Basic Research Paper

Impact of PTEN gene knockout in human breast carcinoma MCF-7 cells on activity of JNK pathway

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Key words: breast neoplasm, JNK pathway, PTEN, MCF-7 cell

Background and Objective: PTEN plays a critical role in the development and progression of many cancers. PTEN may act directly or indirectly to integrate complex signal network systems, to influence downstream signal pathways and target molecules. This study investigates the impact of PTEN gene knockout in human breast carcinoma MCF-7 cells on the activity of the JNK pathway. Methods: MCF-7 cells were transfected with PTEN antisense oligonucleotide, or treated with 10 μmol/L SP600125, or received both transfection and SP600125 treatment. The expression of PTEN protein was detected by confocal spectral microscopy. The early stage cell apoptosis and cell cycle were detected by flow cytometry (FCM). The proliferation of MCF-7 cells was determined by MTT assay. The phosphorylated JNK and downstream substrate ATF-2, C-Jun protein were assayed by western blot. Results: The expression of PTEN protein in MCF-7 cells was effectively blocked by PTEN antisense oligonucleotide. Early apoptosis of MCF-7 cells was induced by SP600125 after knocking down PTEN, with an early apoptosis rate of (32.4 ± 2.4)%; MCF-7 cells were arrested at G1 phase. The proliferation rate was significantly lower in the combination group than in the SP600125 group and the antisense oligonucleotide group (p < 0.05). The phosphorylation levels of JNK, ATF-2 and C-Jun were downregulated in the combination group. Conclusions: PTEN plays an important role in the activation of the JNK pathway in MCF-7 cells. Loss of PTEN results in the activation of the JNK pathway in MCF-7 cells, and improves sensitivity of MCF-7 cells to JNK inhibitor.

Breast cancer is the most common malignant tumor in women.1 To date, the molecular mechanism in the development and progression of breast cancer has not yet been fully clarified. Studies show that the generation of breast cancer is often accompanied by PTEN protein expression as well as decreased or lost expression of this protein.2-3 The JNK pathway plays a critical role in cell differentiation and apoptosis as well as the development and progression of many human diseases.4-6 Some studies have indicated that the activation of the JNK pathway can be used as a marker for breast cancer progression.7 In this study, we knocked out the PTEN gene by antisense oligonucleotide in human breast carcinoma MCF-7 cells and explored the effect of PTEN expression or knockdown on the JNK pathway in these cells. The results obtained provide an important theoretical basis for both clinical guidance and the treatment of JNK pathway-targeted therapy of breast cancer patients.

Materials and Methods

Materials. MCF-7 cells (PTEN+) were purchased from the China Center for Type Culture Collection. JNK inhibitor SP600125 was purchased from Biomol. Rabbit anti-human p-JNK and p-ATF-2 polyclonal antibodies as well as mouse anti-human p-C-Jun monoclonal antibody were purchased from CST Company. Mouse anti-PTEN monoclonal antibody was purchased from Santa Cruz Biotechnology. PTEN antisense oligonucleotide sequence 5'-TTT GAT GAT GGC TGT CAT-3', complementary to the sequence located at position 1–18 of PTEN mRNA translation initiation region, was synthesized by Shanghai Sangon Biological Engineering Technology & Services.

Methods. Cell culture. MCF-7 cells were cultured in DMEM with 10% fetal calf serum at 37°C in humidified air containing 5% CO2.

Transfection with PTEN antisense oligonucleotide and intervention with JNK inhibitor SP600125. MCF-7 cells were divided into four groups, namely, untreated MCF-7 cells, MCF-7 cells treated with SP600125 (SP600125 group), MCF-7 cells infected with PTEN antisense oligonucleotide (antisense oligonucleotide group) as well as those infected with PTEN antisense oligonucleotide and treated with SP600125 (combination group).
MCF-7 cells in the antisense oligonucleotide group were treated as follows. Ten microliters of oligonucleotide (10 μmol/L) and 100 μL of OPTI MEM medium were mixed in one tube (tube A) while 10 μL of liposomal suspension and 100 μL of OPTI MEM medium were mixed in another tube (tube B). The liquids in tubes A and B were then mixed together and placed at room temperature for 30 min. MCF-7 cells at 70% confluence were washed twice with a serum-free medium. After being added with the above-mentioned mixtures and brought to a final volume of 2 mL with a serum-free medium, cells were placed in an incubator for four to six hours. The complete medium was then added. After an overnight culture, SP600125 (10 μmol/L) was further added to MCF-7 cells in the combination and cultured for 24 hours.

