Original Article
AKT2 expression changes impact on invasive ability of breast cancer cells

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Abstract: AKT2 plays an important role in PI3K signaling pathway and directly relates to cell growth. Study showed that AKT2 was associated with tumor occurrence, growth, and invasion. This research aimed to detect AKT2 expression impact on breast cancer cell invasive ability, to provide support for clinical prognosis. AKT2 overexpression vector was constructed using pEGFP-N1 as the carrier and transfected to breast cancer cell line by liposome. RT-PCR and Western blot were applied to test AKT2 expression at 12 h, 16 h, and 24 h after transfection, respectively. Transwell assay was performed to determine cell invasion. AKT2 overexpression vector pEGFP-AKT2 and inhibitory vector AKT2-sh-RNA were successfully constructed. After pEGFP-AKT2 transfection, AKT2 expression increased significantly, and cell invasive ability also enhanced. AKT2-sh-RNA transfection declined AKT2 level and reduced breast cancer cell invasive ability. AKT2 expression changes had certain relationship with breast cancer cell invasive ability. AKT2 overexpression may affect breast cancer prognosis.

Keywords: AKT2, breast cancer cell, transwell assay, cell transfection

Introduction

Breast cancer is a common malignant tumor with the highest incidence among female tumors. There are more than 1 million new cases every year around the world. Our country is also one of the high incidence areas [1, 2]. For breast cancer has various complicated pathogenesis, including genetic factor, environmental factor, psychological factor, and work stress, etc. Its occurrence presents younger trend that brought great burden to society productivity and family. At present, searching for effective treatment and prediction method of breast cancer is the hotspot [3].

Following the development of biological technology, especially the progress of human genomics, people awareness of the disease gradually transferred to genetic level. A variety of genes closely related to disease have been discovered and their mechanism has been elucidated. Meanwhile, targeted therapy also achieved certain progress in clinical treatment [4, 5]. Research suggested that every disease had various genes expression changes. Thus, study the relationship between gene expression and the disease is one of the methods of precision medicine [6].

We selected AKT2 in this study that belongs to AKT family (protein kinase B). As a major kinase of anti-oxidative stress, AKT protein kinase family is an important cell survival promoting factor. It has critical role in cell apoptosis, cell proliferation, cell differentiation, physiological metabolism, senescence, disease, and cancer. The family mainly includes AKT1, AKT2, and AKT3. The former two genes have highly conservative region and similar protein primary structure [7, 8]. AKT2 is the main subtype of AKT, and also is confirmed to have a direct relationship with oncogenesis. Therefore, investigate the relationship between AKT2 expression and breast cancer cell invasive ability can provide new targets for breast cancer clinical gene therapy [9-11]. This study constructed AKT2 overexpression vector based on pEGFP-N1 carrier and transfected AKT2 overexpression vector and AKT2-sh-RNA vector to MCF-7 using liposome in vitro cell transfection technology, respectively. RT-PCR and Western blot were applied to test
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**Materials and methods**

**General information**

Breast cancer cell line MCF-7 was purchased from Shanghai Huayan Bio-Technology co., LTD. RPMI 1640, trypsin, fetal calf serum, and PBS were got from Gibco. Flask and dish were bought from Corning. Penicillin-streptomycin and Western blot related reagents were from Beyotime. Transwell chambers and Matrigel were from BD. Liposome transfection kit was from Invitrogen. AKT2 antibody was produced by Xinlebio. RNA extraction kit (Trizol) and cDNA synthesis kit were from TAKARA. PCR amplification reagents were from Promega. Primers used in the experiment were from Invitrogen. Gene sequencing was performed by BGI. Restriction enzyme and T4 DNA ligase were purchased from NEB. Plasmid extraction kit was from Omega.

**Vector construction**

AKT2 sh-RNA vector was designed and verified by Chongqing Western CO., LTD. AKT2 primer and restriction enzyme cutting site were designed based on CDS sequence published by NCBI and pEGFP-N1 carrier frame sequence. XhoI was added to the upstream, while BamHI enzyme loci was added to downstream. The primers used were as follows: upstream (5’-3’): atgaagaccgagaggccgcggac, downstream (5’-3’): gctcggagatgctggccgagtaggag. The product length was 1260 bp. PCR amplification condition contained 94°C for 3 min, followed by 30 cycles of 94°C for 35 s, 58.7°C for 35 s, and 68°C for 1.5 min, and 68°C for 10 min at last. Agarose gel electrophoresis was performed to recycle the target band. The band together with pEGFP-N1 carrier was double enzyme digested overnight by XhoI and BamHI, and then the enzyme-digested products at 1260 bp and 4700 bp were collected. Next, the products were connected by T4 DNA ligase at 4°C overnight. The connection system included: 1 μl enzyme, 1 μl 10×Buffer, 2 μl carrier recycled product, 4 μl AKT2 PCR recycled product, and 2 μl ddH2O. Then it was converted to Dh5α competent cells in medium containing kanamycin overnight. Monoclonal bacteria was picked to perform PCR verification and sequenced to exclude mutation.

**Cell culture and transfection**

MCF-7 in store was water bathed at 37°C for 5 min and centrifuged at 1000 rpm/min for 3 min. Then the cells were cultured in RPMI 1640 medium containing 10% FBS and passaged after 24 h. Liposome transfection was performed at 16 h after cell adherence according to the kit introduction. Each group had three repeats.

