CLINICAL SCIENCE

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

Flavia R. Mangone,[I,II] Irina G. Bobrovnitchaia,[I,II] Sibeli Salaorni,[I,II] Erika Manuli,[I,II] Maria A. Nagai[I,II]

[I] Faculdade de Medicina da Universidade de São Paulo, Disciplina de Oncologia, Departamento de Radiologia e Oncologia São Paulo/SP, Brazil. [II] Instituto do Câncer do Estado de São Paulo (ICESP) - Laboratório de Genética Molecular do Centro de Investigação Translacional em Oncologia, São Paulo/SP, Brazil.

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-kinase*CA* 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 exons 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.

Mangone FR, Bobrovnitchaia IG, Salaorni S, Manuli E, Nagai MA. *PIK3CA* exon 20 mutations are associated with poor prognosis in breast cancer patients. Clinics. 2012;67(11):1285-1290.

Received for publication on July 9, 2012; **First review completed on** July 17, 2012; **Accepted for publication on** July 23, 2012

E-mail: nagai@usp.br

Tel.: 55 11 3893-3013

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

No potential conflict of interest was reported.

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,	<4.0	44 (51.2)
cm	≥4.0	42 (48.8)
Lymph node	Negative	22 (25.6)
Metastasis	Positive	64 (74.4)
Hormonal	Pre-menopause	30 (34.9)
status	Post-menopause	56 (65.1)
ER	Negative	27 (31.4)
	Positive	53 (61.6)
	Missing	06 (7.0)
PR	Negative	43 (50.0)
	Positive	37 (43.0)
	Missing	06 (7.0)
HER2	Negative	71 (82.6)
	Positive	08 (9.3)
	Missing	07 (8.1)
TP53^{Mut}	No	63 (73.3)
	Yes	10 (11.6)
	Missing	13 (15.1)

TNM: tumor, nodes, and metastases; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

Mutation analysis by polymerase chain reaction-single stranded conformation polymorphism (PCR-SSCP) and direct DNA sequencing

Two sets of primers were used to amplify exon 9 (forward: 5′-CCAGAGGGGAAAAATATGACA-3′; reverse: 5′-CATTTTAGCACTTACCTGTGAC-3′) and exon 20 (forward: 5′-CATTTGCTCCAAACTGACCA-3′; reverse: 5′-TGAGCTTTCATTTTCTCAGTTATCTTTTC-3′) of the *PIK3CA* gene. The PCR products were separated using the GeneGel^{TM} Excel 12.5/24 Kit (GE Healthcare, Sweden) according to the manufacturer's instructions. Gels were stained using the DNA Silver Staining Kit (GE Healthcare) according to the manufacturer's instructions. Samples exhibiting differences in gel band mobility were cloned (TOPO-TA Cloning® Kit, Invitrogen) and then sequenced using a MegaBACE 1000 automatic sequencer (Amersham Biosciences) and the ET Dye Terminator Kit (Amersham Biosciences). All sequences were analyzed using Mutation Surveyor software v3.2 (SoftGenetics).

Statistical analysis

Fisher's exact test and Spearman's rho correlation were used to assess the association and the direction of the association, respectively, among categorical variables. OS and DFS rates were calculated based on the Kaplan-Meier method, and the curves were compared using the log-rank test. OS and DFS rates were determined from the day of the diagnosis to the date of death or to the date on which recurrence was detected, respectively.

Statistical analyses were performed using IBM SPSS Statistics 19.0, 2010 (SPSS Chicago, IL). Differences were considered significant when the *p*-value was less than 0.05.

