**CLINICAL SCIENCE**

**PIK3CA exon 20 mutations are associated with poor prognosis in breast cancer patients**

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**OBJECTIVES:** The phosphatidylinositol 3-kinase/AKT axis is an important cell-signaling pathway that mediates cell proliferation and survival, two biological processes that regulate malignant cell growth. The phosphatidylinositol 3-kinase CA gene encodes the p110α subunit of the phosphatidylinositol 3-kinase protein. There are phosphatidylinositol 3-kinase CA mutations in several types of human tumors, and they are frequently observed in breast cancer. However, these mutations have not been investigated in Brazilian breast cancer patients.

**METHODS:** PCR-SSCP and direct DNA sequencing were performed to identify phosphatidylinositol 3-kinaseCA exon 9 and exon 20 mutations in 86 patients with sporadic breast cancer. The relationships between PIK3CA mutations and patient clinicopathological characteristics and survival were analyzed. The presence of the TP53 mutation was also examined.

**RESULTS:** Twenty-three (27%) of the 86 primary breast tumors contained PIK3CA mutations. In exon 9 and 20, we identified the hotspot mutations E542K, E545K, and H1047R, and we identified two new missense mutations (I1022V and L1028S) and one nonsense (R992X) mutation. Phosphatidylinositol 3-kinase CA exon 20 mutations were associated with poor overall survival and TP53 gene mutations.

**CONCLUSIONS:** Phosphatidylinositol 3-kinase CA mutations are common in tumors in Brazilian breast cancer patients, and phosphatidylinositol 3-kinase CA and TP53 mutations are not mutually exclusive. Phosphatidylinositol 3-kinase CA exon 20 mutations are associated with poor survival, and they may be useful biomarkers for identifying breast cancer patients with aggressive tumors and for predicting the response to treatment with PI3K pathway inhibitors.

**KEYWORDS:** Breast Neoplasm; PIK3CA; TP53; Mutation; Prognosis.

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**INTRODUCTION**

The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway plays an important role in cellular processes, such as proliferation, differentiation, survival, and migration (1,2). Alterations in the components of this signaling pathway, including gain-of-function mutations in the p110 catalytic subunit of PI3K, have been identified in a wide spectrum of human cancers (3,4). Class I PI3Ks are heterodimers composed of catalytic (p110) and regulatory (p85) subunits involved in regulating cell division and in tumorigenesis (5,6).

The PIK3CA gene comprises 20 exons encoding the p110α catalytic subunit. This gene is mutated in a wide range of tumors, including glioblastomas, gastric cancers, lung cancers, ovarian cancers, hepatocellular carcinomas, endometrial carcinomas, brain cancers, and breast cancers (3). The majority of PIK3CA mutations cluster in hotspot regions in exon 9 (the helical domain) and exon 20 (the kinase domain). The most common missense mutations change amino acid residues E542 and E545 to lysine in the helical domain and change H1047 to arginine in the kinase domain. Functional studies suggest that these particular PIK3CA mutations lead to increased PI3K activity (6,7).

The frequency of PIK3CA mutations in breast cancer ranges from 16.4 to 45% (3,8-10). However, the association between PIK3CA mutations and specific clinicopathological features of breast cancer is still a matter of debate. Furthermore, the relationship between the presence of PIK3CA mutations in breast cancer patients and overall survival (OS) and disease-free survival (DFS) is controversial. Some studies have found that breast cancer patients with PIK3CA gene mutations have improved OS and DFS rates compared with breast cancer patients lacking such mutations (9,11-13). Conversely, other studies have found
that the presence of PIK3CA mutations is correlated with poor outcome (14-16).

In the present study, we identified mutations in exons 9 and 20 of the PIK3CA gene in primary breast tumors from Brazilian breast cancer patients, and we analyzed the relationship between mutational status and patient clinicopathological features and outcomes.

