RESEARCH ARTICLE

miRNA-1297 Induces Cell Proliferation by Targeting Phosphatase and Tensin Homolog in Testicular Germ Cell Tumor Cells

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Abstract

To investigate the role of miR-1297 and the tumor suppressor gene PTEN in cell proliferation of testicular germ cell tumors (TGCT). MTT assays were used to test the effect of miR-1297 on proliferation of the NCCIT testicular germ cell tumor cell line. In NCCIT cells, the expression of PTEN was assessed by Western blotting further. In order to confirm target association between miR-1297 and 3'-UTR of PTEN, a luciferase reporter activity assay was employed. Moreover, roles of PTEN in proliferation of NCCIT cells were evaluated by transfection of PTEN siRNA. Proliferation of NCCIT cells was promoted by miR-1297 in a concentration-dependent manner. In addition, miR-1297 could bind to the 3'-UTR of PTEN based on luciferase reporter activity assay, and reduced expression of PTEN at protein level was found. Proliferation of NCCIT cells was significantly enhanced after knockdown of PTEN by siRNA. miR-1297 as a potential oncogene could induce cell proliferation by targeting PTEN in NCCIT cells.

Keywords: Testicular germ cell tumor - phosphatase and tensin homolog - miR-1297 - siRNA - cell proliferation

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Introduction

Testicular germ cell tumor (TGCT) is the most common cancer in young men, with peak incidence among those aged 25 to 34 years in the United States (Chung et al., 2013). It is well known that TGCT is originate from transformed primordial germ cells (PGCs) during early embryonic development (Rajpert-De et al., 2007; Andreassen et al., 2013). TGCT is relatively rare, but the incidence has increased several folds during the last decades in most western countries (Huyghe et al., 2003; 2007).

Although most of patients with newly diagnosed TGCT are eventually cured, the treatment-associated morbidity is still significant at higher stages (Feldman et al., 2008; Cost et al., 2012), specifically in patients who have metastatic disease at diagnosis (Sokoloff et al., 2007). The potential morbidity of current therapies is of special concern in this population. Therefore, a better understanding of the detailed mechanisms might be helpful to find new therapeutic targets and strategies for the treatment of TGCT.

Abnormal PTEN gene are found at high frequency in many sporadic human cancers, such as glioblastomas, endometrial, prostate and breast cancers (Dahia, 2000; Wang et al., 2009; Baig et al., 2011; Mahdi et al., 2013; Verit et al., 2013). Accumulated evidences show that tumor-suppressor gene PTEN is implicated in TGCT pathogenesis (Kimura et al., 2003; Andreassen et al., 2013). The tumor suppressor gene PTEN, encodes a lipid phosphatase for phosphatidylinositol 3, 4, 5-triphosphate and the main function of PTEN is to block the PI3K pathway by dephosphorylating phosphatidylinositol 3, 4, 5-triphosphate to PI-4, 5-bisphosphate thus counteracting PI3K function (Kimura et al., 2003; Molinari et al., 2014). Male mice that lacked PTEN were found bilateral testicular teratoma, which resulted from impaired mitotic arrest and outgrowth of cells with immature characters. Further studies with PTEN knockout PGCs *in vitro* revealed that these cells had greater proliferative capacity and enhanced pluripotent embryonic germ cell colony formation. It is obvious that PTEN plays an important role on germ cell differentiation and genesis of testicular germ cell tumor (Kimura et al., 2003).

MicroRNAs (miRNAs) are involved in most of the cellular processes (Gu et al., 2013), however, the complicated and diversified biological roles of miRNAs are far from being understood. miRNAs are non-coding regulatory RNA consisting of 20-24 nucleotides. More than 2000 miRNA have been identified in humans, and many miRNA alterations have also been found in tumors (Calin et al., 2006; Baer et al., 2013). To the best of our knowledge, there are only two microRNAs which are miR-199a and miR-383 have been investigated in TGCT. The deregulation of miR-199a-3p expression in TGCT was observed which indicated that it may be involved in TGCT carcinogenesis (Chen et al., 2014). Another study showed that downregulation of microRNA-383 is

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associated with male infertility and promotes testicular embryonal carcinoma cell proliferation by targeting IRF1 (Lian et al., 2010).

