The role and correlation analysis of PI3K/AKT pathway in breast carcinoma with lymph node metastasis

Ying Wang1, Xiangguo Dang1, Meng Zhang1, Changran Wei2, Xiangqi Li2


INTRODUCTION

Extensive dissemination of primary breast cancer is the leading cause of death, and axillary lymph nodes is the first step in extensive metastasis, lymph node metastasis has become one of the main criteria for the prognosis of breast cancer patients and treatment options [1]. Clinical experiments show that tumor cells migrating to lymph nodes must pass lymphatic vessels and new lymphangiogenesis process and greatly promote the lymphangiogenesis of tumor. The key protein that induces lymphangiogenesis is vascular endothelial growth factor receptor 3 (VEGFR-3), which is activated by vascular endothelial growth factor C and D (VEGF-C and VEGF-D), creating a new favorable environment for lymphangiogenesis [2]. Phosphatidylinositol 3-kinase/serine protein kinase B (PI3K/AKT) signaling pathway is dysregulated in most human tumors and regulates tumor cell proliferation and apoptosis and is involved in IGF-1 induction VEGF-C up-regulation is an important role in the process of lymphatic metastasis of breast cancer [3]. AKT2 is one of the isoform of AKT, as an oncogene, AKT2 protein is overexpressed in many tumor tissues [4]. However, the mechanism of the interaction between PI3K/AKT expression in breast cancer and lymphatic metastasis is not completely understood. In this dissertation, we examined the relationship between PI3K/AKT protein and VEGFR-3 expression in breast cancer by detecting the expression of PI3K/AKT, to investigate the role of its expression on lymphatic metastasis of breast cancer.

MATERIALS AND METHODS

Specimen

General information: The project has been approved by the Medical Ethics Committee of the Affiliated Hospital of Taishan Medical College. Between 1/1/2015 and 12/31/2017, 236 female patients with pathologically proven invasive ductal carcinoma from the Department of Breast Surgery, Affiliated Hospital of Taishan Medical College. By HE staining pathological diagnosis of invasive ductal carcinoma of breast. It including 128 patients with lymph node metastasis and 108 patients without lymph node metastasis. All patients had no prior radiotherapy or chemotherapy. Based on tumor TNM staging from the International Union Against Cancer (UICC) and American Cancer Society (American Joint Committee on Cancer, AJCC), 38 cases were stage 1, 113 cases were stage II, and 85 cases were stage III. Detailed pathological features are shown in Table 1.

For the experiment purpose, approximately 1.0 cm × 1.0 cm × 1.0 cm fresh specimen from each case was taken and stored at 80°C refrigerator. The remaining specimens were fixed with formaldehyde and embedded in paraffin for pathological diagnosis.

Main reagents: Rabbit anti-human PI3K (PI3K p85) polyclonal antibody, rabbit anti-human AKT (AKT2 isoform) polyclonal antibody, rabbit anti-human VEGFR-3 polyclonal antibody were bought in Signalway Antibody, rabbit anti-human PI3K (PI3K p85) polyclonal antibody, rabbit anti-human AKT (AKT2 isoform) polyclonal antibody, rabbit anti-human VEGFR-3 polyclonal antibody were bought in Signalway Antibody, rabbit anti-human PI3K (PI3K p85) polyclonal antibody, rabbit anti-human AKT (AKT2 isoform) polyclonal antibody, rabbit anti-human VEGFR-3 polyclonal antibody were bought in Signalway Antibody, rabbit anti-human PI3K (PI3K p85) polyclonal antibody, rabbit anti-human AKT (AKT2 isoform) polyclonal antibody, rabbit anti-human VEGFR-3 polyclonal antibody were bought in Signalway Antibody.

