

## Overexpression of *PTEN* Induces Cell Growth Arrest and Apoptosis in Human Breast Cancer ZR-75-1 Cells

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**Abstract** Phosphatase and tensin homolog (*PTEN*) is a tumor suppressor gene located at human chromosome 10q23, might play an important role in cell proliferation, cell cycle and apoptosis of cancer cells. In this study, the eukaryotic expression vectors pBP-wt-*PTEN* (containing a wild-type *PTEN* gene) and pBP-G129R-*PTEN* (containing a mutant *PTEN* gene) were used to transfect breast cancer ZR-75-1 cells. After transfection, ZR-75-1 cells expressing *PTEN* were obtained and tested. The blue exclusion assay showed the growth rate of the cells transfected with pBP-wt-*PTEN* was significantly lower than that of the control cells transfected with pBP-G129R-*PTEN*. Analysis of the cell cycle by flow cytometry showed that the progression from the G<sub>1</sub> to the S phase was arrested in cells expressing wild-type *PTEN*. Some typical morphological changes of apoptosis were also observed in cells transfected with pBP-wt-*PTEN*, but not in those transfected with pBP-G129R-*PTEN*. This study shows that overexpression of *PTEN* in ZR-75-1 cells leads to cell growth arrest and apoptosis.

**Keywords** *PTEN*; tumor suppressor gene; breast cancer; cell growth; apoptosis

*PTEN* is a tumor suppressor gene located at human chromosome 10q23 that encodes a dual substrate-specific phosphatase. This gene is frequently deleted or mutated in a wide range of human tumors and tumor cell lines [1–3]. Previous studies have shown that transient expression of *PTEN* in *PTEN*-null endometrial, melanoma and lymphoid cancers could suppress cell growth and cause cell apoptosis [4,5]. Germline mutations of the *PTEN* gene, including homozygous deletions, have been found in patients with Cowden's disease, an autosomal dominant syndrome carrying elevated risk for cancers of the breast and thyroid [6,7]. Mutations of this gene were reported in two of 26 breast cancer cell lines and in two of 14 primary breast tumors examined [8], indicating that loss of *PTEN* function is probably associated with progression of breast cancers. However, whether overexpression of *PTEN* in

human breast cancer cells affects their growth and apoptosis remains to be investigated.

In the present study, we transfected the *PTEN*-null breast cancer cell line ZR-75-1 with a *PTEN*-expressing vector and examined the effect of *PTEN* overexpression on cell proliferation and apoptosis.

### Materials and Methods

#### Cell culture and transfection

The breast cancer cell line ZR-75-1 was obtained from the Chinese Academy of Medical Sciences (Beijing, China). The breast cancer cells were cultured in RPMI 1640 complete culture medium (GIBCO, New York, USA) supplemented with 15% heat-inactivated fetal bovine serum (FBS; GIBCO), benzylpenicillin (100 kU/L), and streptomycin (100 mg/L) at 37 °C in a humidified incubator, with 5% CO<sub>2</sub> in air. The cells were routinely passaged every 1 or 2 d. For transfection, 5×10<sup>6</sup> cells mixed with

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500  $\mu$ l of RPMI 1640 (supplemented with 15% heat-inactivated FBS, without antibiotics) were seeded into a 24-well plate and incubated at 37 °C for 48 h. Nearly confluent cells were then co-transfected with pBP-wt-*PTEN* plasmid or pBP-G129R-*PTEN* plasmid (gifts of Prof. Frank FURNARI, Ludwig Institute for Cancer Research, San Diego, USA) (2.0  $\mu$ g/well) and pTR-UF5 plasmid (gift of Dr. Nicholas MUZYCKA, Florida University, Gainesville, USA) harboring green fluorescence protein reporter gene (0.2  $\mu$ g/well) using a Lipofectamine 2000-mediated (Invitrogen, Carlsbad, USA) method according to the manufacturer's protocol (ratio of plasmid to Lipofectamine was 1:1). Six hours after transfection, the medium was changed to normal culture medium. Cells were continuously cultured until harvest for analysis.

### Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted 48 h after transfection using Trizol reagent (GIBCO). RT was carried out using a one-step RT-PCR kit (Qiagen, Hilden, Germany). The primers for *PTEN* (amplified products were 373 bp) were 5'-AAAGCTGGAAAGGGACGAAC-3' (forward), and 5'-CAGGTAACGGCTGAGGGAAC-3' (reverse). The house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as an internal standard. The upstream primer of *GAPDH* (amplified products were 226 bp) was 5'-GAAGGTGAAGGTCGGAGTC-3', and the downstream primer was 5'-GAAGATGGTGATGGGATTTTC-3'. Thermal cycle conditions were as follows: 50 °C for 30 min, 95 °C for 15 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide, and detected by ultraviolet irradiation.

