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Study on PI3k gene expression in breast cancer samples and its association with clinical factors and patient survival

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ABSTRACT

Breast cancer is the most common cancer among women in the world. The phosphatidylinositol 3-Kinase (PI3k), which regulates various cellular signaling pathways, is often elevated in human cancers. This study aimed to evaluate the expression of the PI3k gene in breast cancer. In this case-control study, 40 paraffin-embedded tissues of breast cancer and 40 adjacent non-tumor tissues were examined. After total RNA extraction and cDNA synthesis, the relative expression of the gene was obtained using the real-time-PCR method and evaluated by the $2^{-\Delta\Delta CT}$ method. Also, the association of gene expression with clinical factors and survival rate was investigated. Data analysis was performed by SPSS statistical software (version 22), t-test, and ANOVA. A p-value of less than 0.05 was considered significant. The results showed that PI3k expression was significantly increased in breast tumor tissues compared to non-tumor tissues (p = 0001). Consistent with these results, PI3k expression was associated with metastasis (p = 0.008) and high tumor grade (p = 0.01). In addition, increasing PI3k expression decreased overall survival compared to its low expression (p = 0.03). In general, PI3k plays a tumor-enhancing role in the progression of breast cancer. In addition, increased PI3k expression is associated with metastasis and poor prognosis of cancer, so that PI3k may be useful in the diagnosis, treatment, and prognosis of people with the disease. However, further investigation is needed to substantiate this claim.

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Introduction

Breast cancer is one of the most common causes of cancer in women and the second leading cause of cancer death among women worldwide (1). According to published reports, the incidence of this cancer in the United States is one in eight women. Breast cancer is a highly heterogeneous disease, and several genetic and environmental factors are involved in its formation (2). These factors include age, family background, pregnancy, foreign estrogen intake, overweight and obesity, smoking, and alcohol consumption (3).

Therefore, knowledge of the molecular basis of the processes and mechanisms involved in the occurrence and progression of this cancer can be helpful in early diagnosis and treatment (4). In addition, many studies are being conducted today to identify factors that can predict the response to treatment and the ultimate fate of patients (5). The most common of these factors, known as prognostic factors, are tumor size and grade, axillary lymph node involvement, estrogen receptor (ER) and progesterone receptor (PR) status, and Human epidermal growth factor receptor 2 (HER-2) (6, 7). Prognostic factors are biological and clinical markers produced by cancer cells or due to the body's response to cancer and are associated with overall patient survival and disease-free survival (8). Contradictions to the results of the study of these markers and the impact of various demographic and environmental factors on them, it is helpful to identify relevant prognostic factors that can help make clinical decisions and predict the final fate of patients (9). The PI3k gene was selected and studied to investigate as one of these prognostic factors in the present study.

The phosphatidylinositol 3-Kinase (PI3k) is activated by binding to the receptor tyrosine kinase (RTK) coupled to G protein and integrin (10). Activated PI3k phosphorylates phosphoinositol 4,5 bisphosphate (PIP2) to phosphoinositol 3,4,5 trisphosphate (PIP3). The PI3K family is divided into eight distinct catalytic subunits capable of phosphorylating inositol lipids (11).

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This family is classified based on protein domain structure, specific lipid substrate, and regulatory subunits in three classes (I, II, and III). Class I enzymes have been studied more than the other two classes (10). This heterodimeric class consists of two catalytic and regulatory subunits. By binding the ligand to the receptor at the cell surface and activating the tyrosine kinase receptors, the SH2 domain from the P85 regulatory subunit binds to phosphorylated tyrosine in the tyrosine kinase growth factor receptor, and the inhibitory effect of the regulatory subunit is removed from the P110 catalytic subunit (12). Activation of P110 causes phosphorylation and conversion of PIP2 to PIP3, which activates proteins that have a pH domain, such as protein kinase B or serine/threonine kinase (AKT), and phosphoinositide-dependent protein kinase 1 (PDK-1) (13).

The activated form of AKT phosphorylates proteins that regulate metabolism (via glycogen citrate kinase 3), translation control (via P70s6 kinase), and cell survival (via FORKHEAD, BAD, and caspase-4 transcription factors) (14). AKT is also involved in cell cycle regulation through cyclin D1 and E2F. Changes in both catalytic and regulatory subunits can lead to increased enzyme activity and cell carcinoma. But according to previous studies, catalytic subunit changes are more common. The gene encoding the catalytic subunit is called class I, and its enzymatic family mutations are common in various cancers, including breast cancer (15).

According to the above, it could be hypothesized that the PI3k gene can act as a diagnostic and prognostic factor in cancer (16). Therefore, this study aimed to evaluate the expression of the PI3k gene in breast cancer tissue samples, compare its expression with adjacent healthy tissue, and investigate the relationship between the expression level of this gene with clinical factors and the overall survival of patients.