MATERIALS AND METHODS

Tumor samples and genomic DNA extraction

Samples from 86 primary breast tumors were obtained from breast cancer patients diagnosed at the Hospital do Cancer A. C. Camargo, São Paulo, Brazil, from February 1993 to March 1998. The median follow-up time was 63.3 months (range, 25 to 78 months). None of the patients had received any medical treatment related to their breast cancer before the biopsy/mastectomy procedure. After surgical excision, biopsy specimens were immediately frozen and stored in liquid nitrogen until DNA extraction. Histopathological review of the tumor slides was performed to confirm the diagnosis. All tumors were classified according to the World Health Organization Histological Typing of Breast Tumors classification, and the clinical stage of each patient was determined according to the 5th Edition of the UICC TNM classification of malignant tumors. The tumors were all infiltrating ductal carcinomas. The median age of the patients at the time of diagnosis was 55 years (range, 26 to 85 years). The patient and tumor characteristics are shown in Table 1. Tissue specimens were ground to a powder under liquid nitrogen using a Frozen Tissue Pulverizer (Termovac Industries, Copiague, N.Y.), and high-molecular-weight DNA was extracted as previously described (17). This study was approved by the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo Ethics Committee. All subjects were given information about the study and provided written informed consent.

Table 1 - Patient and tumor characteristics (n = 86).

| Variable | Characteristic | n (%) |
|----------|----------------|-------|
| Age, y   | ≤55            | 45 (52.3) |
|          | >55            | 41 (47.7)  |
| Stage, TNM| Early          | 37 (43.0)  |
|          | Late           | 49 (57.0)  |
| Tumor size, cm | ≤4.0           | 44 (51.2)  |
|          | >4.0           | 42 (48.8)  |
| Lymph node | Negative      | 22 (25.6)  |
|          | Positive       | 64 (74.4)  |
| Metastasis | Pre-menopause | 30 (34.9)  |
| Hormonal status | Post-menopause | 56 (65.1)  |
| ER       | Negative       | 27 (31.4)  |
|          | Positive       | 53 (61.6)  |
|          | Missing        | 6 (7.0)    |
| PR       | Negative       | 43 (50.0)  |
|          | Positive       | 37 (43.0)  |
|          | Missing        | 6 (7.0)    |
| HER2     | Negative       | 71 (82.6)  |
|          | Positive       | 08 (9.3)   |
|          | Missing        | 07 (8.1)   |
| TPS3 Mut | No             | 63 (73.3)  |
|          | Yes            | 13 (15.1)  |

Tumor size, lymph node status, ER, PR, and HER2: Available in 78 (89.7%) patients.

RESULTS

We investigated mutations in exon 9 and exon 20 of the PIK3CA gene in 86 primary breast tumors by performing SSCP analysis and DNA sequencing. Of the 86 tumors, 23 (27%) exhibited PIK3CA mutations: 13% in exon 9 and 14% in exon 20. Table 2 lists the PIK3CA variants identified by DNA sequencing. We characterized seven non-synonymous variants, two of which were new (I1022V, L1028S); three synonymous variants, two of which were new (S541S, L1028L); one new stop codon-gain variant (R992X); and one previously known stop codon-lost (X1069W) variant. Figure 1 shows representative electropherograms of the PIK3CA variants characterized in the primary breast tumors.

Table 2 - Observed variations in PIK3CA mutations in exons 9 and 20 in breast tumors (n = 86).

| Nucleotide | Variant ID | Residue | Variation type |
|------------|------------|---------|----------------|
| 70282G>A   | COSM763    | E545K   | Non-synonymous coding |
| 70223A>G   | COSM41783  | E525G   | Non-synonymous coding |
| 70273G>A   | COSM760    | E542K   | Non-synonymous coding |
| 70277T>G   | -          | S541S   | Synonymous coding |
| 86110C>T   | -          | R992X   | Stop codon gained |
| 86171A>G   | COSM27130  | E1012G  | Non-synonymous coding |
| 86200A>G   | -          | L1022V  | Non-synonymous coding |
| 86211C>T   | rs17849079 | T1025T  | Synonymous coding |
| 86218T>C   | -          | L1028L  | Synonymous coding |
| 86219T>C   | -          | L1028S  | Non-synonymous coding |
| 86276A>G   | COSM775    | H1047R  | Non-synonymous coding |
| 86343A>G   | COSM17449  | X1069W  | Stop codon lost |
New variants were considered mutations, as they were not present in the paired normal tissue of the same patients (data not shown). The frequency of the hotspot mutation E545K was 8.1%, corresponding to 63.6% of the helical (exon 9) mutations. The other common helical (exon 9) mutation, E542K, was observed in only one case. The kinase (exon 20) hotspot mutation H1047R was observed at a frequency of 14%, representing 91.7% of the PIK3CA exon 20 mutations.