### Western blot analysis

The cells were washed three times with cold phosphate-buffered saline (PBS) 48 h after transfection, collected by scraping and lysed in 150  $\mu$ l ice-cold Tris buffer (50 mM, pH 8.0) containing 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1.0 mg/L aprotinin, and 0.2 mg/L phenylmethylsulphonyl fluoride for 10 min. The extracts were centrifuged at 12,000 g for 15 min, and the concentration of protein in each lysate was determined with Coomassie Brilliant Blue G-250. Loading buffer was added to each lysate, which was subsequently boiled for 3 min and electrophoresed on a SDS-polyacrylamide gel. Before electrophoresis, the proteins were mixed with 2 $\times$ loading buffer (containing 100 mM Tris-HCl, pH 6.8,

20% glycerin, 4% SDS, 0.05 g/L tetrabromophenol sulfonphthalein, and 10% 2- $\beta$ -mercaptoethanol) by the same volume. Proteins were transferred onto nitrocellulose membranes (Sigma, New York, USA). After blocking with 5% skim milk in Tris-HCl (pH 7.5) at room temperature for 2 h, the nitrocellulose membranes were reacted for 2 h with specific antibodies in the same blocking solution [PTEN antibody and anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, USA) were used at 1:300 and 1:500 dilutions, respectively]. After extensive washing with Tris-HCl containing 0.05% Tween 20, the membranes were reacted with anti-mouse immunoglobulin G-horseradish peroxidase for PTEN protein detection. Finally, protein bands were visualized using an electrochemiluminescence test kit (Santa Cruz Biotechnology). Densitometric analyses were carried out using Scion Image software Version Cot33 (Scion, Frederick, USA).

### Cell growth assay

Cell growth was measured by Trypan blue staining. An equal number of cells were plated into 12-well plates to be transfected with pBP-wt-*PTEN* plasmid or pBP-G129R-*PTEN* plasmid and cultured for various times. After incubation, the medium was removed. Cells were then washed with phosphate-buffered NaCl solution and fixed with 12.5% glutaraldehyde for 20 min at room temperature. Cells were rinsed with distilled water and incubated with 1 mg/ml Trypan blue (Sigma) for 30 min. Cells were then observed and counted under an inverted microscope and growth curves were drawn using the cultured time as abscissa and living cells as ordinate.

### Fluorescence microscopy

ZR-75-1 cells ( $1 \times 10^5$ ) were seeded in a 35 cm Petri dish and cultured in RPMI 1640 supplemented with 15% heat-inactivated FBS without antibiotics. After incubation at 37 °C for 24 h, cells of two dishes were transfected with pBP-wt-*PTEN* plasmid or pBP-G129R-*PTEN* plasmid (the final concentration of plasmid was 2.0  $\mu$ g/ml). After transfection for 6 h, the medium was changed to complete culture medium. Cells were collected 24 h after the transfection and centrifuged for 5 min at 200 g to remove cell debris, and cells were washed three times with 0.9% saline after centrifugation for 5 min at 1000 g to remove culture medium. Then 10  $\mu$ l of 10 mg/L Hoechst 33258 was added to the cell suspension and cells were incubated for 30 min in the dark. Ten microliters of the stained cell suspensions was taken out, dripped on slides and covered with a cover slip. The morphological changes of nuclei in pBP-wt-*PTEN*-transfected ZR-75-1 cells were observed

using fluorescence microscope (400×) to discriminate normal cells, apoptotic cells and necrotic cells. Cells were photographed and the images were processed with Adobe Photoshop software version 7.0 (Adobe, San Jose, USA).

### Flow cytometry analysis

After treatment with pBP-wt-*PTEN* plasmid or pBP-G129R-*PTEN* plasmid for 48 h, ZR-75-1 cells were harvested by centrifugation at 200 g for 5 min to remove cell debris, and washed three times with PBS by centrifugation at 1000 g for 5 min to remove culture medium. The cell suspension was fixed in ice-cold 70% ethanol in PBS, and stored at 20 °C. Prior to analysis, the cells were washed and resuspended in PBS, and incubated with 1 g/L of RNase I and 20 g/L propidium iodide at 37 °C for 30 min. Apoptosis was analyzed with a flow cytometer (Coulter Becton Dickinson, Miami, USA). For each sample, at least  $1 \times 10^4$  cells were analyzed by flow cytometry, and the percentage of apoptotic cells in the sub-G<sub>1</sub> phase was calculated using Multicycle software (Phoenix Flow Systems, San Diego, USA).