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-loss (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	Variation 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
70272T>G	-	S541S	Synonymous coding
86110C>T	-	R992X	Stop codon gained
86171A>G	COSM27130	E1012G	Non-synonymous coding
86200A>G	-	I1022V	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

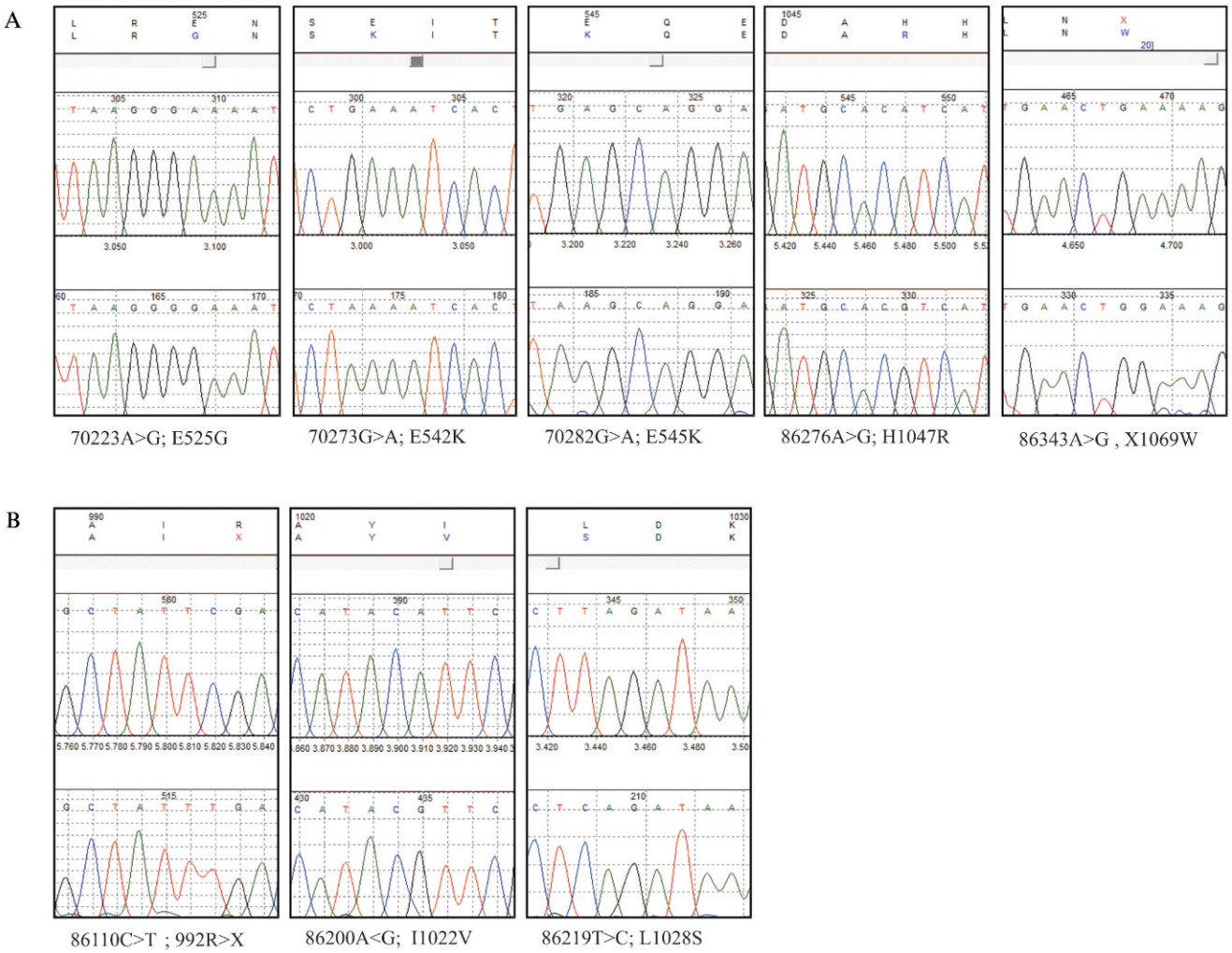

Figure 1 - Representative eletropherograms of the *PIK3CA* mutations characterized in the breast cancer biopsy samples in this study. (A) Known mutations and (B) new mutations.

