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Original Article

Phosphatase and tensin homolog regulates microRNA-182 and AKT to induce apoptosis of breast cancer cells SKBR3

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Abstract: Breast cancer is a disease of high incidence impacting the health of numerous female patients. Phosphatase and tensin homolog (PTEN) is involved in breast cancer cell apoptosis, however, the underlying mechanism is largely unknown. This study aims to explore the role and mechanism of PTEN in breast cancer cell apoptosis. Breast cancer cells SKBR3 were transfected with the overexpression vector of PTEN, and then cell apoptosis was assessed by flow cytometry. The phosphorylated level of v-akt murine thymoma viral oncogene homologs (p-AKT) and the level of microRNA-182 (miR-182) were detected in order to analyze the mechanism of PTEN in regulating SKBR3 cell apoptosis. Besides, miR-182 mimic was transfected to detect changes in apoptosis and AKT activation. Results showed that PTEN overexpression suppressed p-AKT level and induced SKBR3 cell apoptosis ($P < 0.01$). It also down-regulated miR-182 and miR-183 levels ($P < 0.001$), but did not affect miR-96 level ($P > 0.05$). miR-182 mimic promoted p-AKT and suppressed cell apoptosis ($P < 0.05$), which attenuated the effects of PTEN overexpression, implying the involvement of miR-182 in the mechanism of PTEN functions. These findings provide fundamental evidence for the potential application of PTEN in molecular therapy for breast cancer. The mechanism of PTEN in regulating breast cancer cell apoptosis may be related to its suppression on miR-182 and AKT activation. Further research will be necessary to take full advantage of PTEN in managing breast cancer.

Keywords: AKT, apoptosis, breast cancer, microRNA-182, phosphatase and tensin homolog

Introduction

As one of the most common cancers, breast cancer has long been the leading cause of cancer death in women. In 2012, breast cancer alone accounted for almost 15% of female cancer deaths [1]. It has a higher incidence but a lower mortality in developed areas [2], which is largely credited to the breast cancer screening and the neoadjuvant therapy adopted by these countries [3, 4]. Various risk factors may facilitate the development of breast cancer, such as unhealthy lifestyle, ionizing radiation and genetics among others. Mutations in breast cancer (BRCA) genes are responsible for a large proportion of inherited breast cancer cases [5]. Numerous studies have highlighted the important roles of gene expression profiles in treating breast cancer [6].

V-akt murine thymoma viral oncogene homologs (AKT) are pro-tumorigenic factors as revealed in many cancers [7]. The mutation in *AKT1* and *AKT2* is closely associated with breast cancer development [8]. Pathologically, AKT1 monitors the migration of mammary epithelial tumor cells and controls onset of breast cancer *in vivo* [9]. Its high level reduces the sensitivity of breast cancer cells to doxorubicin [10]. Due to the pivotal roles of AKT in breast cancer, the pathways concerning AKT are becoming attractive targets for exploring molecular targeting drugs of this disease [11, 12].

microRNAs (miRNAs), a kind of small non-coding RNAs that regulate gene expression post-transcriptionally via binding to target mRNAs, are being highlighted as crucial modulators of various diseases including breast cancer [13].

For example, members of the miR-183 family are considered as potential biomarkers for disease prognosis and intriguing targets of therapy [14]. Concretely, members of this family miR-96, miR-182 and miR-183 are up-regulated in breast cancer [15], functioning as a cluster to promote epithelial-mesenchymal transition and invasion [16], or individually to regulate cell invasion and sensitivity to infrared radiation in breast cancer [17, 18].