Next, we investigated whether PIK3CA mutations were associated with breast cancer development and progression. The demographic and clinicopathological characteristics of patients with tumors containing PIK3CA mutations were compared with those of patients with tumors lacking PIK3CA mutations. There were no statistically significant differences between the clinicopathological features or steroid hormone receptor status in patients with or without PIK3CA mutations (Table 3).

Using a data set of TP53 mutations published previously by our group (17), we evaluated whether any of the 73 patients had both PIK3CA and TP53 mutations. None of the tumors with exon 9 PIK3CA mutations (PIK9$$^{\text{mut}}$$) also contained TP53 mutations. In contrast, we observed a correlation between the presence of exon 20 PIK3CA mutations (PIK20$$^{\text{mut}}$$) and TP53 mutations (Fisher’s test p-value = 0.05, Spearman’s correlation = 0.03, r = 0.253; Table 3).

We also tested whether PIK3CA mutations were associated with patient OS or DFS. A comparison of patients who had tumors with or without PIK3CA mutations revealed no significant differences in cancer-specific survival. On the other hand, when patients were grouped according to the presence of PIK3CA helical domain (exon 9) or kinase domain (exon 20) mutations, the presence of exon 20 mutations was associated with poorer OS (p = 0.026) and DFS (p = 0.079) (Table 4 and Figure 2). We further analyzed the relationship between survival and exon 20 mutations by conducting Kaplan-Meier analyses. We found that patients with tumors harboring exon 20 mutations had a significantly shorter mean OS and DFS compared with patients lacking exon 20 mutations (median OS: 24.1 months and not reached, respectively, p = 0.007; median DFS: 15.9 months and not reached, respectively, p = 0.025) (Table 4 and Figure 2).

**DISCUSSION**

No previous study has investigated the frequency and spectrum of PIK3CA mutations in primary tumors from...
Brazilian breast cancer patients. In this study, we identified PIK3CA mutations in primary breast tumors from a group of Brazilian breast cancer patients and correlated these mutations with patient clinicopathological features and outcomes. The observed frequency of PIK3CA mutations was 27%, which is in accordance with similar studies that have examined the frequency of exon 9 and 20 mutations (frequency range, 16.4 to 45%) (3,8-10). This result indicates that PIK3CA mutations are quite common genetic events in tumors in Brazilian breast cancer patients. The frequency of the most common missense activating mutations (E542K, E545K, and H1047R) in the primary breast tumors was 82.6%, the same rate previously reported in the literature (11). We also identified three new PIK3CA variants, two missense variants, and one nonsense variant. These variants were considered mutations, as they were not present in the paired normal tissue of the same patients (data not shown).

In our analysis of the relationship between PIK3CA mutations and patient clinicopathological characteristics, we found no significant correlations between PIK3CA mutations and patient age, clinical stage, tumor size, or lymph node metastasis. Some previous studies showed significant associations between PIK3CA mutations and steroid hormone (estrogen and/or progesterone) receptor status in breast cancer patients (13,14,18,19), while others failed to find such associations (12,15). Although the association between PIK3CA mutations and steroid hormone receptor status did not reach statistical significance, we observed a higher frequency of PIK3CA mutations in estrogen receptor-positive tumors compared with receptor-negative tumors, mainly in exon 9.

The association between PIK3CA mutations and breast cancer patient survival remains controversial. In the present work, we found that kinase domain (exon 20) mutations were strongly associated with poorer OS and DFS. Various studies have reported that the presence of PIK3CA mutations is associated with good prognosis (11,13), is associated with poor prognosis (14,16), or has no survival effect (18,20) in breast cancer patients. Kalinsky et al. (13) found a direct association between the presence of mutations in the C2, helical, or kinase functional domains and better DFS or OS. They also found that the H1047R mutation was strongly associated with the absence of lymph node metastasis (13). Similarly, Maruyama et al. (11) described a positive

