Different Prognostic Roles of Mutations in the Helical and Kinase Domains of the *PIK3CA* Gene in Breast Carcinomas

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Abstract

Purpose: In breast cancer, the *PIK3CA* gene is frequently mutated at "hotspots" in exons 9 and 20, corresponding to the helical and kinase domains, respectively. We decided to investigate the association of *PIK3CA* mutations with pathologic features and clinical outcome in a large series of patients with breast cancer.

Experimental Design: Frozen samples from 163 consecutive patients were analyzed for *PIK3CA* mutations using PCR single-strand conformation polymorphism and sequence analyses.

Results: We identified 46 missense mutations, 24 (53%) in exon 9, and 21 (47%) in exon 20. Twelve (50%) of the 24 mutations in exon 9 were of the E542K type and 11 (46%) were of the E545K type. Twenty (95%) of the 21 mutations in exon 20 were H1047R substitutions. Mutations in exon 9 were more frequent in lobular carcinomas (42% of cases) than in ductal carcinoma (11% of cases; $P = 0.002$). At univariate survival analysis, *PIK3CA* exon 20 mutations were associated with prolonged overall and disease-free survival, whereas mutations in exon 9 were associated with significantly worse prognosis. At multivariate analysis, exon 9 *PIK3CA* mutations were the strongest independent factor to predict poor prognosis for disease-free survival ($P = 0.0003$) and overall survival ($P = 0.001$).

Conclusion: Our data show that exon 9 *PIK3CA* mutations are typical of infiltrating lobular carcinomas. In addition, they indicate that *PIK3CA* mutations in different exons are of different prognostic value: exon 9 mutations are independently associated with early recurrence and death, whereas exon 20 *PIK3CA* mutations are associated with optimal prognosis.

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The study of the mechanisms and of the alterations of signal transduction through tyrosine kinase growth factor receptors (TKGFR) is one of the most promising fields of translational research in oncology. The molecular cascade of events which is triggered by TKGFR activation is complex and includes the interplay between several metabolic pathways. In many human tumors, including breast cancer, TKGFR constitutive hyperactivity is a key pathogenetic event of clinical and therapeutic relevance. Specific inhibitors of TKGFR are already in clinical use for several kinds of human tumors and many more are in the preclinical research phase. The development of drugs interfering with the TKGFR downstream molecular events may be an additional strategy to find new agents which could be of great help in cancer therapy (1).

Among the most important downstream molecular events following TKGFR activation is the activation of class A phosphoinositide-3-kinases (PI3K), which catalyze the phosphorylation of inositol lipids to produce phosphatidylinositol-3,4,5-trisphosphate (2). Phosphatidylinositol-3,4,5-trisphosphate activates the serine/threonine kinase AKT which in turn regulates several signaling pathways controlling cell survival, apoptosis, proliferation, motility, growth, and cytoskeletal rearrangement. Gain of function of the PI3K-AKT pathway may have pathogenic effects in human neoplasia, and some of these functional derangements could be candidate targets for cancer therapy (1, 3, 4).

The typical form of PI3K is a heterodimer with an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. The 85 kDa regulatory subunit can bind to phosphotyrosine residues of activated growth factors inducing activation of the lipid kinase activity of the 110 kDa catalytic subunit. The catalytic subunit is encoded by the *PIK3CA* gene, which is amplified, overexpressed, or mutated in several human malignancies and might have oncogenic activity in humans (5). In experimental breast cell line models, *PIK3CA* gene mutations increase kinase activity, promote cell growth and proliferation, are associated with metastatic capability, and confer increased resistance to

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paclitaxel (6–8). In human breast carcinomas, *PIK3CA* is frequently mutated at "hotspots" in exons 9 and 20, corresponding to the helical (E542K and E545G) and kinase (H1047R) domains, respectively (5, 9). Multiple studies have shown that these mutations are observed in 18% to 40% of breast cancers, and recent data suggest that they may be related to poor clinical outcome (10). We have recently shown that mutations in exon 9 are frequent in invasive lobular carcinomas (ILC), although they are rare in infiltrating ductal carcinomas (IDC) and in other subtypes of breast cancers; conversely, mutations in exon 20 are randomly seen in all breast cancer histotypes (9). These differences in association between major histologic types and exon 9 and exon 20 mutations suggest different biological, and possibly, clinical significance of mutations occurring in different functional domains of the PI3K molecule.

