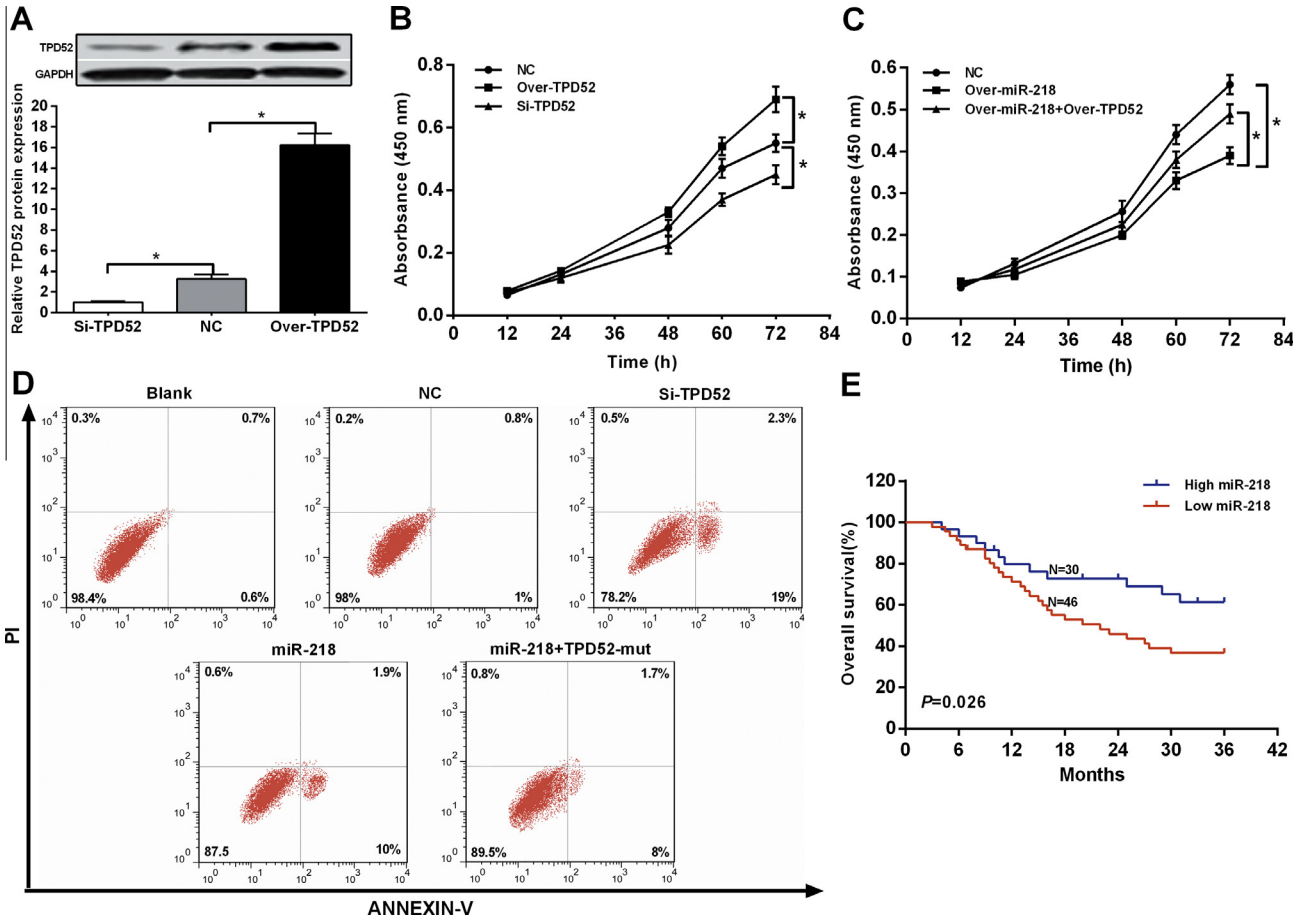

Fig. 4. miR-218 suppresses prostate cancer cell growth through inhibiting TPD52 expression. (A) Western blot showed successful knockdown and overexpression of TPD52. (B) MTT assay showed that TPD52 increased viability of PC3 cells. (C) Suppression of PC3 cell viability by miR-218 could be rescued by transfection of TPD52 expression vector with mutated 3′UTR binding sites of miR-218. (D) Flow cytometry showed that there were similar apoptosis-promoting roles between TPD52 knockdown and miR-218 overexpression. However, transfecting TPD52 vector with mutated 3′UTR specific for miR-218 could significantly attenuated apoptosis rate than miR-218 alone. (E) Kaplan–Meier curves for overall survival and miR-218 expression in group of 76 PC patients. Figure is representative of 3 independent experiments. Data are showed as mean ± S.D. ($n = 5$). $^*P < 0.05$.

