Clinical Cancer Research

PIK3CA Mutations May Be Discordant between Primary and Corresponding Metastatic Disease in Breast Cancer

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Abstract

Purpose: *PIK3CA* mutations are frequent in breast cancer and activate the PI3K/Akt pathway. Unexpectedly, *PIK3CA* mutation appears in general to be associated with better outcome. In a cohort of patients where both primary and metastatic lesions were available, the objective was to assess changes in *PIK3CA* mutations. We wished to discern whether selective pressures occur and the influence of *PIK3CA* mutation on time to recurrence.

Experimental Design: Formalin-fixed paraffin-embedded tumor blocks were obtained from 104 patients with paired samples from primary tumors and corresponding asynchronous metastatic breast tumors. Samples were analyzed for *PIK3CA* mutations (exons 9 and 20) as well as immunohistochemical evaluation for PTEN, pAKT, Ki67, ER, and HER2.

Results: *PIK3CA* mutation was detected in 45% of the primary tumors. Overall, there was a net gain in mutation in metastatic disease, to 53%; nonetheless, there were instances where metastases were wild type in patients with *PIK3CA* mutant primary tumors. Laser capture microdissection on a subset of cases revealed microheterogeneity for *PIK3CA* mutational status in the primary tumor. *PIK3CA* mutants overall showed a significantly longer time to first recurrence than wild type cases ($P = 0.03$).

Conclusion: *PIK3CA* mutations occur at high frequency in primary and metastatic breast cancer; these may not necessarily confer increased aggressiveness as mutants had a longer time to recurrence. Because *PIK3CA* status quite frequently changes between primary and metastatic disease, it emphasizes the necessity of assessing the *PIK3CA* status in the metastatic lesion for selection of *PIK3CA* inhibitor therapy. *Clin Cancer Res; 17(4); 667–77. ©2010 AACR.*

Introduction

The phosphatidylinositol 3-kinase (PI3K) signaling pathway is involved in regulating cellular processes required for tumorigenesis, including protein synthesis, metabolism, cell survival, proliferation, apoptosis avoidance, and angiogensis (1). The class 1A PI3-kinases are a family of lipid kinases that exist as heterodimers composed of a catalytic subunit p110α and a regulatory subunit p85α. The *PIK3CA* gene encodes the catalytic subunit p110α. PI3K is activated by transmembrane receptor tyrosine kinases, including those of the epidermal growth factor family. In breast cancer, several components of the PI3K/PTEN/AKT pathway are often dysregulated, including amplification of HER2, loss of PTEN function, and mutations of the *PIK3CA* gene. A major mechanism for abnormal PI3K pathway activation in cancer is mutation in the *PIK3CA* gene, as this gene is one of the most frequently mutated oncogene in breast cancer (2), implying that *PIK3CA* mutations may play an oncogenic role in breast carcinogenesis. The mutations are activating mutations, resulting in constitutive increased downstream signaling with increased pAKT (3–5).

In recent studies, *PIK3CA* mutations are identified in 20% to 40% of primary breast cancer (2, 6–13). The vast majority, comprising approximately 90% of *PIK3CA* mutations (12), are clustered at 2 hotspot regions in exon 9 (E542K and E545K) and exon 20 (H1047R and H1047L) (10, 14), encoding the helical and kinase domains, respectively.

Inconsistent correlations with *PIK3CA* mutations and clinicopathologic variables have been reported. Several studies show associations with *PIK3CA* mutations and hormone receptor (HR) positive disease (10, 11, 13, 15), HER2 negativity (6, 7, 13) and have been observed with higher frequency in lobular carcinoma than in other histological

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Translational Relevance

This study is the first to report the frequency of *PIK3CA* mutations in primary tumors and corresponding metastases in breast cancer. In a cohort of 104 patients where both primary and metastatic lesions were available, we report that *PIK3CA* mutations occur at high frequency in primary and metastatic breast cancer, and we detected a high occurrence of discrepancy in *PIK3CA* mutations between primary tumors and metastases, with a trend toward gain of mutations in metastatic breast cancer, and speculate that *PIK3CA* mutation can be acquired during disease progression. Furthermore, laser capture microdissection on a subset of cases revealed microheterogeneity for *PIK3CA* mutational status in the primary tumor, indicating intratumoral heterogeneity. These findings have implications for future research as well as clinical practice as it emphasizes the necessity of assessing the *PIK3CA* status in the metastatic lesion for selection of *PIK3CA* inhibitor therapy.

types of breast cancer (6, 9). Others have reported no correlations with clinicopathologic variables (2, 12, 16).