In the present study, we investigated *PIK3CA* status in a new large series of histologically well-characterized samples of human breast carcinomas with long-term clinical data concerning treatments and outcome. We investigated the association of different *PIK3CA* mutations with pathologic and biological features of the tumors, and with clinical outcome. Our study confirms the association of exon 9 *PIK3CA* mutations with the lobular histotype, and shows that *PIK3CA* mutations in different exons are of different prognostic value.

Materials and Methods

Tissue samples. We investigated 163 primary breast carcinomas, consecutively collected at the S. Chiara Hospital in Trento between 1995 and 2001. All cases were received fresh within 10 min from surgical excision, and a fragment of tumor tissue was snap-frozen in liquid nitrogen. The rest of the samples were formalin-fixed and paraffin-embedded. Cases were staged according to the International Union Against Cancer tumor-node-metastasis staging system (6th edition). Pathologic data are reported in Table 1 (*first column*). Informed consent from all patients and approval from the ethical committee were obtained.

Genetic analysis. Frozen material was processed for molecular study after confirmation of the nature of the tissue (tumoral versus normal tissue, tumor type, percentage of tumor cells) using frozen section analyses. Genomic DNA was extracted from frozen samples according to standard procedures (11). Genetic analysis of the *PIK3CA* gene was done by PCR amplification of exons 9 and 20 with flanking intronic sequences and single-strand conformation polymorphism (SSCP) analysis, followed by sequencing of positive cases. Details of the primers used for amplification are available on request. PCR was done in a total volume of 10 μL containing 1× TaqMan buffer, 1.5 mmol/L of $MgCl_2$, 800 μmol/L of deoxynucleotide triphosphates, 300 nmol/L of each primer, 0.3 units of Taq DNA polymerase, and 10 ng of genomic DNA. The thermal cycling conditions included 4 min at 95°C, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, and one cycle of 72°C for 7 min. A nonradioactive SSCP assay was devised to screen for mutations, as previously described (12). Briefly, after completion of the PCR reaction, the product was diluted 1:5 in loading buffer [95% formamide, 2 mmol/L EDTA (pH 8.3)]. Fifteen microliters of the diluted samples were denatured (5 min at 90°C), immediately cooled on ice and loaded onto a 12% nondenaturing polyacrylamide gel in the presence of 5% glycerol. Electrophoresis was carried out for 14 h at 20°C at 3 W. Upon complete migration, the gels were subjected to silver staining using the PlusOne Silver Staining Kit (Amersham Pharmacia Biotech). Cases showing aberrant bands were considered as potentially mutated. To confirm the mutations, these cases were re-amplified starting from genomic DNA and subjected to SSCP and sequencing.

Table 1. *PIK3CA* mutations and clinicopathologic variables of breast carcinoma

Variable	Total cases (*N* = 163)	Mutated (*n* = 45)	Normal (*n* = 118)	*P*
Age				
Years (mean ± SD)		60.5 ± 14.2	62.8 ± 15.5	NS (0.4)
Tumor size				
T_1	79	21 (27%)	58 (73%)	NS (0.86)
T_2	62	19 (31%)	43 (69%)	
T_3	6	1 (17%)	5 (83%)	
T_4	16	4 (25%)	12 (75%)	
N status				
N_0	78	25 (32%)	53 (68%)	NS (0.29)
N_+	85	20 (23%)	65 (77%)	
Histologic type				
Lobular	19	10 (53%)	9 (47%)	0.014
Ductal	144	35 (24%)	109 (76%)	
Mib1 expression				
Low	43	16 (37%)	27 (63%)	NS (0.1)
High	120	29 (24%)	91 (76%)	
ER expression				
Positive	137	38 (28%)	99 (72%)	NS (1)
Negative	26	7 (27%)	19 (73%)	
PgR expression				
Positive	98	27 (28%)	71 (72%)	NS (1)
Negative	65	18 (28%)	47 (72%)	
cErbB2 expression (HercepTest)				
Positive	26	8 (31%)	18 (69%)	NS (0.81)
Negative	137	37 (27%)	100 (73%)	
p53 expression				
Positive	43	11 (26%)	32 (74%)	NS (0.84)
Negative	120	34 (28%)	86 (72%)	

Abbreviation: NS, not significant.