As important, studies have revealed that phosphatase and tensin homolog (PTEN) is involved in regulating apoptosis of breast cancer cells [19]. However, the mechanism of PTEN in breast cancer cell apoptosis remains elusive. This study performed *in vitro* experiments in breast cancer cells SKBR3, aiming to uncover the impact and potential mechanism of PTEN on breast cancer cell apoptosis. PTEN was overexpressed by transfecting its overexpression vector, and then cell apoptosis was assessed. We also up-regulated miR-182 in SKBR3 cells to investigate whether miR-182 was related to the functional mechanism of PTEN. This study was supposed to provide potential molecular therapeutic strategies for the treatment of breast cancer.

Materials and methods

Cells

Human breast cancer cells SKBR3 (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA). The cells were incubated in humid air with 5% CO_2 at 37°C. Medium was changed every 3 d.

Transfection

Overexpression vectors of human PTEN gene were constructed to overexpress PTEN. The complete coding sequence of human PTEN (GenBank: BC005821.2) with Flag tag sequence added to the 5' end was cloned and ligated to pcDNA3.1(+) (Invitrogen, Carlsbd, CA, USA) and transformed into DH5α-T1 (TransGen, Beijing, China). Positive clones were screened by ampicillin (100 μg/mL, Laibio, Shanghai, China) in the solid medium. The correct ligation (Flag-PTEN) was verified by sequencing.

SKBR3 cells were seeded in 24-well plates (1×10^5 cells/well) at 1 d before transfection. When the confluency reached 90%, transfection was conducted using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. The overexpression vector Flag-PTEN (1 μg/well) was transfected to overexpress PTEN and blank vector pcDNA3.1(+) used as a negative control. miR-182 mimic (100 nM) or the mimic control synthesized by Sangon Biotech (Shanghai, China) was transfected to overexpress miR-182. The cells were incubated at 37°C for 48 h for further experiments.

Cell apoptosis assay

At 48 h post transfection, SKBR3 cells were collected and treated using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Kit (BioVision, Milpitas, CA, USA) to detect apoptotic cells. According to the instructions, 1×10^5 cells were collected and resuspended in Binding Buffer. Then 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) were added, and the cells were incubated in the dark for 5 min at room temperature. The percent of apoptotic cells were quantified by flow cytometry using LSRFortessa (BD Biosciences, San Jose, CA, USA). The FITC-positive and PI-negative cells were considered to be apoptotic cells.

Western blot

Total protein samples from transfected cells were extracted and purified using ProteoPrep Total Extraction Sample Kit (Sigma-Aldrich, Shanghai, China) at 48 h post transfection. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the protein was transferred to polyvinylidene fluoride membranes (Invitrogen). The membranes were first blocked in 5% skim milk in phosphate-buffered saline (PBS) for 4 h at room temperature, and then incubated in primary antibodies against Flag tag (#14793, Cell Signaling Technology, Danvers, MA, USA), pan-AKT (#2920), phospho-pan-AKT (p-AKT, ab38449, Abcam, Cambridge, UK) overnight at 4°C. Here GAPDH (ab8245) was used as a control. Membranes were washed in PBS for 5 times and then incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies against rabbit or mouse IgG (ab6721 or ab6789) for 2 h at room temperature. After the membranes were washed in PBS for 5 times,