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 fact, the suppressing effect of miR-218 was also observed in several type of cancers, including oral cancer [16], nasopharyngeal cancer [17] liver cancer [15] and bladder cancer [14]. In oral cancer, miR-218 inhibits cancer growth by targeting the mTOR component Rictor and inhibiting AKT phosphorylation [16]. In nasopharyngeal cancer, tumor suppressing effect of miR-218 is mediated by down-regulation of survivin and the SLIT2-ROBO1 pathway [17]. In liver cancer, the miR-218 could mediate the suppressive effect of 1,6,7-trihydroxyxanthone on liver cancer cells by targeting-Bmi-1 [15]. These results suggest that the molecular targets and regulative networks of miR-218 might be complex and cancer specific.

In prostate cancer, higher TPD52 expression was observed in both tumor and matched adjacent histologically normal tissues than in cancer-free prostate tissues [18]. High TPD52 transcript level was associated with higher -grade localized prostate cancers [4], development of systemic progression and early lethality [19]. Therefore, the level of TPD52 expression has been considered as a prognostic marker of prostate cancer. Previous in vitro analysis based on prostate cancer cells showed higher TPD52 expression is closely related to increased survival, proliferation, migration and invasion [10]. However, how TPD52 expression is regulated in prostate cancer cells is still not clear.

During the past several years, several studies found TPD52 transcript levels could be regulated by miRNAs. Actually, 80% of the TPD52 transcript was originally identified as 3′-UTR, which usually contains targeting sequence of miRNA [11]. Therefore, it is quite natural that TPD52 is a target of miRNAs. Takahashi et al. firstly reported that miR-107 and miR-185 transfection resulted in reduced TPD52 levels in nonsmall cell lung cancer cells [20]. In colorectal cancer cells, TPD52 is a target of miR-34a [12]. miR-34a is a well-known regulator of tumor suppression. Down-regulation of TPD52 is thus one of the pathways of miR-34a in tumor suppression [12]. In prostate cancer, one recent study observed that TPD52 is a target of miR-224 and its expression is upregulated in cancer tissues [13]. Silencing of the TPD52 gene significantly inhibits migration and invasion of prostate cancer cells [13]. However, whether other miRNAs are involved in regulating TPD52 in prostate cancer is not clear. In the current study, we demonstrated that TPD52 is a direct target of miR-218 and there are two effective binding sites between miR-218 and the 3′-UTR of TPD52. Therefore, miR-218 had strong inhibiting effect on TPD52 expression. TPD52 knockdown in PC3 cells led to decreased cell growth and increased apoptotic cell death. Previous studies indicated that TPD52 is required for the survival of ERBB2-positive cancer cells [21]. For example, transient knockdown of TPD52 increased cell death in both LnCaP and C4-2 cell lines, two ERBB2-positive prostate cancer cell lines [6–8]. In fact, PC3 cell is

also an ERBB2-positive cancer cell line [22]. Although further studies are required to confirm the exact role of the association between TPD52 expression and survival of ERBB2 positive prostate cancer cells, the available data at least suggests that TPD52 inhibition might increase the effectiveness of agents targeting this pathway. This study also observed low miR-218 expression is related to poor prognosis in PC patients, which helps to explain why TPD52 expression was also closely related to development of systemic progression within 5 years [5].

In conclusion, our data firstly demonstrated that tumor-suppressive miR-218 directly regulates oncogenic TPD52 in PC cells and the miR-218-TPD52 axis can regulate growth of this prostate cancer cell line. Knockdown of TPD52 resulted in significantly increased apoptosis of PC3 cells. In the future, in vivo study is required to further confirm the function of this axis. Clearly understanding of oncogenic TPD52 pathways regulated by miR-218 might be helpful to reveal new therapeutic targets for PC.

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