Since PTEN plays important role in TGCT, we searched for the microRNAs which target PTEN using bioinformatic algorithms (TargetScan and miRanda database). Among them, PTEN was found to have a putative miR-1297 binding site within its 3'-UTR.

In this study, we observed that miR-1297 promoted proliferation of NCCIT cells and decreased expression of PTEN in NCCIT cells. Furthermore, we showed that PTEN was a direct functional target of miR-1297 in NCCIT cells and proliferation of NCCIT cells was significantly promoted by knockdown of PTEN.

Materials and Methods

Cell culture

The NCCIT cell line was maintained in DMEM/F12 (1:1) medium (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were incubated at 37°C in a humidified chamber supplemented with 5% CO_2. The protocol of animal experiment was approved by the Animal Care and Use Committee at Zhongshan Hospital, Fudan University.

Western blot analysis

NCCIT cells after treatment were washed twice with PBS and protein samples were separated by 10% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto PVDF membranes (Millipore). Membranes were incubated with primary antibodies against PTEN (1:500, abcam, ab32199, 54 kDa) or β-actin (1:2000, Cell Signaling Technology) overnight at 4°C. Blots were washed and incubated for 1 h with HRP-conjugated anti-rabbit secondary antibody. Immunoreactive protein bands were detected using an Odyssey Scanning system. Each band was normalized with respect to its corresponding β-actin band.

MTT assay

NCCIT cells (5000/well) were plated in 96-well plates (BD Biosciences) and incubated at 37°C overnight. The next day, sub-confluent (50-60%) cells were transfected with miR-1297 mimics (50 nmol/L and 100 nmol/L) or PTEN siRNA (50 nmol/L) (sc-29459, Santa Cruz) using Lipofectamine 2000 (Invitrogen), in accordance with the manufacturer's instructions. miR-NC and siRNA control as negative controls were purchased from Santa Cruz. DMEM medium was replaced with DMEM medium supplemented with 10% FBS 5 h post-transfection with miR-1297 mimics or PTEN siRNA. Cell proliferation was assessed at 24, 48 and 72h, using the MTT proliferation assay kit in accordance with the manufacturer's instructions (Sigma). After treatment, cells were incubated with MTT diluted in culture medium for 1 h. Then, DMSO was added to each well, and the optical density at 570 nm was measured under a microtiter plate reader. All experiments were performed in biological triplicate. The relative cell viability was calculated by the following formula: $(OD_{treatment}/OD_{control})\times100\%$.

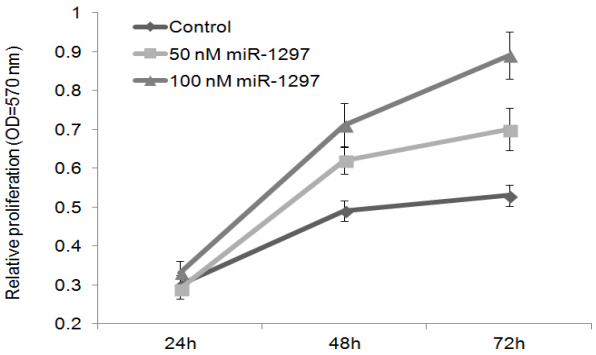

Figure 1. Cell Viability of NCCIT Cells is Enhanced by miR-1297. After transfection with miR-1297 mimic, cell viability of NCCIT cells are elevated at 72h in the presence of either 50 nM or 100nM miR-1297 compared to control group (*p<0.05). MiR-1297 at 100 nM concentration has stronger effect on elevation of cell viability of NCCIT cells than concentration at 50 nM (**p<0.05)

Luciferase reporter assay

The 3' untranslated region (3'-UTR) of PTEN containing the predicted miR-1297 binding site was amplified by PCR in a total volume of 50 μl using the Primer star kit (Takara) in accordance with the manufacturer's instructions. The amplication primers of 3'-UTR of PTEN mRNA: were 5'-AACGTGGGAGTAGACGGATG-3' (sense); 5'-TAGCCCTCAGGA AGAGACCA-3' (antisense).