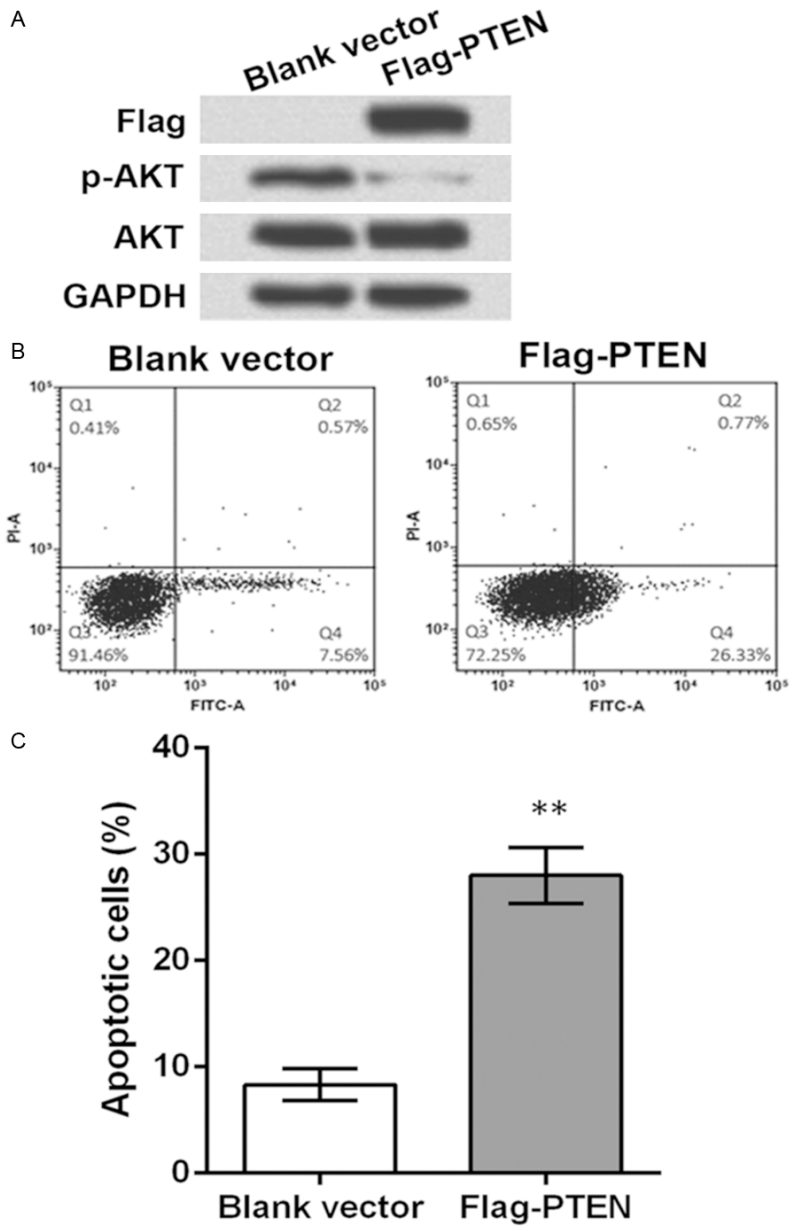

Figure 1. Phosphatase and tensin homolog (PTEN) inhibits the activation of v-akt murine thymoma viral oncogene homolog (AKT) and induces apoptosis of breast cancer cells SKBR3. SKBR3 cells were transfected with the overexpression vector of PTEN with Flag tag (Flag-PTEN) for PTEN overexpression. Blank vector was used as a control. Western blot and flow cytometry were performed at 48 h post transfection. A: Protein levels of PTEN (detected by anti-Flag antibodies), phospho-AKT (p-AKT) and AKT revealed by Western blot. GADPH was used as an internal control. B: Apoptotic cells detected by flow cytometry. Fluorescein isothiocyanate (FITC)-positive and propidium iodide (PI)-negative cells in the lower right quadrant (Q4) indicate apoptotic cells. C: Percent of apoptotic cells based on the flow cytometry results. $^{**}P < 0.01$.

signals were developed by Enhanced Chemiluminescence Detection Kit for HRP (Biological Inductries, Beit-Haemek, Israel). The signal intensity was quantified by ImageJ 1.49 (National Institutes of Health, Bethesda, MD, USA).