Detection of PTEN protein expression by confocal spectral microscopy. MCF-7 cells were harvested and seeded in multi-well plates at a density of 2.5 x 10⁴ cells per well. After putting poly-L-lysine-treated coverslips into the wells, cells were cultured for 24 h and culture fluids were decanted. Cells were then fixed in pre-cooled 100% methanol at -20°C for 5 min, washed three times with 0.2% Triton-PBS and permeabilized with 0.5% Triton-PBS. After being washed three times again with 0.2% Triton-PBS, cells were blocked with 5% BSA for 1 h and incubated overnight with anti-PTEN monoclonal antibody (1:250) at 4°C. Cells were subsequently washed with 0.2% Triton-PBS for 3 x 10 min and incubated with goat anti-mouse IgG-FITC (1:1,000) for 1 h (in a dark place) at room temperature. After being washed again with 0.2% Triton-PBS for 3 x 10 min and treated with PI for nuclear staining for 15 min, cells were mounted and subjected to observation and photography under confocal spectral microscopy.

Flow cytometry analysis of the cell cycle and apoptosis. Cells in each group were digested, resuspended in 0.01 mol/L PBS and fixed overnight with ice ethanol. After RNAase A was added to a final concentration of 50 mg/L, cells were incubated for 1 h in a water bath at 37°C. Propidium iodide (PI) was then added and incubated overnight with anti-PTEN monoclonal antibody (1:250) at 4°C. Cells were subsequently washed with 0.2% Triton-PBS for 3 x 10 min and incubated with goat anti-mouse IgG-FITC (1:1,000) for 1 h (in a dark place) at room temperature. After being washed again with 0.2% Triton-PBS for 3 x 10 min and treated with PI for nuclear staining for 15 min, cells were mounted and subjected to observation and photography under confocal spectral microscopy.

Results

Expression of PTEN protein in MCF-7 cells in each group. The observations under a confocal spectral microscope showed that PTEN protein was positively expressed in MCF-7 cells, predominantly in the cytoplasm. Compared to untreated MCF-7 cells and MCF-7 cells in the SP600125 group, the expressions of PTEN protein in MCF-7 cells in the antisense oligonucleotide group and combination group were significantly suppressed (Fig. 1).

Comparison of the apoptosis rate and cell cycle distribution of MCF-7 cells in each group. In the combination group, MCF-7 cells showed significant early apoptosis and G1 phase arrest, and the early apoptosis rate and the ratio of cells in G1 phase were (32.4 ± 2.4)% and (85.4 ± 4.1)%, respectively, showing significant differences (p < 0.05) when compared with those observed in MCF-7 cells in the antisense oligonucleotide group ([6.3 ± 0.9]%) and (61.1 ± 3.7)%, respectively] and SP600125 group ([5.6 ± 1.1]%) and (59.6 ± 4.2)%, respectively]. In contrast, the early apoptosis rate and the ratio of cells in G1 phase showed no significant difference (p > 0.05) between untreated MCF-7 cells and MCF-7 cells in the antisense oligonucleotide group and SP600125 group (Fig. 2 and Table 1).

Inhibitory effects on the in vitro proliferation of MCF-7 cells in each group. After MCF-7 cells in each group were further cultured for 24, 48 and 72 h, respectively, the proliferation of MCF-7 cells in the combination group was markedly and time-dependently inhibited (especially prominent in cells further cultured for 48 and 72 h), showing significant differences...
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(p < 0.01) when compared with those observed in the SP600125 group and antisense oligonucleotide group. In contrast, no significant difference (p > 0.05) in the inhibitory effects on cell proliferation was noted between untreated MCF-7 cells and MCF-7 cells in the antisense oligonucleotide group and SP600125 group (Fig. 3). Expression of phosphorylated JNK, ATF-2 and C-Jun proteins in MCF-7 cells in each group. After knockout of the PTEN gene in the antisense oligonucleotide group, the phosphorylation levels of JNK, ATF-2 and C-Jun proteins in MCF-7 cells were elevated significantly. In contrast, the phosphorylation levels of these proteins in untreated MCF-7 cells and MCF-7 cells in the SP600125 group were lower. After MCF-7 cells in the combination group were treated with SP600125 for 24 h, the phosphorylation levels of JNK and its downstream substrate ATF-2 and C-Jun proteins were significantly downregulated (Fig. 4).

Discussion

Studies show that PTEN may act directly or indirectly to integrate the complex signal network system. Until now, numerous studies have proved that the PTEN gene plays an important role in the integration of multiple signal pathways and has an impact on target molecules and downstream signal cascades. When PTEN shows structural or functional abnormalities and is not able to effectively control downstream effectors, tumorigenesis is often enhanced even without exogenous stimulation. A variety of signal transduction proteins are encoded by oncogenes or tumor suppressor genes, whose abnormalities are closely related to the development and progression of certain diseases, especially tumors. Another example of oncogene activation involves protein kinases. These kind of enzymes are involved in multiple cell activities, particularly those that can initiate cell cycle progression and regulate other functions through transmitting signals from the cell membrane to the nucleus. PTEN is capable of regulating cell proliferation, differentiation and apoptosis through negatively regulating the MAPK pathway. The JNK pathway is one of the important branch pathways of the MAPK pathway. Its major steps can be roughly summarized as: stresses → germinal center kinase (GCK) → MEKK → SEK1 → JNK → growth arrest and apoptosis. Some studies show that JNK pathway activation is associated with apoptosis-promoting activity in many situations.