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For sequence analysis, PCR products were purified by Multiscreen 384-PCR filter plate (Millipore Corporation) and subjected to bidirectional dye-terminator sequencing using the same primers employed for amplification. Sequencing fragments were detected by capillary electrophoresis using the ABI Prism 3100 DNA analyzer (Applied Biosystems). Sequence chromatograms were analyzed by Mutation Surveyor 3.0 (SoftGenetics), followed by manual review.

Immunohistochemical analysis. Paraffin sections were immunostained for estrogen and progesterone receptor status (6F11 and 1A4; Labvision), Ki67 (MIB1; DakoCytomation), and p53 (D07, Labvision). Cases were considered positive for estrogen receptor (ER), progesterone receptor (PgR), and p53 expression if at least 10% of tumor cells showed nuclear reactivity. The percentage of immunoreacting nuclei Ki67 immunostaining was evaluated: the median percentage was used to separate high versus low labeled cases. Lack of E-cadherin (36B5; Labvision) immunostaining was used to confirm the lobular histotype of selected cases. The HercepTest (DakoCytomation) was used to investigate c-ErbB-2 expression, according to the manufacturer's directions: score 0/1+ cases were recorded as negative and 2+/3+ as positive.

Clinical data. All patients underwent curative tumor resection (quadrantectomy plus postoperative radiotherapy or mastectomy) with axillary lymph node dissection. The age of patients ranged from 17 to 89 (median, 62 years). All patients, except three (due to age >80 years), received adjuvant chemotherapy and/or hormone therapy. Chemotherapy was offered to 29 out of 78 lymph node–negative patients, and in 72 out of 85 node-positive patients. Chemotherapy regimens consisted of cyclophosphamide, methotrexate and 5-fluoruracil (30% of patients) or anthracycline with (9% of patients) or without taxane (17% of patients). Patients with positive hormonal receptors received tamoxifen (68% of patients) or aromatase inhibitors (3% of patients) for 5 years. Seven young patients (<45 years) received, besides tamoxifen, ovarian ablation/suppression, after chemotherapy without treatment-induced amenorrhea.

Data analysis: statistical tests and survival analysis. Association of variables were assessed using Fisher or χ^2 tests and by logistic regression analysis. Disease-free survival (DFS) and overall survival (OS) curves were generated by the Kaplan-Meier method, and differences between survival curves were assessed for statistical significance using the log-rank test. Multivariate analysis was carried out using Cox proportional hazards regression. The *P* values were two-sided, and differences were considered significant at $P \leq 0.05$. Analyses were done with the SPSS software package (SPSS, Inc.).

Results

Incidence and nature of PIK3CA mutations in breast carcinomas. The genomic status of the *PIK3CA* gene was evaluated in 163 primary breast carcinomas by PCR-SSCP analysis of exons 9 and 20. All cases positive by SSCP were subjected to independent PCR amplification and direct sequencing of the PCR product. Bands of mobility shift, indicating the presence of *PIK3CA* mutations, were present in 45 (28%) cases (Table 1). PCR-SSCP analysis of matching normal samples from the same patients showed no evidence of structural aberration of the *PIK3CA* gene. Twenty-four (53%) of the 46 mutations were located in exon 9, and 21 mutations (47%) were in exon 20 (Table 2). Sequence analysis of SSCP-positive cases revealed that all of the mutations observed were missense mutations. Twelve (50%) of the 24 mutations in exon 9 were of the E542K type, and 11 mutations (46%) were of the E545K type. Nineteen (90%) of the 21 mutations in exon 20 were H1047R substitutions (Table 3).

Correlation with clinicopathologic variables. The frequency of *PIK3CA* mutations was significantly higher in lobular carcinomas (53%) than in ductal carcinomas (24%; $P = 0.014$; Table 1). In particular, mutations in exon 9 were more frequent in lobular carcinomas (42% of cases) than in ductal carcinoma (11% of cases; $P = 0.002$), whereas no significant differences were observed in the distribution of mutations in exon 20 in these two histotypes (Table 2). There were no significant correlations between *PIK3CA* mutations and other clinicopathologic and biological variables (Table 1). Analogously, no associations with different clinicopathologic features were seen when cases with mutations in exon 9 or cases with mutations in exon 20 were analyzed separately (data not shown).

The association of the lobular histotype with *PIK3CA* mutations, as a dependent variable, was also evaluated by logistic regression analysis to take into consideration the effects of the other covariates investigated (Table 4). Only the lobular histology of the tumor reached statistical significance as an independent factor associated with the presence of mutations within the *PIK3CA* gene (odds ratio, 6.18; $P = 0.002$).