qRT-PCR

Total RNA samples were extracted from transfected cells by Trizol (Invitrogen), and DNA contamination was degraded by DNase I (Invitrogen). The mature miRNA sequence of hsa-miR-182-5p, hsa-miR-183-5p and hsa-miR-96-5p were retrieved from online database www.mirbase.org [20]. Reverse transcription was catalyzed by PrimeScript Reverse Transcriptase (Takara, Dalian, China) using the specific reverse transcription primers (sequence 5'-CTCAA CTGGT GTCGT GGAGT CGGCA ATTCA GTTGA G-3' plus AGTGTGAG, AGTGAATT or AGCAAAAA to the 3' end) for miR-182, miR-183 or miR-96. qRT-PCR was performed by LightCycler 480 (Roche, Basel, Switzerland) using the reverse primer (5'-TGGTG TCGTG GAGTC G-3') and specific forward primers: 5'-ACACT CCAGC TGGGT TTGGC AATGG TAGAA CT-3' for miR-182, 5'-ACACT CCAGC TGGGT ATGGC ACTGG TAGAA-3' for miR-183 and 5'-ACACT CCAGC TGGGT TTGGC ACTAG CACAT T-3' for miR-96. U6 amplified by forward primer (5'-CTCGC TTCGG CAGCA CA-3') and reverse primer (5'-AACGC TTCAC GAATT TGCGT-3') was used as an internal reference. Data were calculated by $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All the experiments were performed in triplicate. Quantified results of qRT-PCR and flow cytometry were expressed as means ± standard deviation. SPSS 20 (IBM, New York, NY, USA) was used to analyze the data. Significance

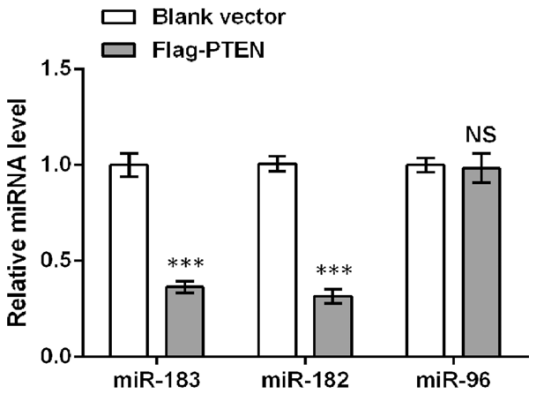

Figure 2. Phosphatase and tensin homolog (PTEN) reduces miR-182 level in breast cancer cells SKBR3. SKBR3 cells were transfected with the overexpression vector of PTEN with Flag tag (Flag-PTEN) for PTEN overexpression. Blank vector was used as a control. qRT-PCR was performed at 48 h post transfection. ***P < 0.001. NS, not significant.

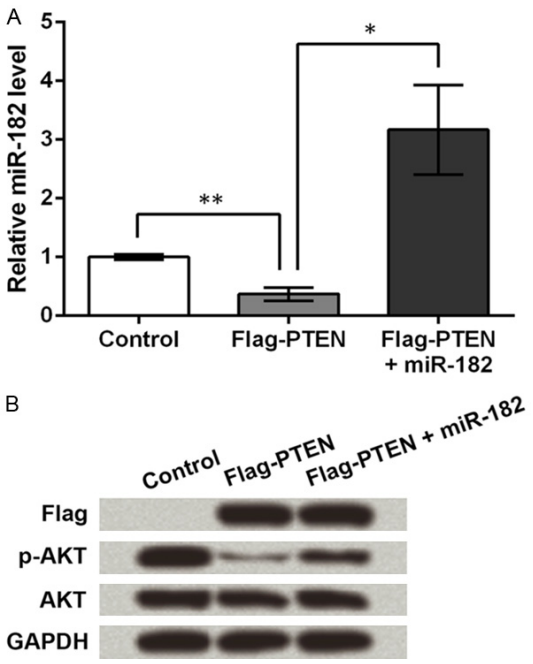

Figure 3. miR-182 overexpression impacts the inhibitory effect of phosphatase and tensin homolog (PTEN) on v-akt murine thymoma viral oncogene homolog (AKT) activation in breast cancer cells SKBR3. SKBR3 cells were transfected with the overexpression vector of PTEN with Flag tag (Flag-PTEN) and miR-182 mimic. Blank vector and mimic control were transfected as controls. qRT-PCR and Western blot were performed at 48 h post transfection. A: Relative miR-182 level in transfected cells detected by qRT-PCR. *P < 0.05. **P < 0.01. B: Protein levels of PTEN (detected by anti-Flag antibodies), phospho-AKT (p-AKT) and AKT revealed by Western blot. GADPH was used as an internal control.

of differences was determined by Student's t test. P < 0.05 was considered to be statistically significant between groups.