The prognostic implication of these activating mutations remains uncertain. Studies have yielded conflicting results, from no significant association between *PIK3CA* mutational status and survival (10), to a less favorable outcome in patients harboring exon 20 (17) mutation or exon 9 mutation (9); to association with a favorable prognostic significance with improved survival for patients with exon 20 mutations (9, 13).

PTEN, a tumor suppressor gene, antagonizes PI3K signaling, resulting in inhibited PI3K downstream signaling. Lack of PTEN function can occur due to loss of protein expression, epigenetic silencing, or inactivating mutations (10).

Few studies have investigated when these mutations occur in the pathogenesis from *in situ* to invasive carcinoma. Two studies demonstrated concordant existence of *PIK3CA* mutations in invasive and DCIS lesions, suggestive of the mutations being an early event (15, 16). To our knowledge, no studies have yet investigated whether *PIK3CA* mutations or PTEN expression is concordant between primary tumor and metastasis in breast cancer.

One of the major issues concerning the PI3K pathway in the clinical setting is that pathway activation may contribute to therapeutic resistance. Several preclinical studies indicate that constitutive signaling through the PI3K pathway mediates treatment resistance to a broad spectrum of therapeutics (3, 18–20). An activated PI3K pathway due to *PIK3CA* mutations is associated with resistance to HER2-targeted therapy (18, 21). Also, reduced PTEN induces resistance to endocrine therapy and HER2-directed therapy for breast cancer patients (21–23).

As treatment in breast cancer is directed toward both subclinical (in the adjuvant setting) and clinical disease is it of crucial importance to know, whether or not the affected pathways in the primary tumors is the same as the affected pathways in the metastases.

In this study, we aimed to assess whether *PIK3CA* mutations and PTEN expression were concordant in paired primary and metastatic breast carcinoma. We also intended to correlate the status of PTEN and *PIK3CA* mutations in the metastases with pAKT to access downstream activation.

Material and Methods

Tumor samples

Formalin-fixed paraffin-embedded (FFPE) tumor blocks were available from 104 patients with paired samples from primary tumors and corresponding asynchronous metastatic lesions. Fifty-four patients were part of a prospective study, in which patients from May 2007 were offered a biopsy from the metastasis, when diagnosed with recurrent breast cancer at the Department of Oncology at Odense University Hospital. An additional 50 archived paired samples collected retrospectively from 1989 to 2005 at the Department of Pathology at Odense University Hospital were included, hence a total of 104 paired samples were obtained. Seven patients were diagnosed at presentation with locally advanced breast cancer and received preoperative chemotherapy. In these cases, the diagnostic core biopsy was used. Patients with primary disseminated disease were excluded, as were patients with bilateral breast cancer. Seventy patients had positive axillary lymph nodes at time of surgery. Because some of the positive lymph nodes consisted of micrometastases or were not available, lymph nodes from a total of 47/70 patients were included. Hematoxylin–eosin (HE) sections were prepared from paraffin-embedded tissue blocks from primary tumors, axillary lymph node metastases, and metastases to confirm the presence of tumor tissue. Patients were defined as having recurrence when disease occurred more than 3 months after primary diagnosis. The study was approved by the Ethical Committee of Region Syddanmark and the Danish Data Protection Agency.

Tissue microarrays

Five-micrometer HE sections were generated from each formalin-fixed paraffin-embedded tissue block. The HE sections were reviewed by a pathologist to confirm the presence of invasive carcinoma, and areas with representative tumor tissue were sampled by two 1 mm cores for tissue microarray (TMA) preparation and DNA extraction.