According to the Kaplan-Meier survival curves, the 5-year survival rate in the series of patients examined was 82%. The median time to recurrence was 35 months (range, 6-79 months) and the recurrence rate was 36 of 164 (22%).

Univariate survival analysis showed no significant correlation between OS or DFS and *PIK3CA* gene mutations (exon 9 + exon 20; Table 5). However, analyzing the significance of mutations in the two different hotspots, we observed that patients with exon 20 mutations showed significantly better OS and DFS as compared with patients without any *PIK3CA* mutation (5-year OS rates for normal versus exon 20–mutated *PIK3CA* were 83% and 100%, respectively; $P = 0.011$; 5-year DFS rates for normal versus exon 20–mutated PIK3CA were 77% and 100%, respectively; $P = 0.010$). On the other hand, patients with exon 9 mutations were associated with worse prognosis as compared with patients without *PIK3CA* mutations (5-year OS rates for patients with normal versus exon 9–mutated PIK3CA were 83% and 62%, respectively; $P = 0.018$; 5-year DFS rates for patients with normal versus exon 9–mutated PIK3CA were 77% and 61%, respectively; $P = 0.040$; Fig. 1). Highly significant differences were observed between the OS and DFS of patients with exon 9 or exon 20 mutations in their tumors ($P = 0.0018$ and $P = 0.0017$, respectively).

Table 2. Functional type of PIK3CA mutations *PIK3CA* mutations and histologic types of infiltrating breast carcinomas

Tumor type	No. of cases	Exon 9 PIK3CA mutations (%)	*P*	Exon 20 PIK3CA mutations (%)	*P*
Lobular	19	8 (42)		2 (10)	
Ductal NOS	144	16 (11)	$P = 0.002$	19 (13)	NS, $P = 1$
Total	163	24 (15)		21 (13)	

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Table 3. Type of *PIK3CA* mutations

PIK3CA mutations		Histologic type	
Exon	Nucleotide change (amino acid change)	Lobular (19 cases)	Ductal (144 cases)
9	G1624A (E542K)	5	7
9	G1633A (E545K)	3	8
9	A1634G (E545G)	—	1
20	A3140G (H1047R)	2	17
20	A3140T (H1047L)	—	2
	Total (%)	10 (53)	35 (24)

In univariate analysis, we observed a significant correlation between OS or DFS and T status and node status (Table 5). The joint effect of the different variables were examined using stepwise Cox regression. The multivariate analysis confirmed that *PIK3CA* mutations at exon 9 were the strongest factors to predicting poor prognosis for both DFS ($P = 0.0003$) and OS ($P = 0.001$; Table 6). *PIK3CA* mutations at exon 20 were not subjected to Cox regression analysis due to the absence of events in the series of patients with *PIK3CA* mutations in this exon.

Discussion

PI3K-AKT pathway alterations have been shown to be linked to cancer development, and *PIK3CA* mutations are among the most commonly mutated genes identified in human cancers (8, 4) and are among the most frequent mutational events in breast cancer (5, 13). In breast carcinomas, *PIK3CA* is mutated in 18% to 40% of cases. Almost all *PIK3CA* mutations involve hotspots on exons 9 and 20, and are mutually exclusive with other gene alterations involved in the same pathway, supporting the important pathogenetic role of this pathway in breast cancer (14).

In the present study, we confirm the high frequency of *PIK3CA* mutations in human breast cancer, occurring in 28% of our cases, in keeping with literature data (5, 9, 10, 13–15). In our series, the frequency of exon 9 and exon 20 mutations were similar (15% and 13%), but mutations in exons 9 and 20 were differently distributed among different morphologic tumor types. Although exon 20 mutations were seen at similar frequencies in ILCs and in IDCs, exon 9 mutations were more frequent in ILC, in keeping with our previous observation in another breast cancer series from a different institution (9) and with the study of Maruyama et al. (15). Our data show that exon 9 *PI3KCA* mutation is one of the most frequent genetic lesions of ILC, occurring in 42% of cases, and seems characteristic of this tumor type. Molecular differences between ILC and IDC are of relevance in understanding the different clinical and pathologic characteristics of these types of breast cancers. Compared with IDC, ILCs are significantly more likely to occur in older patients, to be larger in size and multicentric, to recur and involve contralateral breast, to involve regional lymph nodes with single or small clusters of tumor cells, and to metastasize to the gastrointestinal tract and ovary. Besides histomorphologic differences between ILC and IDC (lower grade and single cell infiltrative pattern in ILC), ILCs are usually more frequently ER- and PgR-positive, lack Her2/neu overexpression/amplification, have a low frequency of p53 gene mutation, low vascular endothelial growth factor expression, and lack expression of the E-cadherin molecule (16–18). Our data supporting the important role of PI3K-AKT pathway alterations in breast cancer, suggest that additional studies should focus on its role in the pathogenesis of different breast tumor types, focusing not only on infiltrating carcinomas but also on benign proliferative lesions and *in situ* ductal and lobular carcinomas.