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 ($PIK9^{mut}$) also contained *TP53* mutations. In contrast, we observed a correlation between the presence of exon 20 *PIK3CA* mutations ($PIK20^{mut}$) 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

Table 3 - Association between the presence of *PIK3CA* mutations and patient and tumor characteristics.

Variable	Category		PIK^{mut}		*p*-value*	$PIK9^{mut}$		*p*-value*	$PIK20^{mut}$		*p*-value*
		n	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		35	6		35	6	
Stage,	Early	37	28	9	0.81	31	6	0.52	34	3	0.22
TNM	Late	49	35	14		44	5		40	9	
Tumor size,	<4.0	39	31	8	0.33	34	5	1.00	36	3	0.21
Cm	≥4.0	47	32	15		41	6		38	9	
Lymph node	Negative	22	17	5	0.78	18	4	0.46	21	1	0.28
metastasis	Positive	64	46	18		57	7		53	11	
Hormonal status	Pre-menopause	30	25	5	0.13	28	2	0.31	28	2	0.53
	Post-menopause	56	38	18		47	9		47	9	
ER	Negative	27	22	5	0.19	26	1	0.09	23	4	1.00
	Positive	53	35	18		43	10		45	8	
PR	Negative	43	33	10	0.45	39	4	0.33	37	6	1.00
	Positive	37	25	12		30	7		32	5	
HER2	Negative	71	54	17	0.20	63	8	1.00	62	9	0.10
	Positive	8	4	4		7	1		5	3	
$TP53^{mut}$	No	63	44	19	0.71	52	11	0.34	55	8	0.05
	Yes	10	6	4		10	0		6	4	

*Fisher's exact test.

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

Table 4 - Association between *PIK3CA* mutations and patient survival.

Category		*n*	Survival rate	Overall survival		Disease-free survival	
				Median	Log rank *p*-value	Median	Log rank *p*-value
PIK^{mut}	No	50	70.0	NR	0.163	NR	0.257
	Yes	22	54.5	NR		NR	
PIK^{mut}	No	50	70.0	NR	0.026	NR	0.079
	PIK9	10	70.0	NR		NR	
	PIK20	12	41.7	24.1		15.9	
$PIK9^{mut}$	No	62	64.5	NR	0.548	NR	0.531
	Yes	10	70.0	NR		NR	
$PIK20^{mut}$	No	60	70.0	NR	0.007	NR	0.025
	Yes	12	41.7	24.1		15.9	

Median survival reported in months; NR: not reached.

