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. AKE2 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
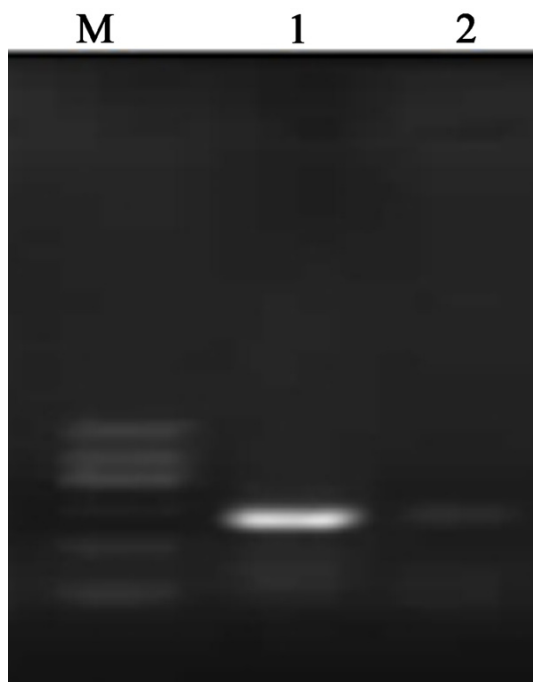
Figure 1. M, DNA marker; 1 and 2, AKT2 CDS PCR amplification fragment.

AKT2 expression at 12 h, 16 h, and 24 h after transfection, respectively. Transwell assay was performed to determine cell invasion.

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 T4DNA 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 BamH I enzyme loci was added to downstream. The primers used were as follows: upstream (5'-3'): atgaagaccgagaggccgcgac, downstream (5'-3'): gctcgcggatgctggccgagtaggag. 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 BamH I, 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 ddH$_2$O. 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 precooled 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
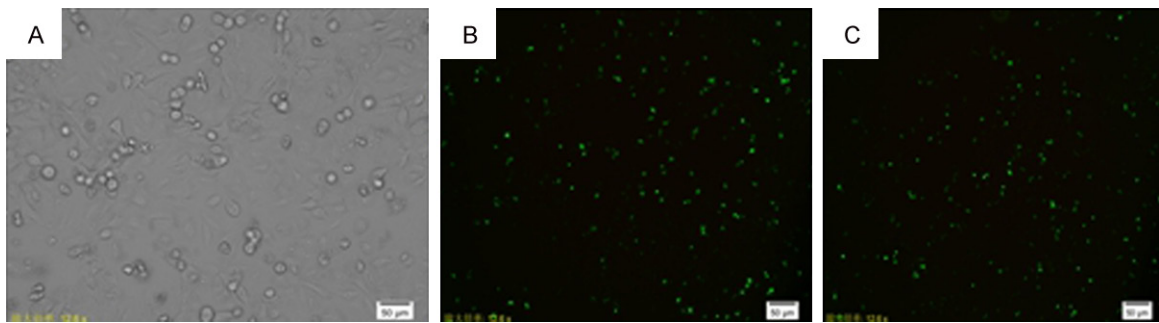
Figure 2. Cell culture and transfection (10×). A. Normal MCF-7; B. 12 h after pEGFP-AKT2 transfection; C. 12 h after AKT2-sh-RNA and pEGFP-N1 co-transfection.

Table 1. RT-PCR detection gray level

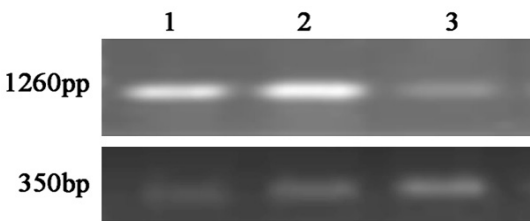| Group	Normal	Co-inhibition	pEGFP-AKT2
AKT-2	890	332	2664
β-actin	324	348	340

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.
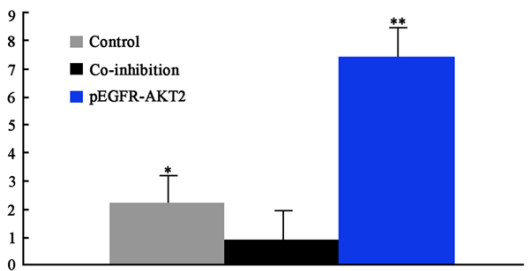

Figure 4. RT-PCR gray level analysis. *P<0.05, **P<0.01, compared with co-inhibition group.