The association of *PI3KCA* mutation with pathologic and biological features of breast carcinomas is still a matter of debate: in the present study, we did not find any relation with

Table 5. Univariate survival analysis

Variable	Overall survival, *P*	Disease-free survival, *P*
PIK3CA, all mutations	NS (0.754)	NS (0.613)
PIK3CA, exon 20 mutations	0.011	0.01
PIK3CA, exon 9 mutations	0.018	0.04
Tumor size, T_{1-2}/T_{3-4}	0.018	0.024
Node status, N_0/N_+	0.044	0.019
Histologic type, ductal/lobular	NS (0.718)	NS (0.671)
ER expression, positive/negative	NS (0.154)	NS (0.1)
PgR expression, positive/negative	NS (0.058)	NS (0.05)
HER2 expression, positive/negative	NS (0.420)	NS (0.407)
p53 expression, positive/negative	NS (0.365)	NS (0.286)

Table 4. Association between mutations in exon 9 of the *PIK3CA* gene and independent covariates computed by logistic regression analysis

Covariates	β Coefficient	Estimated OR (95% CL)	*P*
Tumor size, T_1/T_2	0.83	2.30 (0.61-8.70)	NS (0.21)
N status, N_0/N_+	0.23	1.26 (0.47-3.38)	NS (0.65)
Histologic type, ductal/lobular	1.82	6.18 (1.93-19.82)	0.002
Mib1 expression, low/high	1.05	2.87 (0.93-8.85)	NS (0.07)
ER expression, positive/negative	0.12	1.12 (0.16-7.98)	NS (0.91)
PgR expression, positive/negative	0.43	1.54 (0.49-4.80)	NS (0.46)
HER2 expression, positive/negative	0.82	2.27 (0.34-15.19)	NS (0.34)
p53 expression, positive/negative	0.03	1.03 (0.26-4.15)	NS (0.96)

Abbreviations: OR, odds ratio; CL, confidence limits; NS, not significant.

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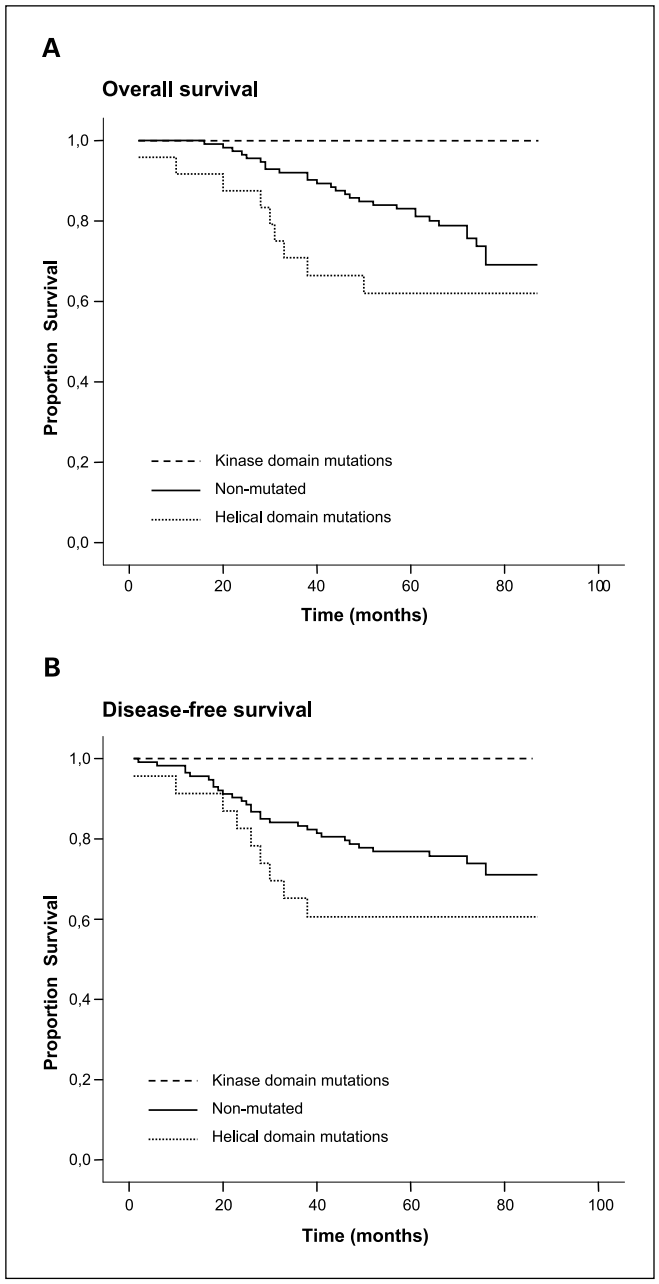