| Variable          | Category  | PK\textsuperscript{mut} | p-value* | PK9\textsuperscript{mut} | p-value* | PK20\textsuperscript{mut} | p-value* |
|-------------------|-----------|-------------------------|----------|--------------------------|----------|--------------------------|----------|
|                   | n         | No  | Yes | No  | Yes | No  | Yes | No  | Yes |
| Age, y            | ≤55       | 45  | 34  | 11  | 0.63 | 40  | 5  | 0.75 | 39  | 6  | 1.00 |
|                   | ≥55       | 41  | 29  | 12  | 0.81 | 31  | 6  | 0.52 | 34  | 3  | 0.22 |
| Stage             | Early     | 37  | 28  | 9   | 0.33 | 34  | 5  | 1.00 | 36  | 3  | 0.21 |
|                   | Late      | 49  | 35  | 14  | 0.78 | 18  | 4  | 0.46 | 21  | 1  | 0.28 |
| TNM               | ≤4.0      | 39  | 31  | 8   | 0.19 | 28  | 2  | 0.31 | 28  | 2  | 0.53 |
|                   | >4.0      | 47  | 32  | 15  | 0.45 | 39  | 4  | 0.33 | 37  | 6  | 1.00 |
| Tumor size, cm    | <4.0      | 64  | 46  | 18  | 0.20 | 63  | 8  | 1.00 | 62  | 9  | 0.10 |
|                   | ≥4.0      | 56  | 38  | 18  | 0.71 | 52  | 11 | 0.34 | 55  | 8  | 0.05 |
| Lymph node        | Negative  | 22  | 17  | 5   | 0.12 | 21  | 1  | 0.31 | 20  | 2  | 0.46 |
|                   | Positive  | 44  | 37  | 7   | 0.44 | 36  | 6  | 0.79 | 35  | 4  | 1.00 |
| Hormonal status   | Pre-menopause | 30  | 25  | 5   | 0.19 | 28  | 2  | 0.31 | 28  | 2  | 0.53 |
|                   | Post-menopause | 56  | 38  | 18  | 0.71 | 52  | 11 | 0.34 | 55  | 8  | 0.05 |
| PR                | Negative  | 27  | 22  | 5   | 0.19 | 26  | 1  | 0.09 | 23  | 4  | 1.00 |
|                   | Positive  | 53  | 35  | 18  | 0.45 | 39  | 4  | 0.33 | 37  | 6  | 1.00 |
| ER                | Negative  | 43  | 33  | 10  | 0.20 | 63  | 8  | 1.00 | 62  | 9  | 0.10 |
|                   | Positive  | 71  | 54  | 17  | 0.71 | 52  | 11 | 0.34 | 55  | 8  | 0.05 |
| HER2              | Negative  | 8   | 4   | 4   | 0.71 | 52  | 11 | 0.34 | 55  | 8  | 0.05 |
|                   | Positive  | 10  | 6   | 4   | 0.71 | 52  | 11 | 0.34 | 55  | 8  | 0.05 |

*Fisher’s exact test.
correlation between the presence of mutations in any domain of the PIK3CA gene and better relapse-free survival. Taken together, these studies suggest a protective role for these mutations. On the other hand, similar to our study, two other studies reported that exon 20 mutations were associated with poorer OS (14,16). It is difficult to compare these studies because of the studies’ population heterogeneity and because there may have been differences in the therapeutic strategies not mentioned in the publications.

Mutations in TP53 and PIK3CA are frequent in breast cancer (21). In the present study, we found a positive correlation between the presence of PIK3CA exon 20 and
mutations, with four samples exhibiting mutations in both genes. This result suggests that the presence of these mutations is not mutually exclusive, as was proposed by Boyault et al. (19). We previously reported that patients with tumors harboring TP53 mutations affecting amino acids involved directly in DNA or zinc binding had a poor prognosis (17). Interestingly, in this study, we found that the presence of PIK3CA exon 20 mutations could be used to stratify patients into distinct prognostic groups, regardless of whether a TP53 mutation was present.

In summary, this is the first study to report that PIK3CA mutations are common in tumors in Brazilian breast cancer patients. We found that PIK3CA exon 20 mutations were significantly associated with TP53 mutations, indicating that PIK3CA mutations and TP53 mutations are not mutually exclusive. Our finding that PIK3CA exon 20 mutations were associated with more aggressive breast cancer and poor outcomes, regardless of the treatment regimen, has important clinical implications.

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AUTHOR CONTRIBUTIONS

Nagai MA conceived the study’s aims and design and performed the data analysis, manuscript preparation, manuscript editing and review. Bobrovitchaia IG, Salaorni S, and Manuli E carried out the experiments and data acquisition. Mangone FR carried out the literature research, data acquisition, data analysis, statistical analysis, and manuscript preparation. All authors read and approved the manuscript.

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