Results

PTEN inhibits AKT activation and induces apoptosis in SKBR3 cells

Out of the crucial functions of AKT in the pathogenesis of breast cancer, we detected the activation of AKT after overexpressing PTEN in SKBR3 cells. Western blot showed that the expression of Flag tag after transfection (**Figure 1A**), suggesting the effective cell transfection to overexpress PTEN. With the PTEN overexpression, AKT protein level did not change obviously, but the activated form p-AKT was markedly increased, indicating that PTEN was able to promote the activation of AKT.

SKBR3 cell apoptosis was assessed by flow cytometry and was found to be increased by PTEN overexpression (**Figure 1B**). Quantitative results indicated that the percent of apoptotic cells was significantly higher after PTEN overexpression (P < 0.01, **Figure 1C**), which suggested that PTEN might promote apoptosis of SKBR3 cells.

PTEN down-regulates miR-182

Since previous studies have found the credible involvement of miR-182 in breast cancer, we therefore quantified miR-182 level in the transfected SKBR3 cells. qRT-PCR results showed that miR-182 level was significantly suppressed by PTEN overexpression (P < 0.001, **Figure 2**). As controls, miR-183 level was also markedly inhibited (P < 0.001), but miR-96 was barely changed (P > 0.05). These results suggested that PTEN might regulate several miRNAs such as miR-182, which was likely to be related to its function in SKBR3 cells.

miR-182 impacts the effects of PTEN on AKT and cell apoptosis

Next we tried to examine whether miR-182 was involved in the functional mechanism of PTEN in SKBR3 cells. The overexpression vector of PTEN and miR-182 mimic were co-transfected into SKBR3 cells, and qRT-PCR showed that miR-182 level was suppressed by PTEN expression (P < 0.01, **Figure 3A**) in consistence with