Materials and methods

In this case-control study, 80 tissue samples were randomly selected from 40 breast cancer patients (40 tumor tissues and 40 adjacent non-tumor tissues) from the hospital's pathology department. Inclusion criteria were confirmation of breast cancer by a pathologist, and cases with benign or suspected masses were excluded from the study. For molecular experiments, small sections were prepared from paraffin blocks and placed in sterile microtubes. The samples were kept at -20 °C until the experiments were performed. The Patients completed informed consent forms. In addition, age, pathological and clinical information of patients such as tumor size, metastasis, and tumor grade were determined.

RNA extraction and cDNA synthesis

The total cell RNA was extracted by RNA extraction MN kit (Nucleo Spintotal RNA FFPE, Germany). The RNA quality and quantity of the samples were evaluated and confirmed by a spectrophotometer (Nanodrop 2000, ThermoFisherScientific, Wilmington, DE, USA). We used a synthesis kit (Takara, Clontech) to amplify complementary DNA (cDNA). This kit was designed based on the reverse transcriptase enzyme, and the recommended instructions consisted of two steps. The first step was to add random hexamer and oligo-dT primers with reverse transcriptase enzyme (Prime Script RT), and the second step was incubation at 37°C for 15 minutes and then for 5 seconds at 85°C.

Evaluation of gene experssion

Primer design for PI3k gene and beta-actin gene as internal reaction control was performed according to the sequence obtained from Ensemble database by Oligo V.7.0 software. BLAST server was used to ensure the specificity of the primer connection (Table 1).

Table 1. The primer Sequences of PI3k (A) and β-actin gene (B)

Gene		Sequence	Product Length
A	Forward	5'-TCCAGAGGGGAAAAATATGAC-3'	272 bp
	Reverse	5'-TATGGTAAAAACATGCTGAG-3'	
B	Forward	5'-AGAGCTACGAGCTGCCTGAC-3'	184 bp
	Reverse	5'-AGCACTGTGTTGGCGTACAG-3'	

Gene expression was assessed by 6000 Rotor-gene and SYBR Green I dye. The reaction for the beta-actin reference gene and the 20-µl PI3k gene was as follows:

SYBR premix Ex taqII (YTA) 10µl, forward and reverse primers (10 picomolar) each 0.3µl, and 2µl of diluted cDNA, eventually reaching the desired volume with nuclease-free distilled water. The reaction temperature in the device was as follows: first,

activation of polymerase enzyme and initial denaturation were performed at 95°C for 10 minutes and then 40 cycles of the amplification reaction, which includes 15 seconds of denaturation at 95°C for 20 seconds. The annealing of primers was at 61°C for 20 seconds, and extension was done at 72°C. The melting step for the products was performed at 72-95°C. For each sample, two repetitions in each cycle were considered. Finally, the Delta Ct method and formula $2^{-\Delta\Delta CT}$ analyzed the reaction data (17).

At the beginning of the study, 60 paraffin samples were selected, and after reviewing their clinical and demographic information, about 15 samples were removed due to defects in clinical data. Five pieces were excluded from subsequent studies due to the lack of RNA quality. In the remaining samples, due to the completeness of information such as tumor grade, tumor size, and lymph node metastasis in all samples, analysis was performed on the remaining samples. Metastasis to areas far from the tumor was not reported in most samples, and therefore only lymph node metastasis was considered.

To evaluate the survival rate in breast cancer and the relationship with the expression of the PI3k gene, patients' information was used in TCGA Bank, and the Kaplan Meyer diagram was drawn by COX regression analysis (results not reported). In this study, two groups of samples with high and low PI3k expression were analyzed, and the survival rate was evaluated for 20 years.

Statistical analysis

Data were analyzed using SPSS statistical software (version 22). According to the normal distribution of data, a t-test was used to assess the expression of PI3k and beta-actin genes in tumor and non-tumor samples, age groups, tumor size, and metastasis. ANOVA test was used to evaluate the relationship between gene expression and tumor grade. All graphs were plotted by GraphPad Prism version7.01 software, and survival was assessed by the Cox regression method and Kaplan Meyer curve. A p-value of less than 0.05 was considered significant.

Results and discussion

In the present study, we examined 40 tumor tissues of breast cancer along with adjacent healthy tissue. Prepared samples belong to breast cancer patients with a mean age was 51.82 ± 1.42 years. Clinical information of the studied samples is shown in Table 2.

Table 2. Clinicopathological information related to the studied samples

Clinical information of patients		Number (percent)
Mean tumor size	< 4 cm	12 (30%)
	≥ 4 cm	28 (70%)
Lymph node metastasis	Positive	21 (52.5%)
	Negative	19 (47.5%)

Quantitative Real-Time PCR was used to evaluate gene expression. The melting curve of the PI3k gene and internal control gene (beta-actin) was obtained as a single peak, indicating the designed primers' specific performance. After ensuring the optimal reaction conditions and product specificity, the gene expression was examined for all samples, and the relative expression was determined for each sample.