Fig. 1. Overall (*A*) and disease-free (*B*) survival curves in the 163 breast carcinoma patients according to the mutational status of the *PIK3CA* gene in primary tumors. Curve differences are statistically significant (see text for details).

grade, size, nodal status, ER and PgR status, proliferative activity, and Her2/neu overexpression. Data from literature are conflicting: in some, but not all studies, *PI3KCA* mutations are associated with positive ER and PgR status, low Ki67/MIB1 labeling, negative Her2/neu expression, and positive nodal status (9, 10, 14). However, these associations are frequently not very robust and may be influenced by the size and characteristics of the examined series of cases, as observed for example in the study of Saal et al., who compared the results obtained from two different series of cases (14). Some of these discrepancies could be related to different proportions of ILC in the examined series, and could be related to the typical phenotype of ILC. In fact, in our previous study, *PI3KCA* mutations were associated with negative Her2/neu expression and low Ki67 labeling, but using logistic regression analysis, we showed that these relations were dependent on the association of *PI3KCA* mutations with the ILC histotype (9).

In the present series, we show that individual *PIK3CA* mutations have profoundly different clinical significance: the 21 cases with exon 20 mutations had an excellent prognosis with no recurrences or deaths in the observed period, whereas among the 24 cases with exon 9 mutations, there were 10 recurrences and 9 deaths. Exon 9 mutations were of independent prognostic value at multivariate analysis, with a hazard ratio for recurrence and death which was almost twice the hazard ratio for nodal status. Our data are intriguing, as the different clinical outcomes of patients with mutations in the helical (exon 9) or kinase (exon 20) domains of *PIK3CA* could indicate different biological effects of the two types of mutations, and could be relevant in terms of potential therapeutic approaches. *In vitro* and *in vivo* studies suggest that both kind of mutations enhance kinase activity and promote cell transformation and metastatic behavior. However, the mechanisms by which the activity of PIK3 is increased and the effects of such mechanisms may be different in the two different mutants. PI3K is a heterodimer with an 85 kDa regulatory subunit (p85) and a 110 kDa (p110) catalytic subunit. A profound effect on PI3K enzymatic activity is caused by intersubunit serine phosphorylation. Indeed, it has been shown that the intrinsic protein kinase activity of PI3K is capable of phosphorylating the p85α subunit, resulting in a marked decrease in PI3K activity. This is likely to represent a feedback mechanism to shut off the growth factor signaling. It has been shown that the helical domain mutants (exon 9, E542K, E545K) of p110 bind to p85, but are not inhibited by p85. Thus, E542K and E545G mutations might cause a disruption of the p85 nSH2-p110 interface, leading to a loss of p110 inhibition and constitutive PI3K activity (19). On the other hand, kinase domain mutants (H1047R) may directly activate the catalytic subunit of PI3K (7, 8), but in this case, the p85 inhibitory mechanism may still be active. In keeping with this hypothesis, it has recently been reported that the activation of the PI3K pathway *in vitro* is slightly lower when cells harbor kinase domain mutations (7, 20). In addition, to explain our findings of lower aggressiveness for exon 20–mutated cases, it could be hypothesized that exon 20 mutation *in vivo* causes a disruption or acquisition of an abnormal protein-protein interaction, which might have different effects from the ones observed in experimental models.