The PCR fragment was inserted into psiCHECK2 vector within XhoI and NotI restriction sites (Promega). The mutant constructs were generated by mutation. Lipofectamine 2000 (Invitrogen) was used for the transfection of wild type 3'-UTR of PTEN (WT), the mutated 3'-UTR of PTEN (Mutant), miR-1297 mimic, or the control mimic (Control) in accordance with the manufacturer's instructions. Forty eight hour after transfection, cells were collected and luciferase activity was analyzed using a dualluciferase reporter assay system (Promega).

Statistical analysis

Data are presented as the mean±standard deviation from at least three independent experiments. Two-tail Student's t-test and ANOVA was performed to analyze the data using SPSS 12.0. p<0.05 was considered statistically significant.

Results

Regulation of NCCIT cell proliferation by miR-1297

To explore the effect of miR-1297 on testicular germ cell tumor proliferation, miR-1297 mimics were transfected into the human NCCIT cell line, and proliferation was assessed by MTT assay. We found that cellular proliferation gradually increased following transfection with miR-1297, in a concentration-dependent manner. Treatment of cells with 50 or 100 nM miR-1297 led to an increase in NCCIT cell growth at 48 h and 72 h (p<0.05) compared with the negative control (Figure 1). This promotion effect was significantly enhanced

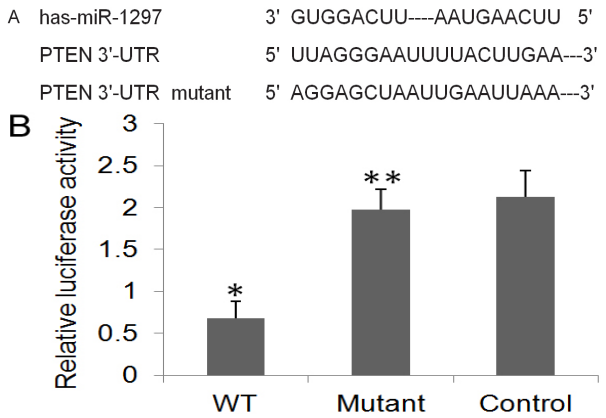

Figure 2. PTEN is a Direct Target of miR-1297 in NCCIT Cells. (Figure 2A) Consequential pairing of target region of PTEN 3'-UTR and miR-1297 were predicted using bioinformatic algorithms. (Figure 2B) Luciferase activity was much lower following co-transfection of psiCHECK-2/PTEN wild type 3'-UTR (WT) and miR-1297 mimic when compared to psiCHECK-2/PTEN mutated 3'-UTR (Mutant) and empty 3'-UTR vector (control) (*$p<0.05$). However, there is no difference between mutant group and control (**$p>0.05$)