Based on the results of the t-test, it was found that the mean relative expression of the PI3k gene in tumor tissue compared to healthy adjacent tissue has a significant increase. So that gene expression in tumor samples was almost three times higher than healthy samples, and there was a significant difference between them (p = 0.0001) (Fig. 1).

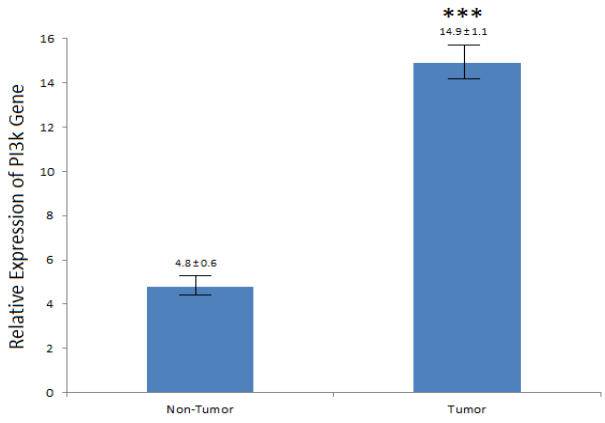

Figure 1. Comparison of mean relative expression of PI3k gene in tumor and non-tumor tissues in breast cancer; *** Indicates a significant difference between the two groups with a value of p less than 0.001.

In addition, we studied gene expression in different clinical conditions. Since the average size of tumor specimens was approximately 4 cm, all tumor specimens were divided into two categories smaller than 4 cm and larger than or equal to 4 cm, and a

significant increase in expression was observed in tumors larger than 4 cm. However, there was no statistically significant relationship for this difference in gene expression (p = 0.059) (Fig. 2a). Also, in the study of the relationship between PI3k gene expression and lymph node metastasis, gene expression in metastatic tumors increased in comparison with non-metastasis, and this difference in expression between the two groups showed a statistically significant difference (p = 0.008) (Fig. 2b). Considering the association between gene expression and tumor grade, an obvious increase in expression was observed in tumor grades 2 and 3 compared to tumor grade 1, which was statistically significant (p = 0.01) (Fig. 2c).

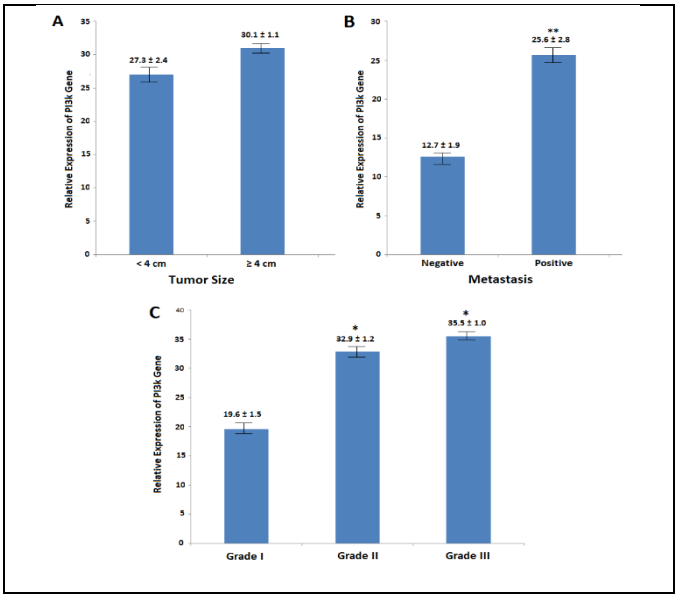

Figure 2. Comparison of the relative expression of PI3k gene: A. In tumors of smaller than 4 cm, and equal size and larger than 4 cm; B. In tissues with positive and negative metastases; C. In different tumor degrees. * Indicates a significant difference between the two groups with a p-value of less than 0.05. ** Indicates a significant difference between the two groups with a p-value of less than 0.01. *** Indicates a significant difference between the two groups with a value of p less than 0.001. Graphs are marked with a standard deviation.

Cox analysis and Kaplan Meyer diagram were used to investigate the relationship between gene expression and overall survival in patients. The OncoLnc database plotted the graph, and it was found that the increase in PI3k gene expression is accompanied by a decrease in patient survival (p = 0.039) (Fig. 3).