### Statistical analysis

All statistical analyses were carried out using one-way ANOVA tests. Values of  $P < 0.05$  were considered significant.

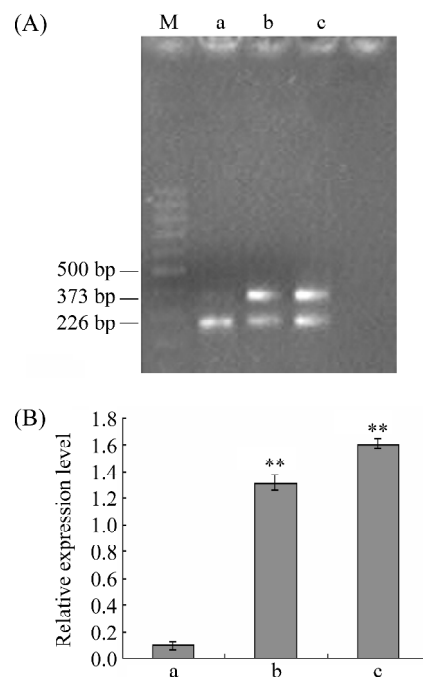
## Results

### Overexpression of *PTEN* in transfected cells

To verify the expression status of *PTEN* in the ZR-75-1 cells transfected with pBP-wt-*PTEN* and pBP-G129R-*PTEN*, the mRNA and protein levels of *PTEN* in the transfected cells were determined by RT-PCR and Western blot analysis, respectively. RT-PCR analysis showed that *PTEN* mRNA was abundant in cells transfected with either pBP-wt-*PTEN* or pBP-G129R-*PTEN*, but undetectable in the untreated cells (Fig. 1). Accordingly, an appreciable amount of PTEN protein was found in cells transfected with pBP-wt-*PTEN* or pBP-G129R-*PTEN*, but not in the untreated (control) cells (Fig. 2). These data clearly indicate that transfection with pBP-wt-*PTEN* or pBP-G129R-*PTEN* resulted in overexpression of *PTEN* in ZR-75-1 cells.

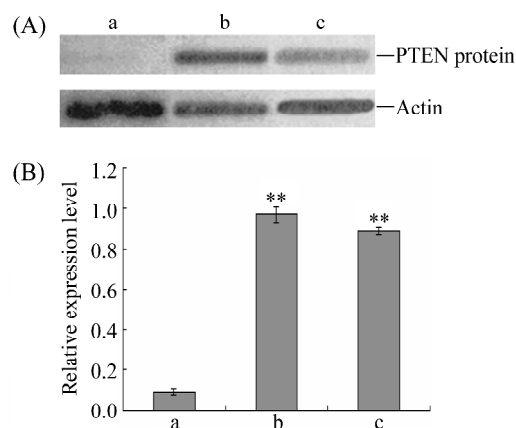
### Effect of *PTEN* overexpression on cell growth

To investigate whether increased levels of PTEN in ZR-75-1 affect cell growth, the number of cells at each time point after transfection was determined by Trypan blue staining. Compared with untreated cells, the cells



**Fig. 1** Reverse transcription-polymerase chain reaction analysis of tumor suppressor gene *PTEN* mRNA levels in ZR-75-1 breast cancer cells after transfection with plasmid pBP-wt-*PTEN* or pBP-G129R-*PTEN* for 48 h

(A) Gel electrophoresis of PCR products of *PTEN* gene in three cell groups. (B) Relative expression level of *PTEN* gene in different treated group cells. a, untreated group; b, pBP-wt-*PTEN* (expressing wild-type *PTEN*); c, pBP-G129R-*PTEN* (expressing mutant *PTEN*); M, 100 bp DNA marker.  $n=3$ . The results in Fig. 1(B) are the mean $\pm$ SD; \*\* $P < 0.01$  versus control.