Figure 5. Western blot detection of AKT2 expression in transfected cells.
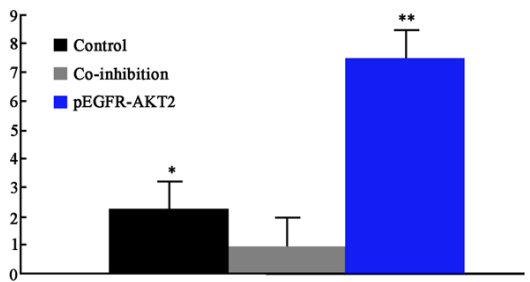
Figure 6. Western blot results analysis. *P<0.05, **P<0.01, compared with co-inhibition group.

g and 4°C for 5 min, the RNA was solved in 20 μl DEPC water after dried for 10 min. RNA concentration was measured, and RNA was stored at -80°C.

Protein extraction: The cells were washed by PBS after transfection and added with 50 μl RIPA. Then the cells were collected to an EP tube and vortexed for 3-4 times. After ce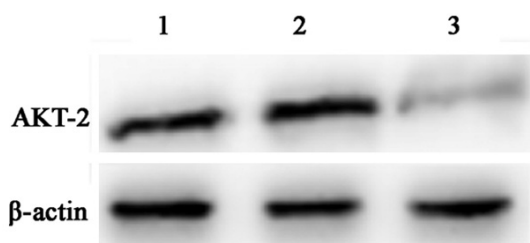ntrifuged at 14000 g and 4°C for 15 min, the supernatant was moved to a new EP tube. The protein was quantified by BCA and stored at -80°C.

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 $1×10^5$/ml, whereas 1000 μl complete medium was added to the lower

Figure 7. Comparison of transmembraned cell numbers. *P<0.05, **P<0.01, compared with co-inhibition group.

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% CO_2 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

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 unfrezen 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 transcripted to cDNA for PCR detection. Image J software was used for data analysis and

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

[1] Berardi DE, Flumian C, Rodriguez CE, Diaz Bessone MI, Cirigliano SM, Bal de Kier Joffe ED, Fiszman GL, Urtreger AJ, Todaro LB. PKCdelta Inhibition Impairs Mammary Cancer Proliferative Capacity But Selects Cancer Stem Cells, Involving Autophagy. J Cell Biochem 2016; 117: 730-40.

[2] Daswani VP, Ayesa U, Venegas B, Chong PL. Concentration-induced j-aggregate formation causes a biphasic change in the release of trans-combretastatin a4 disodium phosphate from archaeosomes and the subsequent cytotoxicity on mammary cancer cells. Mol Pharm 2015; 12: 3724-3734.

[3] Gruppi F, Hejazi L, Christov PP, Krishnamachari S, Turesky RJ, Rizzo CJ. Characterization of nitrogen mustard formamidopyrimidine adduct formation of bis(2-chloroethyl)ethylamine with calf thymus DNA and a human mammary cancer cell Line. Chem Res Toxicol 2015; 28: 1850-1860.

[4] Song H, Jung JI, Cho HJ, Her S, Kwon SH, Yu R, Kang YH, Lee KW, Park JH. Inhibition of tumor progression by oral piceatannol in mouse 4T1 mammary cancer is associated with decreased angiogenesis and macrophage infiltration. J Nutr Biochem 2015; 26: 1368-78.

[5] Accornero P, Miretti S, Bersani F, Quaglino E, Martignani E, Baratta M. Met receptor acts uniquely for survival and morphogenesis of EGFR-dependent normal mammary epithelial and cancer cells. PLoS One 2012; 7: e44982.

[6] Arumugam A, Parada J, Rajkumar L. Mammary cancer promotion by ovarian hormones involves IGFR/AKT/mTOR signaling. Steroids 2012; 77: 791-797.