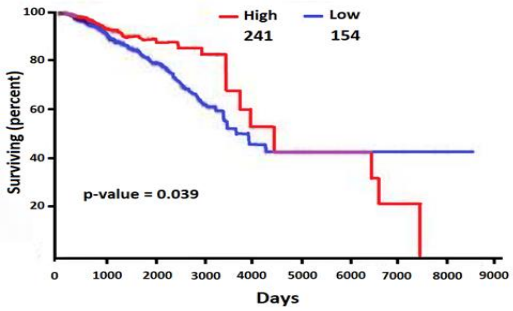

Figure 3. Comparison of relative expression of PI3k gene with overall survival per unit time

Breast cancer is the most common type of malignancy in women and is the second leading cause of cancer-related death (18). Stopping angiogenesis and cell migration in the tumor, exacerbating and inducing cell death by apoptosis, and manipulating cell signaling pathways are promising new strategies in the treatment and control of cancer (19). Cancer cells can break down tissue boundaries and migrate to nearby and distant tissues. This process (metastasis) is the worst form of cancer. Matrix metalloproteinase (MMP) is one of the types of proteinases involved in tumor development (20).

Inhibition of angiogenesis and destruction of basement membrane and extracellular matrix is a valuable and modern treatment method for treating solid tumors and preventing tumor cell invasion. Specific objectives of such projects include growth factor kinase receptor inhibitors and MMPs (21). The role and position of PI3K are crucial in the transmission of messages for the production and activation of MMPs. The data indicate that the PI3K pathway modulates the activity of gelatinases (22).

Although most studies of the PI3K message pathway have been performed on the classic P110-P85 (now known as Class I of the PI3K family), over the past ten years, it has been found that the PI3K superfamily (EC2.7.1.137) belongs to a large family of structurally similar enzymes, but their phosphatidyl inositol substrates are different (23). From the human genome project data, it appears that eight different types of PI3K catalytic subunits are capable of phosphorylating inositol lipids. These eight members can be divided into three different categories based on protein structure, lipid substrate properties, and regulatory subunits (24). Class I includes P110α, P110β, P110δ, and P110γ. Class II has PI3K-C_2α,

PI3K-$C_2\beta$, and PI3K-c2 γ. Class III includes Vps34p which has the types of lipid products produced by these enzymes, ptdIns3P, (3,4) P_2, (3,5) P_2, (3,4,5) P_3. These groups are involved in transmitting intracellular messages in a variety of ways (24, 25). For example, δ and P110γ isoforms are involved in cell migration. Group I isoforms are involved in cell motility, cell size and survival, and cell proliferation. In addition, the lipid products resulting from the activity of these enzymes cause polarization in mammalian cells and function in the invasive mechanisms of leukocytes (25). Category II isoforms are primarily membrane-dependent, and the integrative messages of growth factors and cytokines stimulate the activity of this class (26). Class III isoforms are involved in the intracellular traffic of materials and molecules and are associated with inner membranes. The lipid products of the superfamily PI3K enzymes control a variety of message pathways within the cell (27). Part of this derivation results from a wide range of proteins affected by PI3K lipids, and at another level, the diversity of PI3K isoforms responsible for this diversity in function (28).

The PI3K message pathway is inherently oncogenic and is overexpressed in many types of human cancer. According to the above, it may be hypothesized that the PI3K gene and its transcription factor can be considered an effective biomarker in the diagnosis and prognosis of breast cancer (29). In the present study, the role of the PI3K gene in breast cancer was evaluated, and the results showed that PI3K gene expression is significantly increased in breast cancer tumor tissues compared to surrounding healthy tissues. Regarding the relationship between gene expression and clinical data, studies have shown that the expression of the PI3K gene in metastatic tumors has increased significantly compared to no metastasis. The present study also showed a direct relationship between the expression of the PI3K gene and the size and degree of breast cancer tumors. In confirmation of the results of this study, Liang et al. (30), in the analysis of PI3K gene expression and its relationship with the occurrence of metastasis in patients with lung adenocarcinoma, showed that increased expression of this gene is associated with cancer cell metastasis to lymph nodes. These findings are consistent with the results of other studies showing that PI3K has an influential role in the spread of tumor tissues (31). Genetic studies have been performed on many other types of cancer (32-35).

Also, in the present study, the relationship between PI3k gene expression and the overall survival of patients was investigated. As shown in Figure 3, people with higher PI3k gene expression have a shorter lifespan and survival. In contrast, individuals with lower levels of PI3k gene expression have higher overall longevity and survival. Therefore, it can be concluded that the decrease in PI3k expression is significantly associated with an increase in overall patient survival. Similar to the present study, other studies have been performed to investigate the relationship between PI3k gene expression and the overall survival time of patients (36, 37). In cancer research, the survival rate is considered one of the disease's leading prognostic indicators (38). In addition to various demographic factors, this index is affected by the cancer stage at the time of diagnosis, facilities, and diagnostic and therapeutic measures. The 5-year survival rate for breast cancer in the United States has risen from 63% in the early 1960s to 90% in 2020. The improvement of these cases in developed countries indicates the progress of diagnostic and therapeutic interventions (39).

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