Table 1  Cell cycle of MCF-7 cells after different treatments detected by flow cytometry

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell proportion (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G0/G1</td>
<td>S</td>
</tr>
<tr>
<td>Control</td>
<td>53.3±1.5</td>
<td>29.2±2.0</td>
</tr>
<tr>
<td>SP600125</td>
<td>59.6±4.2</td>
<td>24.6±1.8</td>
</tr>
<tr>
<td>PTEN antisense oligonucleotide</td>
<td>61.1±3.7</td>
<td>24.8±1.7</td>
</tr>
<tr>
<td>PTEN antisense oligonucleotide combined SP600125</td>
<td>85.4±4.1</td>
<td>8.0±1.9</td>
</tr>
</tbody>
</table>

All values are presented as mean ± SD of three experiments. *p < 0.05, vs. SP600125 group, antisense oligonucleotide group and control group.
sensitivity of tumor cells to JNK inhibitor. Western blot analysis indicated that the abnormal activation of the JNK pathway was related to the knockout of PTEN. Moreover, the phosphorylation levels of ATF-2 and C-Jun proteins, two downstream substrates of JNK, were also related to PTEN expression. SP600125 could significantly downregulate the phosphorylation levels of JNK protein and its substrates (ATF-2 and C-Jun proteins) in MCF-7 cells with PTEN gene knocked out. The observation that SP 600125 could inhibit not only the expression of c-Jun protein but also the expression of JNK protein may be due to the inhibitory effect of SP600125 on the phosphorylation of JNK downstream gene MKK4. After analyzing these results, we speculate that PTEN knockout in MCF-7 cells can activate the JNK pathway via a mechanism that may be associated with a loss of negative regulatory impact of PTEN on the JNK pathway and resulting changes in the transcription of JNK downstream target genes. The observation that the expression levels of PTEN were related with the sensitivity of MCF-7 cells to JNK pathway inhibitor suggests that JNK plays a key role in the regulation of the PTEN/PI3K pathway. For this reason, JNK is a potential target for the treatment of breast cancer.

PTEN is the point of convergence of multiple signaling pathways and is involved in multiple antitumor effects such as anti-invasion, anti-proliferation and anti-metastasis. Therefore, our future studies will focus on clarifying the intermediate steps that result in ultimate effects and elucidate the relationship among various signaling pathways.

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For these reasons, in our study, we used antisense oligonucleotide technology to knock out the PTEN gene in MCF-7 cells expressing high-level PTEN and observed the impact of JNK inhibitor SP600125 on MCF-7 cells with a knockout of the PTEN gene. We found that, after MCF-7 cells with an effective knockout of the PTEN gene were treated with JNK inhibitor SP600125, their growth was significantly suppressed, indicating that the sensitivity of MCF-7 cells to SP600125 was significantly enhanced. The underlying mechanism is associated with SP600125-induced significant increase in early apoptosis and G1 phase arrest. This result is similar to the conclusion drawn by Vivanco that a decrease in PTEN expression could enhance the sensitivity of tumor cells to JNK inhibitor. Western blot analysis indicated that the abnormal activation of the JNK pathway was related to the knockout of PTEN. Moreover, the phosphorylation levels of ATF-2 and C-Jun proteins, two downstream substrates of JNK, were also related to PTEN expression. SP600125 could significantly downregulate the phosphorylation levels of JNK protein and its substrates (ATF-2 and C-Jun proteins) in MCF-7 cells with PTEN gene knocked out. The observation that SP 600125 could inhibit not only the expression of c-Jun protein but also the expression of JNK protein may be due to the inhibitory effect of SP600125 on the phosphorylation of JNK downstream gene MKK4. After analyzing these results, we speculate that PTEN knockout in MCF-7 cells can activate the JNK pathway via a mechanism that may be associated with a loss of negative regulatory impact of PTEN on the JNK pathway and resulting changes in the transcription of JNK downstream target genes. The observation that the expression levels of PTEN were related with the sensitivity of MCF-7 cells to JNK pathway inhibitor suggests that JNK plays a key role in the regulation of the PTEN/PI3K pathway. For this reason, JNK is a potential target for the treatment of breast cancer.

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**References**