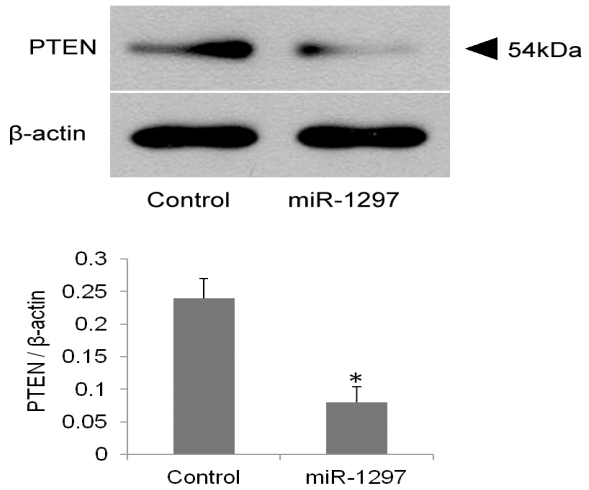

Figure 3. Expression of PTEN in NCCIT Cells. NCCIT cells were transfected with mirVana® miR-1297 mimic and mirVana™ miRNA Mimic, Negative Control #1 using Lipofectamine 2000. The PTEN protein expression was downregulated in NCCIT cells treated with miR-1297 mimic when compared to miR-negative control. PTEN protein bands were quantitated by densitometry (*$p<0.05$)

following transfection with 100 nM miR-1297 at 48 h and 72 h ($p<0.05$) compared with the effect of 50 nM miR-1297. In summary, these results indicated that proliferation of NCCIT cells was enhanced by miR-1297.

MiR-1297 binds to 3'-UTR of PTEN in NCCIT cells

To investigate the mechanism of miR-1297 on regulation of NCCIT cell proliferation, bioinformatic algorithms (TargetScan and miRanda database) were used to analyse the putative targets of miR-1297. Among them, PTEN was found to have a putative miR-1297 binding site within its 3'-UTR. To validate miR-1297 binding to this predicted site, we cloned the 3'-UTR of PTEN containing the putative miR-1297 binding site into a luciferase reporter construct (Figure 2A).

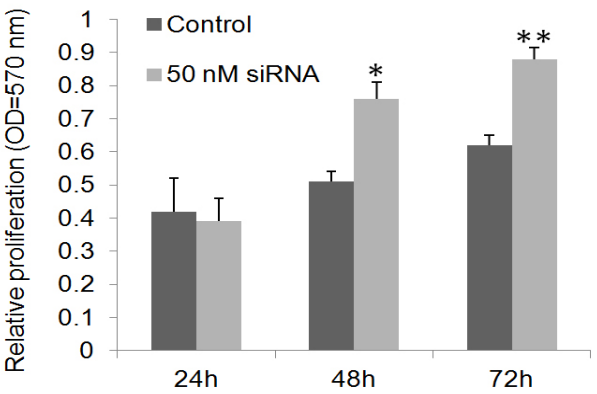

Figure 4. Knockdown of PTEN Significantly Inhibits the Cell Viability of NCCIT Cells. NCCIT cells were transfected with PTEN siRNA and Negative Control siRNA using Lipofectamine 2000. Relative cell viability was tested by MTT assay. Relative cell viability of NCCIT cells transfected with 50 nmol/L PTEN siRNA was increased significantly at both 48 h (*$p<0.05$) and 72 h (**$p<0.05$) when compared with Negative Control siRNA

Luciferase activity was significantly decreased following co-transfection of psiCHECK-2/PTEN wild type 3'-UTR (WT) with miR-1297, compared with psiCHECK-2/PTEN mutated 3'-UTR (Mutant) or the miR-negative control (Control) (Figure 2B). These results indicate that miR-1297 specifically binds to the 3'-UTR of PTEN in NCCIT cells.

Expression of PTEN regulated by miR-1297 in NCCIT cells

The effect of miR-1297 transfection on endogenous PTEN mRNA and protein expression was subsequently measured in NCCIT cells by Western blot. As shown in (Figure 3), the expression of protein was decreased in NCCIT cells after transfect with 100 nM miR-1297 mimic compared with miR-negative control. These results further suggest that miR-1297 directly targets PTEN in NCCIT cells. Since overexpression of miR-1297 elevated the cell viability of NCCIT cells, and given that PTEN is a direct target of miR-1297, we hypothesized that the elevated effect of miR-1297 on NCCIT cells might be achieved via targeting PTEN. However, before we make this conclusion, whether cell viability of NCCIT cells could be enhanced by PTEN loss must be investigated.