[7] Ayoub NM, Akl MR, Sylvester PW. Combined gamma-tocotrienol and Met inhibitor treatment suppresses mammary cancer cell proliferation, epithelial-to-mesenchymal transition and migration. Cell Prolif 2013; 46: 538-553.

[8] Cotrim CZ, Fabris V, Doria ML, Lindberg K, Gustafsson JA, Amado F, Lanari C, Helguero LA. Estrogen receptor beta growth-inhibitory effects are repressed through activation of MAPK and PI3K signalling in mammary epithelial and breast cancer cells. Oncogene 2013; 32: 2390-2402.

[9] Fan H, Guan JL. Compensatory function of Pyk2 protein in the promotion of focal adhesion kinase (FAK)-null mammary cancer stem cell tumorigenicity and metastatic activity. J Biol Chem 2011; 286: 18573-18582.

[10] Feigin ME, Akshinthala SD, Araki K, Rosenberg AZ, Muthuswamy LB, Martin B, Lehmann BD, Berman HK, Pietenpol JA, Cardiff RD, Muthuswamy SK. Mislocalization of the cell polarity protein scribble promotes mammary tumorigenesis and is associated with basal breast cancer. Cancer Res 2014; 74: 3180-3194.

[11] Furth PA, Nakles RE, Millman S, Diaz-Cruz ES, Cabrera MC. Signal transducer and activator of transcription 5 as a key signaling pathway in normal mammary gland developmental biology and breast cancer. Breast Cancer Res 2011; 13: 220.

[12] Jones PH, Mahauad-Fernandez WD, Madison MN, Okeoma CM. BST-2/tetherin is overexpressed in mammary gland and tumor tissues in MMTV-induced mammary cancer. Virology 2013; 444: 124-139.

[13] Lamartiniere CA, Jenkins S, Betancourt AM, Wang J, Russo J. Exposure to the endocrine disruptor bisphenol A alters susceptibility for mammary cancer. Horm Mol Biol Clin Investig 2011; 5: 45-52.

[14] Li J, Cho YY, Langfald A, Carper A, Lubet RA, Grubbs CJ, Ericson ME, Bode AM. Lapatinib, a preventive/therapeutic agent against mammary cancer, suppresses RTK-mediated signaling through multiple signaling pathways. Cancer Prev Res (Phila) 2011; 4: 1190-1197.

[15] Lyu H, Huang J, Edgerton SM, Thor AD, He Z, Liu B. Increased erbB3 promotes erbB2/neu-driven mammary tumor proliferation and co-targeting of erbB2/erbB3 receptors exhibits potent inhibitory effects on breast cancer cells. Int J Clin Exp Pathol 2015; 8: 6143-6156.

[16] Miwa HE, Koba WR, Fine EJ, Giricz O, Kenny PA, Stanley P. Bisected, complex N-glycans and galectins in mouse mammary tumor progression and human breast cancer. Glycobiology 2013; 23: 1477-1490.

[17] Nakles RE, Kallakury BV, Furth PA. The PPARgamma agonist efatutazone increases the spectrum of well-differentiated mammary cancer subtypes initiated by loss of full-length BRCA1 in association with TP53 haploinsufficiency. Am J Pathol 2013; 182: 1976-1985.

[18] Shibata MA, Iinuma M, Morimoto J, Kurose H, Akamatsu K, Okuno Y, Akao Y, Otsuki Y. alpha-mangostin extracted from the pericarp of the mangosteen (garcinia mangostana linn) reduces tumor growth and lymph node metastasis in an immunocompetent xenograft model of metastatic mammary cancer carrying a p53 mutation. BMC Med 2011; 9: 69.

[19] Thompson MD, Mensack MM, Jiang W, Zhu Z, Lewis MR, McGinley JN, Brick MA, Thompson HJ. Cell signaling pathways associated with a reduction in mammary cancer burden by dietary common bean (phaseolus vulgaris L.). Carcinogenesis 2012; 33: 226-232.

[20] Tiwari RV, Parajuli P, Sylvester PW. γ-Tocotrienol-induced autophagy in malignant mammary cancer cells. Exp Biol Med (Maywood) 2014; 239: 33-44.