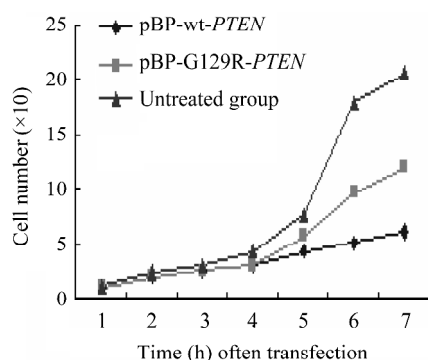
**Fig. 2** Western blot analysis of tumor suppressor gene *PTEN* protein levels in ZR-75-1 breast cancer cells after transfection with plasmid pBP-wt-*PTEN* or pBP-G129R-*PTEN* for 48 h

(A) Expression of PTEN protein in three cell groups. (B) Relative expression level of PTEN protein in different treated cells. a, untreated group; b, pBP-wt-*PTEN* (expressing wild-type *PTEN*); c, pBP-G129R-*PTEN* (expressing mutant *PTEN*). The results in Fig. 2(B) are the mean $\pm$ SD;  $n=3$ . \*\* $P < 0.01$  versus control.

transfected with pBP-wt-*PTEN* (expressing wild-type *PTEN*) showed a significantly lower growth rate, whereas the cells transfected with pBP-G129R-*PTEN* (expressing mutant *PTEN*) showed no difference in their growth rate (Fig. 3). Flow cytometry analysis indicated that there were a significant increase in the number of cells at the G<sub>1</sub> phase and a decrease in the number of cells at the S phase in the pBP-wt-*PTEN*-transfected cells, but not in the pBP-G129R-*PTEN*-transfected cells, suggesting that overexpression of wild-type *PTEN* could induce G<sub>1</sub> arrest in ZR-75-1 cells.

### Effect of *PTEN* overexpression on cell apoptosis

To examine whether overexpression of *PTEN* could induce cell apoptosis, detection of apoptotic cells using Hoechst 33258 staining and flow cytometry was carried out after transfection of ZR-75-1 cells with pBP-wt-*PTEN*

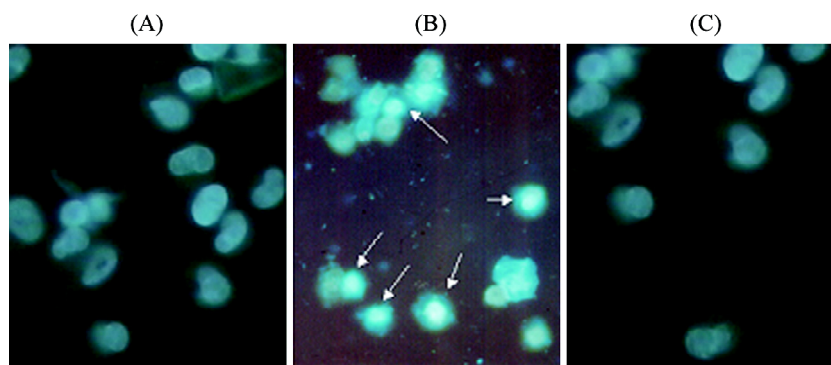


**Fig. 3** Number of ZR-75-1 breast cancer cells after transfection with plasmid pBP-wt-*PTEN* (expressing wild-type *PTEN*) or pBP-G129R-*PTEN* (expressing mutant *PTEN*) for different time periods

or pBP-G129R-*PTEN*. As shown in Fig. 4, there were very few apoptotic cells in the untransfected and pBP-G129R-*PTEN*-transfected cells (expressing mutant *PTEN*). In contrast, many more apoptotic cells, characterized by apoptotic bodies and fragmentation of nuclei, were observed in the pBP-wt-*PTEN*-transfected cells (expressing wild-type *PTEN*). Flow cytometry analysis showed that approximately 7% of pBP-wt-*PTEN*-transfected cells were apoptotic (Fig. 5). These results suggest that overexpression of wild-type *PTEN* in ZR-75-1 cells could induce apoptosis.