Proliferation of NCCIT cells regulated by PTEN

In order to know the effect of PTEN on cell viability of NCCIT cells, targeted knockdown of PTEN by siRNA and MTT assay were employed further. Treatment of NCCIT cells with 50 nmol/L PTEN siRNA markedly enhanced cell viability at 48 h and 72 h, respectively, compared with negative control siRNA ($p<0.05$) (Figure 4). This result demonstrated that reduced expression of PTEN contributed to the elevated cell survival of NCCIT cells.

Discussion

Although testicular germ cell tumors (TGCT) account for only 1% of malignancies in males, it is the most common malignancy among men aged 20-35 years[17].

The overall incidence of TGCT has increased from 3.35 per 100, 000 to 4.84 per 100, 000 men from 1973 to 2002 (Edwards et al., 2005).

The tumor suppressor PTEN is a lipid phosphatase, which plays important roles in the process of cellular proliferation, differentiation, apoptosis, adhesion and migration (Tamura et al., 1999). Evidences showed that PTEN is one of the most frequently mutated tumor suppressor genes in human cancer, and the overall frequency of PTEN mutations in sporadic human cancers is similar to that of the tumor suppressor p53 (Kimura et al., 2003).

Loss of PTEN function in embryonic stem (ES) cells and human cancer cell lines led to PtdIns (3, 4, 5) P3 accumulation and the activation of its downstream signaling molecule, Akt/PKB. Further studies showed that activation of the PI3K/Akt pathway by the loss of PTEN stimulates various biological functions, such as cell cycle progression, cell survival and cell migration (Kimura et al., 2003). In our study, the role of PTEN on cell viability of NCCIT cells was evaluated by transfection of PTEN siRNA. The result showed that knockdown of PTEN by siRNA significantly enhanced the cell viability of NCCIT cells, which suggest that targeting PTEN may be a valuable strategy to prevent the proliferation of TGCT.

MicroRNAs are a class of endogenous noncoding RNAs of 20-22 nucleotides. Processed mature miRNA can interact with the 3'-UTR of target mRNA causing degradation and/or translation repression (Buchan, 2014). A couple of microRNAs, such as miR-199a and miR-383 have been implicated in the pathogenesis of TGCT (Lian et al., 2010; Chen et al., 2014). Although PTEN plays important role in TGCT genesis, there are no literatures investigating PTEN in TGCT regulated by microRNA. In the MTT assay, we found that cell viability of NCCIT cells was elevated by miR-1297. Given that both miR-1297 and PTEN could regulate the cell viability of NCCIT cells, it is important to know whether PTEN is a direct target of miR-1297 in NCCIT cells. To explore whether regulation of miR-1297 on NCCIT cells viability is mediated by PTEN, we used bioinformatic algorithms (TargetScan and miRanda database) to predict gene targets for miR-1297. Among them, PTEN was found to have a putative miR-1297 binding site within its 3'-UTR. This prediction by software is supported by test of luciferase reporter activity. Overexpression of miR-1297 reduced expression of PTEN by 3'-UTR luciferase report assay, while this effect was not appearing by mutation of the PTEN 3'-UTR. Studies on Laryngeal squamous cell carcinoma discovered that PTEN was directly regulated by miR-1297 which supported our results. In addition, downregulation of miR-1297 in Hep-2 cells was shown to inhibit cancer cell proliferation, migration, and tumor genesis (Li et al., 2012). Our further experiment showed that overexpression of miR-1297 promoted proliferation of NCCIT cells which is consistent with previous literature (Li et al., 2012).

On the whole, our data suggest that downregulation of PTEN results in elevated proliferation of NCCIT cells and miR-1297 promotes viability of NCCIT cells by targeting PTEN, which could be employed as a potential therapeutic strategy in TGCT.

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