JBUON 2021; 26(3): 747-752
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Investigation of somatic *PIK3CA* gene mutations in breast cancer patients

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Summary

Purpose: *Mutations of the PI3K/AKT/mTOR signaling pathway occur in 70% of all breast cancers and represent a clinically useful marker for disease prognosis and patient management. The purpose of this work was to study the main somatic PIK3CA gene mutations in breast cancer patients and the search for a relationship with the main clinical and pathological characteristics and the effect of neoadjuvant chemotherapy (NAC).*

Methods: *The study involved 29 patients with luminal B breast cancer. DNA was isolated from samples of tumor tissue before and after treatment using the QIAamp DNA mini Kit (Qiagen, Germany). Samples were prepared for sequencing by amplification with primers containing TruSeq index and adapter sequences (Illumina, USA) using Encyclo polymerase.*

Resuts and conclusion: *We found 5 different somatic changes in 28% of patients: c.3140A>G (p.His1047Arg), c.3140A>T (p.His1047Leu), c.1624G>A (p.Glu542Lys), c.1633G>A (p.Glu545Lys), c.3145G>C (p.Gly1049Arg). In the group of patients with mutations, 50% showed PIK3CA gene amplifications. The c.3140A>T (p.His1047Leu) mutation was associated with low disease-free survival rates. PIK3CA gene mutations were observed in 38% of patients with HER2-subtype, and metastasis-free survival rates were, on average, 1.5 times higher than in patients with normal gene status.*

Key words: *mutations, PIK3CA, neoadjuvant chemotherapy, breast cancer*

Introduction

Phosphoinositide 3-kinases (PI3K) are a family of three different classes of lipid kinases involved in the processes of cell growth and survival in various types of breast cancer and are part of the PI3K/AKT/mTOR signaling pathway [1]. This is a universal pathway for most cells responsible for cell growth, proliferation, metabolism and avoiding apoptosis. About 60% of tumors have mutation leading to hyperactivation of this signaling pathway and, subsequently, tumor development [2]. Somatic mutations in genes encoding components of the PI3K pathway occur in more than 70% of breast cancer cases. These include mutations or amplification of *PIK3CA* (p110α subunit), *PIK3CB* (p110β subunit), and *PIK3R1* (p85α subunit), which are PI3K catalytic subunits [3]. The most common mechanism of pathological activation of the PI3K/AKT/mTOR pathway is mutation or amplification of the *PIK3CA* gene, which leads to breast cancer oncogenesis and tumor resistance [4-6]. Mutations

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Received: 10/02/2021; Accepted: 08/03/2021

in this gene are found in 40% in the early tumor stages with positive hormone receptor (HR +) and negative epidermal growth factor receptor (HER2-) [7]. Most of the *PIK3CA* gene mutations are localized in specific regions of exons 9 and 20, which correspond to amino acids E542K and E545K in exon 9 of the helicase domain and in exon 20 of the kinase domain - H1047R and H1047L [8]. The most common somatic *PIK3CA* gene mutations are c.1624G>A, c.1633G>A, c.3140A>G, and c.3140A>T, accounting for more than 90% of all mutations and affecting pathogenesis.

The presence *PIK3CA* gene mutations in breast cancer is associated with a more favorable prognosis and less aggressive forms of disease like luminal forms that are positive for hormone receptors (HR +) and negative for epidermal growth factor receptors (HER2-).

According to a 2014 meta-analysis [9], the presence of *PIK3CA* gene mutations was significantly correlated with high rates of unprecedented survival (HR 0.76, 95% CI 0.59-0.98, p=0.03). However, the study did not show the effect of the presence of an aberrant component on survival (HR 0.62, 95% CI 0.25-1.59; p=0.32). The *PIK3CA* gene mutations have been most widely studied with the hormone receptor status of the tumor. A meta-analysis involving 26 studies showed a significant association between the presence of *PIK3CA* mutations and the presence of estrogen (ER) and progesterone receptors (PR) (OR 1.92, 95% CI 1.65-2.23; OR 1.88, 95% CI 1.61-2.20, respectively) [10]. A 2018 study found that patients with HR+HER2- breast cancer with mutations in the *PIK3CA* gene and receiving therapy without PI3K inhibitors showed lower metastasis-free survival rates compared with patients with a normal *PIK3CA* gene [11]. Thus, the predictive significance of *PIK3CA* gene mutations in patients with HR+HER2- breast cancer remains controversial.

The aim of this work was to study the main somatic mutations of the *PIK3CA* gene in patients with breast cancer and search for a relationship with the main clinical and pathological characteristics and the effect of neoadjuvant chemotherapy (NAC).