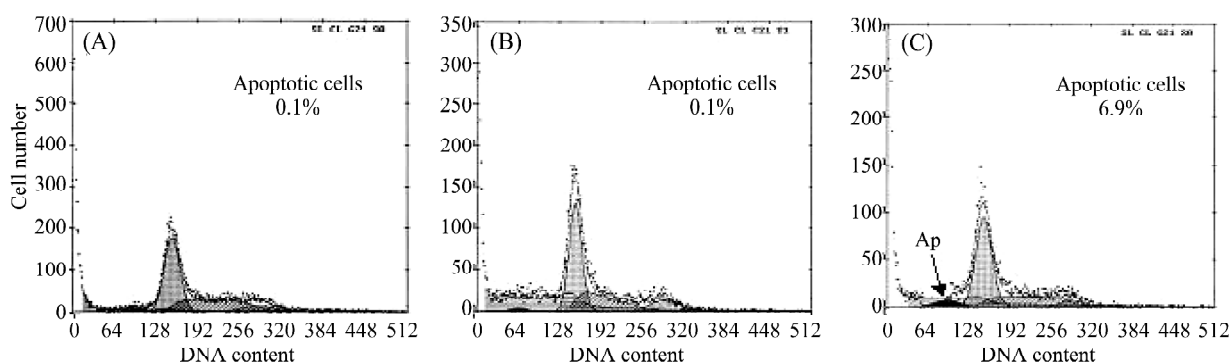
### Discussion

Recently, with the development of molecular biology and its application in oncology, it has been recognized that the activation of oncogenes or the inactivation of cancer suppressor genes plays a great role in the development and progression of cancer. According to published studies, somatic mutations or deletion of cancer suppressor genes such as *TP53* and *p21* were closely correlated to the occurrence and development of tumor [9]. *PTEN* is a major tumor suppressor gene identified on human chromosome 10q23 which encodes a protein of 403 amino acids that includes a phosphatase core motif and two potential tyrosine phosphate acceptor motifs. It is considered as a candidate tumor suppressor gene based on the finding that mutation or loss of this gene has been linked to a variety of common human cancers, including breast, prostate, and brain cancer. *PTEN* is frequently deleted or mutated in a wide range of human tumors and tumor cell lines such as glioblastoma and melanoma, and lymphoid, lung, and endometrial cancers. Furthermore, germline *PTEN*



**Fig. 4** Fluorescence micrographs of Hoechst 33258 stained ZR-75-1 breast cancer cells after transfection with plasmid pBP-wt-*PTEN* or pBP-G129R-*PTEN* for 48 h

(A) Untreated group. (B) pBP-wt-*PTEN* (expressing wild-type *PTEN*). (C) pBP-G129R-*PTEN* (expressing mutant *PTEN*). The arrows point to apoptotic cells (400×).



**Fig. 5** Flow cytometry analysis of ZR-75-1 breast cancer cells after transfection with plasmid pBP-wt-*PTEN* or pBP-G129R-*PTEN* for 48 h

(A) Untreated group. (B) pBP-G129R-*PTEN* (expressing mutant *PTEN*). (C) pBP-wt-*PTEN* (expressing wild-type *PTEN*). Ap, apoptotic peak.

mutations have been found in patients with juvenile polyposis coli, Cowden's disease, a multiple hamartoma syndrome with a high risk of breast and thyroid cancer, and the related hamartomatous polyposis syndrome, Cowden's syndrome [10], suggesting that inactivation of *PTEN* plays an important role in tumorigenicity.

In this study we have examined the effect of overexpression of *PTEN* on the growth and apoptosis of *PTEN*-null ZR-75-1 cells. Our results have shown an obvious correlation between the number of apoptotic cells, observed by Hoechst 33258 staining and flow cytometry analysis, and the increased level of *PTEN* mRNA, determined by semiquantitative RT-PCR and Western blot analysis. The change in the mRNA and protein levels in pBP-wt-*PTEN*-transfected cells was obvious, and apoptotic cells were also observed in the same group of cells 48 h after transfection. This strongly suggests that overexpression of *PTEN* by transfection is responsible for the apoptosis in ZR-75-1 cells. The data from semiquantitative RT-PCR and Western blot analysis also show a parallel or corresponding change between *PTEN* mRNA and *PTEN* protein in the pBP-wt-*PTEN*-transfected cells. Furthermore, our flow cytometry results also show that overexpression of *PTEN* in ZR-75-1 cells caused growth suppression mediated initially by G<sub>1</sub> arrest, followed by cell death, in agreement with previous reports for other cancers [11].

In the present study, both pBP-wt-*PTEN* plasmid and a phosphatase-inactivating type plasmid pBP-G129R-*PTEN* were used to transfect cells to provide a helpful control. According to our results, overexpression of *PTEN* was found in the pBP-G129R-*PTEN*-transfected group, but apoptotic cells were nearly absent, and no detectable change in the cell cycle was observed compared with the

untreated group, indicating that the phosphatase activity of *PTEN* is required for the inhibition of cell growth and induction of apoptosis [12,13].

Our finding that overexpression of *PTEN* induced cell growth arrest and apoptosis in the ZR-75-1 cell line suggests a role for *PTEN* as a tumor suppressor gene in the prevention and treatment of breast cancer. Our data also support the notion that *PTEN* might be a new target for cancer gene therapy.

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