Only a few other studies have investigated the clinical significance of *PIK3CA* mutations, and have produced conflicting results. Li et al. suggest that mutations in any part of the gene may be related to poor clinical outcome, but their prognostic value is not independent from other pathologic and biological variables (10). Conversely, the recent study of Maruyama et al. (15), suggests that *PIK3CA* mutations are significantly and independently associated with favorable relapse-free survival. In the light of our present results, these discrepancies could be related to the different proportions of cases mutated in different exons included in both studies.

Our present study is of relevance concerning potentially therapeutic approaches, as it suggests that *PIK3CA* exon 9 mutations could be the most important targets for PI3K-inhibiting

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Table 6. Multivariate analysis of prognostic variables for DFS and OS

Variable	Overall survival				Disease-free survival			
	β	SE	HR (95% CI)	*P*	β	SE	HR (95% CI)	*P*
Exon 9 mutations, positive/negative	1.67	0.46	5.32 (2.14-13.22)	0.0003	1.53	0.48	4.61 (1.80-11.82)	0.001
Tumor size, T_1/T_2	0.85	0.50	2.34 (0.88-6.17)	NS (0.09)	0.66	0.50	1.94 (0.72-5.21)	NS (0.19)
N status, N_0/N_+	0.94	0.41	2.57 (1.16-5.69)	0.02	1.05	0.42	2.85 (1.26-6.42)	0.012
Histologic type, ductal/lobular	1.17	0.67	3.23 (0.87-11.97)	NS (0.08)	1.07	0.67	2.90 (0.78-10.78)	NS (0.11)
ER expression, positive/negative	0.06	0.55	1.06 (0.36-3.11)	NS (0.92)	0.19	0.53	1.22 (0.43-3.41)	NS (0.71)
PgR expression, positive/negative	1.02	0.40	2.78 (1.27-6.09)	0.01	0.97	0.40	2.64 (1.20-5.82)	0.02
HER2 expression, positive/negative	0.68	0.62	1.98 (0.59-6.62)	NS (0.27)	0.75	0.62	2.12 (0.63-7.16)	NS (0.23)
p53 expression, positive/negative	0.13	0.42	1.13 (0.50-2.56)	NS (0.76)	0.13	0.41	1.14 (0.51-2.52)	NS (0.75)

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval; NS, not significant.

drugs. Because mutational activation of *PIK3CA* is essential for tumor growth, the search for pharmacologic inactivation of mutant forms of *PIK3CA* is becoming a very attractive field of research. Targeting PI3K with appropriately designed drugs could be a new therapeutic strategy, which could also be combined with other drugs interfering with the same pathway, such as rapamycin-based mTOR inhibitors (1). Promising preliminary results have been reported with the broadly acting PI3K inhibitor, LY294002, which preferentially inhibits mutated *PIK3CA* as compared with wild-type PI3K *in vitro* (6). However, because the PI3K pathway is a key part of the body's metabolic processes, these drugs may have side effects that will need to be considered and managed. An effort to treat cancer by inhibiting PI3K may also affect the patient's response to insulin, and therefore, nontoxic small molecule inhibitors that specifically target major mutant forms of *PIK3CA* should be developed (4). It can also be hypothesized to combine classical chemotherapy with targeted therapy: classical chemotherapy, such as doxorubicin and taxol-based therapies, targets cellular events such as DNA replication and cell division which are downstream of the targets of signal transduction pathway inhibitors. Combining classical chemotherapy with targeted therapy, it could be possible to enhance therapeutic benefits and lower the effective concentrations of chemotherapeutics necessary for the effective elimination of a particular tumor (21).

In summary, our study confirms the high frequency of PI3K mutations in breast cancer, showing that exon 9 and exon 20 mutations were differently distributed among different morphologic tumor types. Although exon 20 mutations were seen at similar frequencies in ILCs and in IDCs, exon 9 mutations were more frequent in ILCs. Mutations in the two different hotspots have also profoundly different clinical significance because exon 9 mutations are independently associated with poor prognosis, whereas exon 20 mutations are associated with prolonged DFS and OS. These data suggest that *PIK3CA* exon 9 mutations could be the most important targets for PI3K-inhibiting drugs.

The high frequency of PI3K mutations in breast cancer, their possible therapeutic manipulation, and the suggested different roles of mutations in different exons, should prompt additional studies on larger and more homogeneous series of patients in order to verify whether these data could effectively be translated from the bench to the bedside.

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