Methods

The study involved 29 patients with luminal B HER2-negative breast cancer of stages IIA–IIIB with morphologically verified diagnosis, aged 27-66 years (average 46.8±0.7) (mean±SE) (Table 1). In accordance with the Consensus Conference on Neoadjuvant Chemotherapy in Carcinoma of the Breast, April 26-28, 2003, Philadelphia, Pennsylvania [12], all patients received 4-8 courses neoadjuvant chemotherapy with AC (adriamycin 50 mg/m^2 and cyclophosphamide 600 mg/m^2 1 per 3 week) or ACT (adriamycin 50 mg/m^2, cyclophosphamide 600 mg/m^2, taxotere 75 mg/m^2), CP (cyclophosphamide 1080 mg/m^2, cisplatin 135 mg) or monotherapy taxotere (100 mg/m^2 1 h infusion per day. After 3-5 weeks NAC all patients had a radical or subcutaneous mastec-

Table 1. Clinical and pathological characteristics of patients

Clinical and pathological characteristics	*Number of patients n (%)*
Age (years)	
≤45	15 (51.7)
>45	14 (48.3)
Menstrual status	
Premenopause	24 (82.7)
Menopause	3 (10.3)
Postmenopause	2 (6.0)
Histological type	
Invasive ductal carcinoma	3 (10.3)
Invasive lobular carcinoma	6 (20.7)
Non-specific invasive carcinoma	14 (51.7)
Other types	6 (20.7)
Tumor size	
T_1	2 (7.0)
T_2	23 (79.0)
T_3	2 (7.0)
T_4	2 (7.0)
Lymph node metastasis	
N_0	10 (34.5)
N_1	14 (48.3)
N_2	4 (13.7)
N_3	1 (3.5)
Estrogen receptors	
+	26 (89.6)
–	3 (10.4)
Progesterone receptors	
+	23 (79.3)
–	6 (20.7)
HER2	
0/+	24 (82.7)
++	4 (13.8)
+++	1 (3.5)
Histological forms	
Unicentric	16 (55.2)
Multicentric	13 (44.8)
NAC scheme	
AT	3 (10.3)
ACT	5 (17.2)
Taxotere	7 (24.1)
CP	14 (48.4)
NAC effect	
Stabilization	6 (20.7)
Partial regression	17 (58.6)
Complete regression	6 (20.7)

tomy, radical resection, sectoral resection with axillary lymphadenectomy or other type of organ-preserving surgery, then the patients underwent radiation and/or hormonal or targeted therapy (herceptin for HER2 + status according to indications. Clinical and pathological characteristics of patients are presented in Table 1.

We analyzed biopsy tumor samples before treatment (~10 mm^3 volume), obtained under the guidance of ultrasound and surgical samples after NAC (~30-90 mm^3 volume). Tumor samples were placed in an RNAlater solution (Ambion, USA) and stored at -80°C (after a 24-h incubation at +4°C) for further DNA isolation.

DNA extraction

DNA was isolated from 30 samples of tumor tissue using the QIAamp DNA mini Kit (Qiagen, Germany). DNA concentration and purity of isolation were evaluated on a NanoDrop-2000 spectrophotometer (Thermo Scientific, USA) (from 50 to 190 ng/µl, A_{260}/A_{280}=2.05-2.20; A_{260}/A_{230}=1.95-2.20). DNA integrity was assessed by capillary electrophoresis on a TapeStation instrument (Agilent Technologies, USA); DNA fragments had a mass of more than 60 kbp.

Microarray analysis

Microarray analysis was performed on high density microarrays (DNA chips) of Affymetrix (USA) CytoScanTM HD Array which contains 3000,670 thousand markers – 1,000,900 thousand non-polymorphic markers for the analysis of copy number aberrations (CNA) and more than 700,000 single nucleotide polymorphisms. The presence of polymorphic markers also makes it possible to identify areas with loss of heterozygosity. Sample preparation, hybridization, and scanning procedures were performed in accordance with the manufacturer's protocol on the Affymetrix GeneChip® Scanner 3000 7G system (Affymetrix, USA). The Chromosome Analysis Suite 4.3 software (Affymetrix, USA) was used to process the microchipping results, which was specially developed for analyzing the results of microchipping on the CytoScanTM HD Array. The number of copies of the studied gene *PIK3CA* was three copies per genome.

Next generation sequencing (NGS)

PCR amplification was performed using Encyclo polymerase (Evrogen) according to the manufacturer's recommendations. Then, samples were prepared for sequencing by amplification with primers containing TruSeq index and adapter sequences (Illumina, USA) using Encyclo polymerase. DNA concentration was determined using the dsDNA broad range assay kit, samples were equimolarly mixed and sequenced on the MiSeq platform (Illumina).