Full sections

Samples with scant tumor tissue had full sections made for immunohistochemistry (IHC) and DNA extraction. This was the case in the majority of the metastatic samples as the biopsies were often very small. Patients with locally advanced disease at diagnosis had full sections done on the

diagnostic core biopsy prior to chemotherapy. The full sections were macrodissected before mutation analysis to ensure invasive tissue in sufficient quantities.

Clinical data

Clinical data were assessed from the patients' medical files. All patients except one with primary inoperable disease underwent intended curative surgery. Ninety-one had a biopsy from their first recurrence, and 13 had a biopsy from a later recurrence with 30 biopsies originating from a locoregional recurrence and 74 from a distant recurrence. Median time to biopsy was 59 months (range 8–325). Thirty-five patients received adjuvant chemotherapy with CMF, antracycline- or taxane-containing regimens whereas half of the patients received adjuvant endocrine treatment ($n = 54$) and 2 received adjuvant Herceptin. Date of first recurrence was recorded as the date of biopsy confirmation of the recurrence, and in the patients with a biopsy from a later recurrence, the date of first recurrence was taken from the medical files.

DNA isolation and mutation detection method

FFPE tumor tissue cores were deparaffinized prior to DNA recovery using QuickExtract FFPE DNA extraction kit (Epicentre Biotechnologies) according to manufacturer's instructions. Tissues cores were incubated in extraction buffer for 60 min at 56°C followed by 2 min at 98°C. *PIK3CA* has been reported to have hotspot mutations at codon E542, E545, and H1047 and can therefore be analyzed by SNaPshot genotyping assay (also known as primer-extension or minisequencing). PCR was performed using the Advantage HF2 PCR kit (Clontech) to amplify genomic DNA. PCR thermocycling conditions were: 94°C for 30 s; 10 cycles of 94°C for 20 s, 65°C for 20 s, and 68°C for 20 s, with the annealing temperature decreasing by 1°C/cycle (touchdown PCR); 40 cycles of 94°C for 10 s, 55°C for 20 s and 68°C for 20 s; 68°C for 6 min; 4°C; hold. Primer sequences and extension primers (Supplementary Table S1). PCR clean up was performed with ExoSAP-IT to remove excess primers and free nucleotides. Primer extension reaction was set up with ABI Prism SNaPshot Multiplex kit and extension primers. Primer extension reaction was treated with shrimp alkaline phosphatase (SAP) and analyzed by capillary electrophoresis using ABI 3730xl DNA Analyzer. Genotyping data were analyzed by GeneMapper v4.0 (Applied Biosystems). Genomic DNA from cell line BT483, MCF7, and T47D, which harbor mutation E542K, E545K, and H1047R, respectively, were run along with tumor DNA samples as positive controls. No false positive results were observed in 48 pancreatic neuroendocrine tumor control samples without mutations, demonstrating the reliability of the procedure. Analysis of a subset of samples with DxS PI3K mutation test kit (Qiagen/DxS, Manchester, UK) was performed on a Bio-Rad CFX96 real time PCR system according to manufacturer's instructions.

Immunohistochemistry

The paired material assembled into TMAs and full sections was examined with IHC and chromogenic *in situ* hybridization (CISH). CISH analysis was performed for all HER2 IHC equivocal cases. The analysis was performed using Dako DuoCISH reagents according to manufacturer's directions. Five micrometers sections were cut for immunohistochemical analysis for ER, HER2, Ki67, PTEN, and pAKT. Immunostains were performed on Ventana Discovery XT machines using laboratory standard protocols. Details of the antibodies and conditions used (Supplementary Table S2).

Immunohistochemical scoring and interpretation

Biomarker expression from IHC assays was scored by 2 pathologists. ER, PTEN, and pAKT staining were scored semiquantitatively using the histo-score (H-score) based on staining intensity and percentage of positive cells. Intensity was scored as 0: none, 1: weak, 2: moderate, or 3: strong. H-score was calculated as follows: H score = [fraction of cells with intensity grade 1(%)] + [fraction of cells with intensity grade 2(%) × 2] + [fraction of cells with intensity grade 3(%) × 3].