TABLE 1

<table>
<thead>
<tr>
<th>lymph node metastasis</th>
<th>n</th>
<th>PI3K Positive</th>
<th>χ2</th>
<th>P</th>
<th>AKT2 Positive</th>
<th>χ2</th>
<th>P</th>
<th>VEGFR-3 Positive</th>
<th>χ2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>lymph node metastasis</td>
<td>128</td>
<td>95 (74.22)</td>
<td>6.6802</td>
<td>0.0097</td>
<td>91 (71.09)</td>
<td>9.3474</td>
<td>0.0022</td>
<td>121 (92.97)</td>
<td>5.3676</td>
<td>0.0205</td>
</tr>
<tr>
<td>without lymph node metastasis</td>
<td>108</td>
<td>63 (58.33)</td>
<td>57 (52.78)</td>
<td>86 (79.63)</td>
<td>0.0020</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

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trypsin, streptavidin-horseradish peroxidase (SP) kit (sp-9001) and concentrated DAB color kit were purchased from Beijing Golden Bridge Biotechnology Co., Ltd., one-step RT-PCR kit was purchased from Beijing Tiangen Biochemical Technology Co., Ltd.. Chemiluminescence reagents were purchased from Santa Cruz. The pre-stained protein marker was purchased from Solarbio. Trizol reagents were purchased from Invitrogen, and the RT-PCR primers were synthesized by Invitrogen (Shanghai).

PI3K and AKT2 and VEGFR-3 protein detection

Specimens from 236 patients were embedded in paraffin and sectioned 4μm thick. Immunohistochemical SP method was used to remove the endogenous peroxidase. Antigen retrieval was achieved by high temperature and high pressure and non-specific binding was blocked with goat serum. The slides were then incubated with primary antibodies at room temperature, followed by a biotin-labeled secondary antibody. Tissues were then stained with 3,3′-diaminobenzidine (DAB). Finally, tissues were counterstained with hematoxylin, dehydrated, and mounted. The slides were reviewed under the light microscope within 48 hours. We used PI3K and AKT2-positive sections provided by the reagent company as positive controls. For negative controls, we incubated slide with PBS instead of primary antibodies.

Interpretation of staining results

PI3K and AKT2 located in the cytoplasm were stained as yellow to brown granules, while VEGFR-3-positive cells showed brown particles in cytoplasm or on cell membrane. For each group, known positive tissue was used as positive control and PBS was used instead of one antibody as negative control. Two pathologists evaluated the slides by double-blind method. Three fields from each slide were randomly selected and the positive cells per 100 tumor cells per field were recorded at X400. The final result from each slide was the average of the three fields. Grading standards were based on reference [5] with modification: (1) Staining intensity scores: no stain: 0 point, light yellow: 1 point, brown yellow: 2 points, brown: 3 points; (2) Percentage of positive cells: Positive cells <25% were counted as 0 points; 26%–50% is 1 point; 51%–75% is 2 points; >75% is 3 points, and the final criterion is the score value of the product of the two points. Immunohistochemical results: 0~1 is divided into the negative (-), and 2~3 into the positive (+~+), more than 4 points for strong positive (+ + +).

PI3K and AKT2 and VEGFR-3 mRNA expression

Total RNA was extracted from fresh specimens (236 cases) by TRIzol method. Total RNA (2μg) from each case was reversely transcribed using Reverse transcription polymerase chain reaction (RT-PCR) and bet-2microglobulin (B2M) was used as internal references. The gene of PI3K, AKT2, VEGFR-3, and internal polymeric chain reaction (RT-PCR) and bet-2microglobulin (B2M) was used as internal references. The gene of PI3K, AKT2, and VEGFR-3 mRNA expression and correlation

PI3K and AKT2 and VEGFR-3 mRNA expression

The expressions of PI3K, AKT2, and VEGFR-3 protein in breast invasive duct carcinoma (A-D): PI3K; B-E: AKT2; (C-F): VEGFR-3

Figure 1) The expressions of PI3K, AKT2, and VEGFR-3 protein in breast invasive duct carcinoma (A-D): PI3K; B-E: AKT2; (C-F): VEGFR-3
DISCUSSION