Statistics

The quality control of the sequences was carried out using the fastQC program. Mapping and alignment of sequence reads to the reference human genome assembly Ensembl GRCh37 was performed using BWA-MEM. Sorting and indexing of mapped readings took place in the SamTools program. VarDictJava and FreeBayes programs were used to search for variants of nucleotide sequences. Annotation was carried out using the snpEff program. P value <0.05 was considered significant.

Results and discussion

We first we evaluated the genomic balance in the q26.2 chromosomal region containing the *PIK3CA* gene using a microarray study. Allelic imbalance was detected in 8 samples (28%), followed by identification in 2 patients *PIK3CA* deletions and 6 amplifications.

For a more detailed analysis, the samples were screened for the most common mutations (p.E542K c.1624G> A, p.E545K c.1633G>A, p.H1047R c.3140A>G, p.H1047L c.3140A>T) in the *PIK3CA* gene. We analyzed 58 tumor samples before and after NAC from 29 patients with luminal B breast cancer and blood from each patient was used as a control (for the absence of changes in the studied gene).

As a result, we found that 21 patients had no changes in the *PIK3CA* gene. The remaining patients (28% of all cases) had 5 different somatic changes: c.3140A>G (p.His1047Arg), c.3140A>T (p.His1047Leu), c.1624G>A (p.Glu542Lys), c.1633G> A (p.Glu545Lys), c.3145G>C (p.Gly1049Arg) (Table 2). In the group of patients with mutations, 50% of

Table 2. Mutations of the PIK3CA gene

Patient	*Mutation*	*Type*	*State PIK3CA*
A1	c.3140A>G (p.His1047Arg)	Pathogenic	Amplification
B1	c.1624G>A (p.Glu542Lys)	Pathogenic	Amplification
D1	c.1633G>A (p.Glu545Lys)	Pathogenic	Deletion
D2	c.3145G>C (p.Gly1049Arg)	Likely pathogenic	Normal
K1	c.3140A>G (p.His1047Arg)	Pathogenic	Amplification
K2	c.3140A>G (p.His1047Arg)	Pathogenic	Amplification
K3	c.3140A>T (p.His1047Leu)	Pathogenic	Normal
Sh1	c.3140A>G (p.His1047Arg)	Pathogenic	Normal

patients showed amplification of the *PIK3CA* gene (4/8 patients), 37.5% had a normal gene (3/8 patients), and one patient had a deletion of the studied gene (Table 2).

The most common mutation was a pathogenic single nucleotide polymorphism at position 178952085, which can be in two variants: c.3140A> G (p.His1047Arg) and c.3140A> T (p.His1047Leu). At the same time, in a patient with this mutation, the *PIK3CA* gene was in an amplified state in 60% of cases (Table 2). The c.3140A> G mutation was detected in 4 patients (A1, K1, K2, III) with a good response to therapy. Metastasis-free survival in these patients averaged 43.5 months (from 32 to 70 months). At the same time, the c.3140A> T gene mutation was detected in only one patient K3, who had a relapse of disease after 22 months, despite the high efficacy of chemotherapy.

The remaining mutations: c.1624G>A (p.Glu542Lys), c.1633G>A (p.Glu545Lys), c.3145G> C (p.Gly1049Arg) were found in isolated cases in patients B1, D1, and D2, respectively. All three patients had more than 80% response to chemotherapy. The patient D2 with the opportunistic c.3145G>C mutation developed metastatic disease after 72 months.

Mutations remained the same after chemotherapy, except for one case. Patient D1 with partial regression of disease (response to NAC was 94%) showed complete elimination of the pathogenic mutation c.1633G> A (p.Glu545Lys) (Table 2).

Despite the fact that many sources show a relationship between the main clinical and morphological parameters and the presence of the *PIK3CA* gene mutation, this relationship was not observed in our study. This made it possible to carry out further analysis without taking into account the chemotherapy regimen. More than 70% of all breast cancers are hormone receptor (estrogen or progesterone receptor) (HR) positive and HER2 negative [1]. 40% of patients with HR+HER2- have mutations in the *PIK3CA* gene, including hyperactivation of the catalytic unit alpha isoform (p110α) phosphatidyl inositol 3-kinase (PI3K) [1].

All patients were analyzed for the relationship between the presence of ER, HER2, with *PIK3CA* gene mutations. Regardless of the presence of hormonal receptors, patients with the HER2 subtype of breast cancer had a higher frequency of mutations in the *PIK3CA* gene compared with the HER2+ breast cancer group (p=0.0265). In a more detailed study of patients with HER2-subtype, 38% (7/18 patients) of patients had mutations in the *PIK3CA* gene, while metastasis-free survival rates were on average 1.5 times higher than in patients with normal gene status (40 versus 26.5 months). 4/18 patients developed metastases. In two patients without mutations (C1,P1) metastases developed after 11 and 17 months, while patient K3 with c.3140A>T mutation developed metastases after 22 months, and patient D2 with c.3145G> C mutation after 72 months.