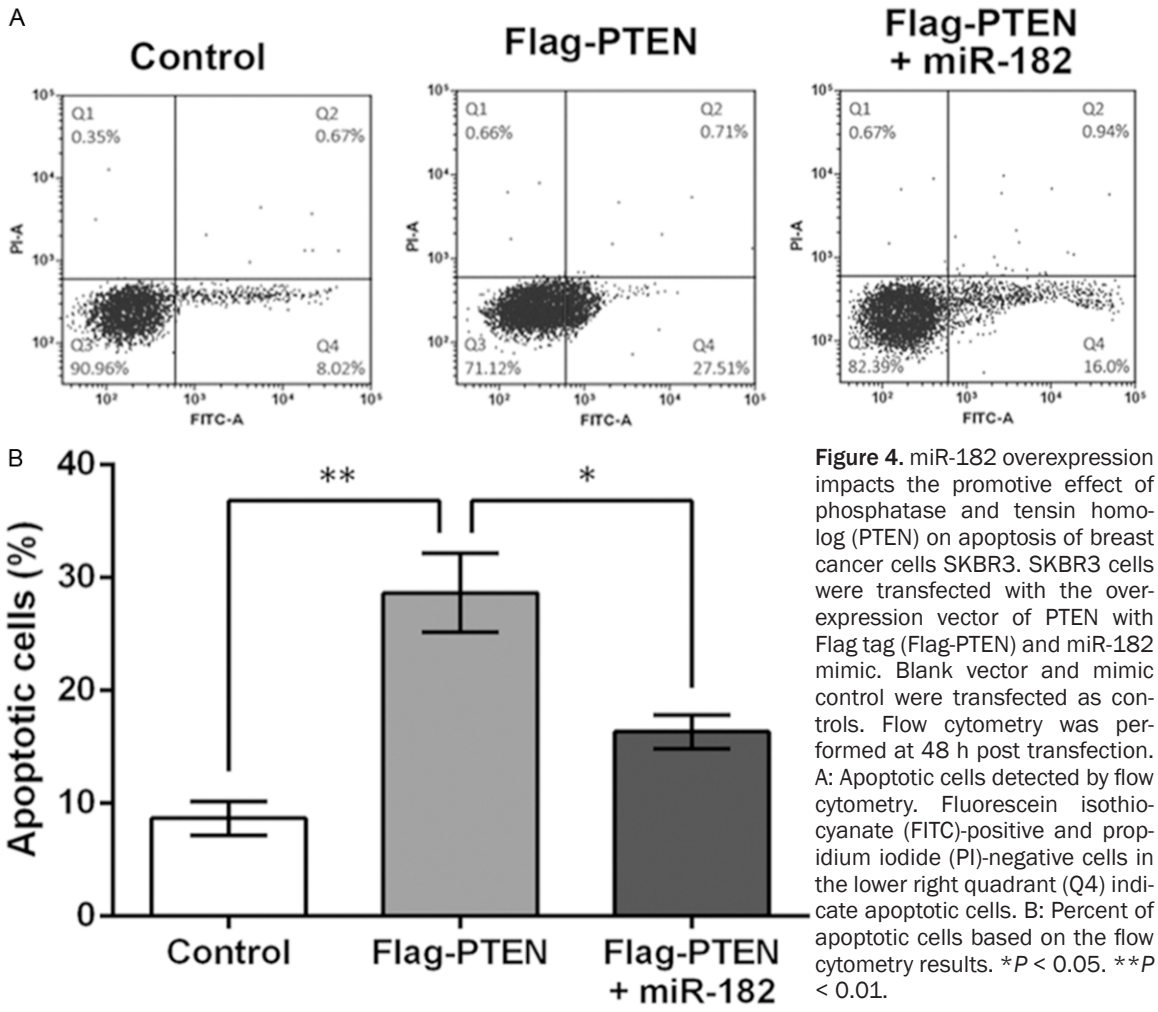

Figure 4. miR-182 overexpression impacts the promotive effect of phosphatase and tensin homolog (PTEN) on apoptosis of breast cancer cells SKBR3. SKBR3 cells were transfected with the overexpression vector of PTEN with Flag tag (Flag-PTEN) and miR-182 mimic. Blank vector and mimic control were transfected as controls. Flow cytometry was performed at 48 h post transfection. A: Apoptotic cells detected by flow cytometry. Fluorescein isothiocyanate (FITC)-positive and propidium iodide (PI)-negative cells in the lower right quadrant (Q4) indicate apoptotic cells. B: Percent of apoptotic cells based on the flow cytometry results. $*P < 0.05$. $**P < 0.01$.

the above results (**Figure 2**) and then elevated by miR-182 mimic ($P < 0.05$), indicating the successful co-transfection. Then Western blot was performed to detect protein levels. PTEN overexpression vector brought out the signal of Flag (**Figure 3B**), but miR-182 mimic did not greatly change the intensity of Flag, suggesting that miR-192 might not influence PTEN expression. However, miR-182 increased p-AKT, impairing the promotive effects of PTEN on AKT activation.

We further examined the cell apoptosis changed by miR-182. Flow cytometry found that PTEN promoted cell apoptosis, which was attenuated by miR-182 (**Figure 4A**), with significant difference between groups ($P < 0.01$ or $P < 0.05$, **Figure 4B**). Thus miR-182 might also suppress cell apoptosis to impair the effect of PTEN. Collectively, miR-182 could attenuate the effects of PTEN on AKT activation and apoptosis in SKBR3 cells, implying its potential involvement in the function of PTEN.