Breast cancer is one of the most common malignant tumors in women from Europe and the United States. According to the latest statistics, China has a relatively low incidence of breast cancer as compared to the United States and Canada, with 2.16 breast cancer cases per million females. Cancer, while the mortality rate reached 15.74% [6]. Lymphatic metastasis of breast cancer is based on lymphangiogenesis. VEGFR-3 is a key protein that induces lymphangiogenesis and a specific marker of lymphatic endothelial cells. VEGFR-3 is an important factor of VEGF-C/D / VEGFR-3 signaling pathway. Experiments confirmed that VEGFR-3 is a receptor tyrosine kinase, VEGFR-3 overexpression and VEGF-C and VEGF-D binding, through the signal transduction, induced lymphatic endothelial cell proliferation, migration and lymphangiogenesis, promoting breast cancer cell survival and metastasis [7]. The spread of the tumor to the lymph nodes and lymphatic system is an important pathway. The expression of VEGF-C induces lung lymphangiogenesis and promotes the lung metastasis of breast cancer. This pattern of metastasis suggests that the VEGF-C / VEGFR-3 pathway not only serves as a preventive measure to the goal of metastasis but also the treatment to established metastatic disease [8].

PI3K / AKT signaling pathway in most human cancers dysregulation, not only can regulate tumor cell proliferation and apoptosis, but also tumor angiogenesis, lymphangiogenesis and invasion and metastasis. All kinds of breast cancer subtypes have different degrees of PI3K / AKT signaling pathway activation and the activation of the pathway indicates a poor prognosis. PI3K is activated in breast cancer tissue which an important component of breast cancer signal transduction pathway [9]. Among the two signals, AKT2 is an important cross between multiple signaling pathways. Under physiological conditions, AKT2 exists in the cytoplasm and stays low activity. Under the stimulation of various factors, activated AKT2 and phosphorylation (p-AKT2) plays a role in affecting multiple downstream effector molecules as well as in the occurrence, development, infiltration and metastasis of breast cancer. Especially in estrogen receptor-positive breast cancer, AKT2 activation is very common, which has a clear correlation with histological grade and lymph node metastasis, suggesting that the high expression of AKT2 may play a role in the malignant transformation and metastasis of breast cancer Important role, also indicates the endocrine therapy resistance [10]. However, AKT2 morphology functions specially in different stages of breast cancer progression. AKT1 participates in local tumor growth and AKT2 participates in distant tumor transmission. AKT2 has a low prognostic value and is therefore a valuable target for therapy [11]. With the exception of involvement in cell transformation, tumorigenesis, progression of cancer, and resistance to breast cancer; mutations in the PI3K/AKT pathway are evident in breast cancer [12]. The relationship between PI3K/AKT expression with clinicopathological features and the prognosis of breast cancer patients was found to be significantly correlated with axillary lymph node metastasis [13]. In invasive ductal carcinoma of the breast,
AKT2 also up-regulates the expression of VEGF-C, thereby promoting lymph node metastasis in breast cancer. It is speculated that PI3K/AKT/VEGF-C pathway may play a role in lymphatic metastasis of invasive ductal carcinoma of breast [14].

Of the 236 breast cancer cases, the expression of PI3K, AKT2 and VEGFR-3 in breast cancer tissues with lymph node metastasis was significantly higher than that in patients without lymph node metastasis group (P<0.05 or P<0.01 ). RT-PCR also showed that the relative expression of PI3K, AKT2 and VEGFR-3 mRNA in invasive ductal breast cancer with lymph node metastasis were significantly higher than that without lymph node metastasis (P<0.05 or P<0.01). Moreover, the relative expressions of PI3K mRNA/AKT2 mRNA and VEGFR-3 mRNA in breast cancer were positively correlated (P<0.001). It is speculated that the up-regulation of PI3K and AKT2 in breast cancer may promote the over-expression of VEGFR-3 in breast cancer cells and induce the lymphatic metastasis through the PI3K/AKT pathway system in lymphatic endothelial cells and induce lymphangiogenesis.

The occurrence and development of breast cancer are not the result of a single gene effect, nor are they involved in the PI3K/AKT pathway. The future treatment of breast cancer should also be a combination of inhibition or activation of multiple gene pathways in order to achieve the desired effect [15]. We suggest that the PI3K/AKT and VEGF-C and D/VEGFR-3 pathways play a common role in the lymphatic metastasis of invasive ductal carcinoma of the breast and regulate the pathways and lymphangiogenesis, thereby promoting lymphatic endothelial cell growth and lymph node metastasis occur in breast cancer. As a result, it will turn hopes into reality that the PI3K/AKT signal pathway and related proteins as targets for therapy of breast cancer, inhibit the lymphatic metastasis of breast cancer, to improve the patient’s survival.

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