The phosphatidylinositol-3-kinase (PI3K) pathway activation is an important step in oncogenesis and plays a role in the development of treatment resistance for both ER positive breast cancer and HER2 [4]. The *PIK3CA* gene mutations are found in 30-40% of cases of HR+HER2- subtype of breast cancer [1,13]. In our study, in 65.5% of all patients, HR+HER2- subtype of breast cancer was verified and 42% had somatic mutations c.3140A> G (p.His1047Arg), c.3140A>T (p.His1047Leu), c. 1624G>A (p.Glu542Lys), c.1633G>A (p.Glu545Lys), c.3145G>C (p.Gly1049Arg), which confirms the world data.

According to a research study [14] it was shown that somatic mutations rather than amplifications are the main genetic change in breast cancer. Frequent and clustered mutations in *PIK3CA* and assessment of his expression make it an attractive molecular marker for early diagnosis and prognosis of breast cancer [15]. Inhibition of *PIK3CA* can activate apoptosis in cells with mutations in this gene, making *PIK3CA* a promising therapeutic target for breast cancer [14]. The presence of *PIK3CA* mutations is associated with a less aggressive course of disease in patients with luminal breast cancer. Patients with *PIK3CA* mutations had higher non-metastatic survival [16]. The results of this work correlate with the data that were obtained in the course of our study.

Another study found that *PIK3CA* mutations were associated with age at diagnosis, hormone receptor positivity, HER2 negativity, lower tumor grade and stage, and lack of lymph node metastasis. And patients with tumors with the *PIK3CA* mutation showed an increase in overall survival (p=0.004) [17].

Data presented in the article [18] correlates with the results of our work. They found two point mutations in exon 9 - (Glu542Lys) and (9Glu545Lys) and also a mutation in exon 20 - (His1047Arg). They also characterized one synonymous mutation (Ala995Ala) and a new frame shift mutation (C. 3195_3202delTGCATTGA). Their results showed that the *PIK3CA* mutation status was not significantly associated with age at diagnosis, tumor size, lymph node involvement, tumor stage, and ER status, while *PIK3CA* mutations were more often present in PR + (p=0.06), and HER2– tumors (p=0.09).

There is a tendency (p=0.07) in patients with *PIK3CA* to a higher expression of AR (androgen receptors), which may indicate an increase in relapse-free survival in patients with early stages of ER+/PR+ tumors after adjuvant hormonal therapy [19]. Another study showed that *PIK3CA* mutations were more common in larger tumors and in ER+ and PR+ tumors. Survival analysis showed that the presence of *PIK3CA* mutations was associated with significantly worse survival (p=0.004) [20]. In addition, it was found that the *PIK3CA* mutation was significantly associated with lymph node-negative status (p=0.012). There was also a tendency towards an increase in the frequency of *PIK3CA* mutations in elderly women (p=0.0535). This study did not find any evidence that *PIK3CA* mutations are associated with sensitivity to chemotherapy in breast cancer, anthracycline-containing regimens or anthracycline/paclitaxel [21].

In addition to next generation sequencing, identification of somatic *PIK3CA* gene changes, microarray analyses were performed. According to the literature data, amplification of the *PIK3CA* gene is observed in various tumors: in lung cancer in 33-42% of the cases, ovarian cancer in 15-27%, esophageal cancer in 14-19%, cervical cancer in 10-17% and 2-16% of head and neck tumors. However, the functional role of *PIK3CA* amplification remains unknown so far [2, 6, 22]. In our study *PIK3CA* gene was amplified in 28% of all cases (6/29 patients). Moreover, in 83% of cases in this state, the gene showed various somatic changes.

Conclusion

The most frequent mutation among all patients was pathogenic single nucleotide polymorphism c.3140A>G (p.His1047Arg) which connected with NAC response. Mutation c.3140A>T (p.His1047Leu) was associated with low metastatic survival rates. Patients with HER2- subtype have *PIK3CA* gene mutations in 38% of the cases, metastatic survival rates were 1.5 times higher than in patients with a normal gene state.

Funding

This work was supported by the Russian Science Foundation grant No. 19-75-00027 «Study of the somatic status of the BRCA1 gene in a breast tumor for personalized treatment purposes».

Compliance with ethical principles

The work carried out complied with the ethical standards developed in accordance with the WMA Declaration of Helsinki «Ethical principles for medical research involving human subjects» in 2000 and the «Rules of Clinical Practice in the Russian Federation» approved by the Order of the Ministry of Health of Russian Federation dated June 19, 2003 No. 266. Informed consent was obtained from all persons participating in the study.

Conflict of interests

The authors declare no conflict of interests.

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