Discussion

Given the reported function of PTEN in the development of several diseases, this study further finds evidence that PTEN induces apoptosis of breast cancer cells SKBR3. PTEN overexpression down-regulates miR-182 level and suppresses the activation of AKT. Furthermore, the effects of PTEN on SKBR3 cell apoptosis and AKT activation can be attenuated by miR-182 overexpression.

Based on existed reports, PTEN has a relatively conserved function of inducing apoptosis in various cell types, leukemia cells and bladder cancer cells for instance [21, 22]. In breast cancer, particularly, PTEN overexpression triggers cell cycle arrest and promotes apoptosis

[19], and its induction by some bicyclic N-fused aminoimidazoles may lead to apoptosis [23]. Further, some researchers maintain that the tumor suppressive function of PTEN is dependent on the induction of autophagy [24]. In concordance with the former studies, our experiments showed that overexpression of PTEN in breast cancer cells SKBR3 accelerated apoptosis, which supports that PTEN may also act as a tumor suppressor in breast cancer.

Out of the aforementioned participation of AKT in modulating breast cancer, we detected AKT activation along with the overexpression of PTEN, and found that PTEN was able to reduce the level of p-AKT, which indicated the suppressed AKT activation. Similar results have been reported in previous studies that AKT factors are activated to suppress cell apoptosis. For example, activation of AKT2 and its upstream phosphoinositide 3-kinase (PI3K) reduces breast cancer apoptosis [25, 26]. Furthermore, the suppression of PTEN on AKT activation has been highlighted in gastrointestinal carcinoid tumors and rat Sertoli cells to regulate cell apoptosis [27, 28]. Thus it is reasonable to speculate that the regulated AKT activation by PTEN in SKBR3 cells is related to the cell apoptosis induced by PTEN.

We further observed that miR-182 level was inhibited by PTEN overexpression, which imply the possibility that miR-182 may participate in the functional mechanism of PTEN in SKBR3 cell apoptosis. Evidence of miR-182 suppressing apoptosis has been well documented in cervical cancer, bladder cancer and prostate cancer [29-31]. Besides, this study further found that miR-182 overexpression could impede the suppressed AKT activation and induced SKBR3 cell apoptosis by PTEN. Collectively, it is plausible that PTEN inducing SKBR3 cell apoptosis may partly depend on miR-182. At the same time, miR-183 was also found inhibited by PTEN, which requires further investigation to reveal its relationship with PTEN in breast cancer cells.

It has been suggested that depletion of miR-182 reduced level of activated AKT via directly target branched chain amino acid transaminase 2 in mouse cardiomyocytes [32], which supports the findings in SKBR3 cells that the PTEN-induced miR-182 down-regulation was accompanied by suppressed p-AKT, while miR-182 overexpression increased p-AKT. With respect to PTEN and miR-182, it is tempting to speculate that PTEN may inhibit the transcription process of miR-182 directly or with the assistance of other factors, since the transcription of miRNAs from the genome is modulated by various transcription factors [33]. Meanwhile, PTEN has the ability to repress transcription of RNA polymerase I by modulating disruption of SL1 complex [34]. However, the possibility that miR-182 inhibits PTEN cannot be excluded. A study has predicted *PTEN* to be a potential target of miR-183 [35], and similarly, *PTEN* was predicted to be a target of miR-182 via http://www.targetscan.org/vert_71/ [36]. These speculations invite a more extensive investigation into the regulatory relationship between PTEN and miR-182.

In conclusion, PTEN overexpression induces apoptosis of breast cancer cells SKBR3, thus being a promising therapeutic strategy for the molecular treatment of breast cancer. The functional mechanism of PTEN in regulating SKBR3 cell apoptosis may involve miR-182 and AKT, which is worthwhile topic in future research.

Disclosure of conflict of interest

None.

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