**RNA and protein extraction and detection**

**Total RNA and protein were extracted after cell transfection**

RNA extraction: The cells were washed by pre-cooled PBS for 2-3 times and treated by 1 ml Trizol for 5 min. Then the cells were moved to an EP tube and added with 0.2 ml chloroform. After centrifuged at 15000 g and 4°C for 15 min, the upper layer was moved to a new EP tube and added with equal amount of isopropanol for 10 min. After centrifuged at 15000 g and 4°C for 15 min, the supernatant was removed and the RNA was treated washed by 75% ethanol twice. After centrifuged at 15000
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Figure 2. Cell culture and transfection (10×). A. Normal MCF-7; B. 12 h after pEGFP-AKT2 transfection; C. 12 h after AKT2-sh-RNA and pEGFP-N1 co-transfection.

Table 1. RT-PCR detection gray level

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal</th>
<th>Co-inhibition</th>
<th>pEGFP-AKT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT-2</td>
<td>890</td>
<td>332</td>
<td>2664</td>
</tr>
<tr>
<td>β-actin</td>
<td>324</td>
<td>348</td>
<td>340</td>
</tr>
</tbody>
</table>

Figure 3. PCR detection of AKT-2 after transfection. 1, normal AKT2; 2, AKT2 expression after pEGFP-AKT2 transfection; 3, AKT2 expression after AKT2-sh-RNA and pEGFP-N1 co-transfection.

Figure 4. RT-PCR gray level analysis. *P<0.05, **P<0.01, compared with co-inhibition group.

Figure 5. Western blot detection of AKT2 expression in transfected cells.

Figure 6. Western blot results analysis. *P<0.05, **P<0.01, compared with co-inhibition group.

Supernatant was moved to a new EP tube. The protein was quantified by BCA and stored at -80°C.

1 μg RNA was used for RT-PCR, while 40 μg protein was used for Western blot detection. β-actin was chose as internal reference.

Transwell assay

The upper chamber of Transwell chamber (bore diameter 8 μm) was coated by Matrigel (BD). 70 μl Matrigel was added to the chamber and incubated at 37°C for 60 min. Cell suspension in FBS-free medium containing BSA was added to the upper chamber at 1×10⁵/ml, whereas 1000 μl complete medium was added to the lower
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chamber. After transfection, the chamber was stained by 0.1% crystal violet for 15 min and photographed under inverted microscope. Three different fields of view were randomly selected under 100× light microscope to calculate the cell number.

Results

Vector construction

AKT2 CDS sequence was successfully obtained by PCR amplification. Agarose gel electrophoresis results were showed in Figure 1. After double enzyme digestion and connected to pEGFP-N1 carrier, the PCR fragment was sequenced by BGI and compared in NCBI blast. The results showed that two fragments sequence appeared mutation, which was in accordance with NCBI published sequence. The plasmid was stored at -80°C.

Cell culture and transfection

MCF-7 cells were unfrozen and cultured in incubator. As shown in Figure 2, the cells appeared adherence phenomenon after 4-5 h. 5 μg pEGFP-AKT2 plasmid was transfected to MCF-7 cells, while AKT2-sh-RNA and pEGFP-N1 vector were co-transfected to MCF-7 cells after mixing. Green fluorescence confirmed transfection success (Figure 2).

RT-PCR detection of AKT2 expression

Total RNA was extracted from cells and reverse transcribed to cDNA for PCR detection. Image J software was used for data analysis and β-actin was treated as internal reference. As shown in Table 1, Figures 3 and 4, AKT2 expression significantly increased to 2.78 times after pEGFP-AKT2 transfection compared with normal control, while it reduced to 0.36 time after AKT2-sh-RNA and pEGFP-N1 co-transfection. It suggested that vector plays proper role after transfection.

Western blot detection of AKT2 expression

Western blot was applied to test AKT2 expression in transfected cells. As shown in Figures 5 and 6, AKT2 protein expression was similar to RT-PCR results. AKT2 expression significantly increased to 3.36 times after pEGFP-AKT2 transfection compared with normal control (P<0.01), while it reduced to 0.42 time after AKT2-sh-RNA and pEGFP-N1 co-transfection (P<0.05). It indicated that vector plays a proper role after transfection to regulate AKT2 expression.

Transwell Boyden assay detection of cell invasive ability

The chamber was incubated in 37°C and 5% CO₂ for 12, 18, or 24 h, respectively. Then the chamber was stained by 0.1% crystal violet for 15 min and photographed under inverted microscope. Three fields of view were randomly selected to count the transmembraned cell number. pEGFR-AKT2 group presented significant difference with normal control and co-inhibition group (Figure 7).

In conclusion, tumor makes the body gradually losing its normal regulation function especially in gene level, leading to local cell abnormal proliferation or differentiation. It forms neoplasm under in vivo and in vitro factors [12-14]. Once formed, neoplasm will not stop growth because of carcinogenic factors elimination. It is featured as rapid growth and out of control, further destroying adjacent normal tissues and organs, namely the cancer cells invasion [15, 16].

Breast cancer also complies with the above laws. Abnormal gene regulation may result in
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cancer cell proliferation and metastasis that lead to serious consequences [17, 18]. Therefore, study invasion related gene expression and clarify its relationship with cancer cell invasion has great significance [19, 20]. Our study selected AKT2 gene that has been confirmed as cell survival promoting factor in the body. It plays an important role in cell apoptosis, proliferation, differentiation, physiological metabolism, senescence, and cancer. It also has certain impact on oncogenesis, invasion, and metastasis. As the main subtype of protein kinase B, AKT2 is validated to relate to tumorigenesis. Thus, explore the relationship between AKT2 expression and breast cancer cell invasive ability, and further clarify the association between AKT2 and tumorigenesis can provide new target for breast cancer gene therapy. Our results revealed that AKT2 expression was successfully controlled in MCF-7 cells in vitro. Further investigation showed that AKT2 overexpression can improve MCF-7 invasive ability, while its level inhibition may weaken MCF-7 invasion. It suggested that AKT2 has a role in regulating MCF-7 cell invasion.

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Disclosure of conflict of interest

None.

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References


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