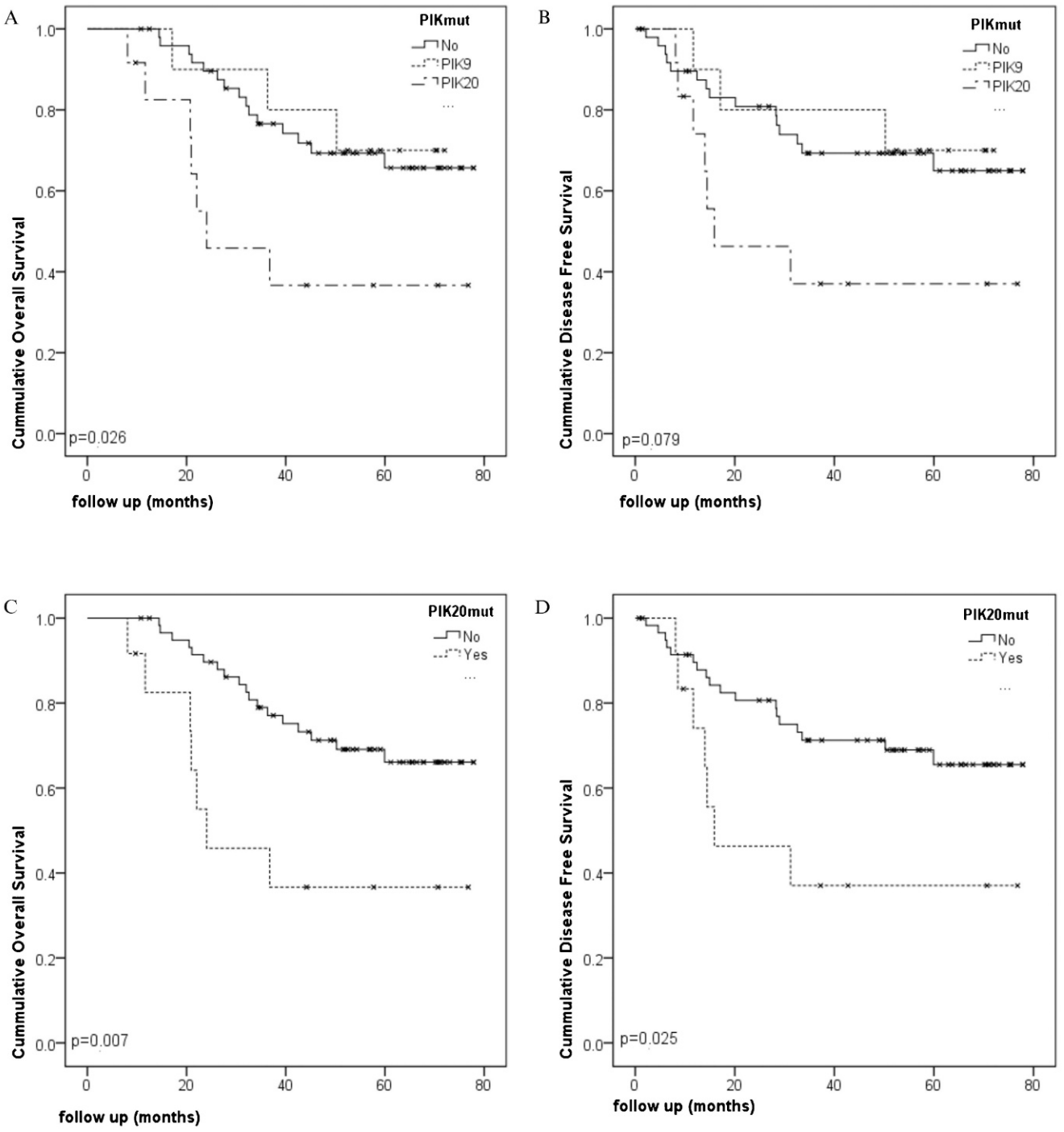

Figure 2 - Kaplan-Meier curves showing long-term survival in primary breast cancer patients, stratified according to *PIK3CA* mutation status. (A) Overall survival and (B) disease-free survival curves were calculated for the stratified patient groups. 'No' indicates patients with tumors with no *PIK3CA* mutations; 'PIK9' indicates patients with tumors with *PIK3CA* mutations in exon 9; and 'PIK20' indicates patients with tumors with *PIK3CA* mutations in exon 20. (C) Overall survival and (D) disease-free survival curves were calculated for the stratified patient groups. 'No' indicates patients with tumors with no *PIK3CA* mutations in exon 20, and 'Yes' indicates patients with tumors with *PIK3CA* mutations in exon 20. *p*-values were calculated using the log-rank 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

TP53 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.

ACKNOWLEDGMENTS

This study was funded by a grant from the Departamento de Ciência e Tecnologia-Ministério da Saúde/Conselho Nacional de Desenvolvimento Científico e Tecnológico (grant number 577587/2008-0 DECIT/CNPq) and by a CNPq grant (grant number 305408/2009-7).

AUTHOR CONTRIBUTIONS

Nagai MA conceived the study's aims and design and performed the data analysis, manuscript preparation, manuscript editing and review. Bobrovnitchaia 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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