Tumors were considered negative for ER if weak immunostaining was observed in less than 1% of tumor nuclei (H-score < 1; ref. 24). For the purposes for examining the relationship between proliferation index and mutation status a cut off for Ki67 of 20% was chosen ($\leq$20% corresponding to a low Ki67 proliferation index), as this cut point seems to correlate with prognosis (25) and is in the range of what has been found and validated in several studies (25–30). PTEN staining was located mainly in the cytoplasm. Because cut off levels for reduced PTEN expression by using immunohistochemical methods are not standardized, the H-score was applied. The lower quartile corresponding to an H-score of $\leq$10 was used as the cut off point to designate PTEN loss. Previous reports on loss of PTEN expression are in the range of 22% to 43% using IHC staining (7, 21–23, 31, 32). Phospho-AKT demonstrated predominantly cytoplasmic staining, although nuclear staining was apparent. The cytoplasmic score was taken as a more direct indicator of pathway activation and used for analyses. The interpretation of the HER2 staining was made according to standardized guidelines for HER2 IHC interpretation (33). Tumors were considered positive for HER2 if immunostaining was scored as 3+, or had an amplification ratio for CISH of 2.2 or more being the cut point that was used to segregate immunohistochemical equivocal tumors (scored as 2+).

Statistical analysis

Paired tests were used when comparing *PIK3CA* status and PTEN expression in primary tumors and metastases. McNemar's test was used when analyzing the binary outcome of the *PIK3CA* status. The nonparametric Wilcoxon signed rank sum test was used analyzing PTEN as a continuous variable as PTEN difference depart from a normal distribution. Associations between *PIK3CA* mutations and

clinicopathologic categorical variables were assessed by chi-square test or Fisher's exact test. A *t*-test was used to compare mean age between *PIK3CA* normal and mutant groups. Logistic regression was used to test for associations with *PIK3CA* and the clinicopathologic variables. For the univariate analysis, time to recurrence (TTR) curves were generated by the Kaplan–Meier method, and differences between recurrence curves were assessed for significance by the log-rank test. To evaluate the independent prognostic value of *PIK3CA* status, a multivariate analysis was carried out by multiple regression. All patients had an event and there was no censoring. TTR was defined as the time interval from surgery to date of biopsy from first relapse or date of first recurrence from the medical files in the 13 patients not having a biopsy from their first recurrence. In the correlation analyses of "PI3K pathway activity" and pAKT H-score, the nonparametric Wilcoxon Mann–Whitney test was used. All tests were 2-tailed and $P \leq 0.05$ was considered significant. All statistical analyses were performed using STATA software version 11.

Results

PIK3CA mutations

Paired samples from 104 patients were analyzed, 101 patients had results in the primary tumors, and 103 in the metastases, respectively. The frequency of *PIK3CA* mutations (exon 9 and/or exon 20) in the primary tumors was 45% (45/101), with 15% (15/101) exon 9 mutations (E542K/E545K), and 36% (36/101) exon 20 mutations (H1047R/H1047L). Six patients had both mutations.

The frequency of *PIK3CA* mutations (exon 9 and/or exon 20) in the corresponding synchronous axillary nodes was 34% (16/47). Among 46 patients with positive axillary nodes with paired *PIK3CA* results, there were discrepancies between primary tumor and axillary nodes in 6/46 (exon 9) patients of whom 3 were mutated in the axillary node whereas the primary tumor was wild type. In exon 20, discrepancies were observed in 6/46 patients of whom one was mutated in the axillary node and wild type in the primary tumor.

In the corresponding metastatic tumors, 53% (55/103) had mutations (exon 9 and/or exon 20), with 20% (21/103) in exon 9 and 42% (43/103) in exon 20. Nine patients had both mutations. Paired results were available for 100 patients (Table 1 and SNaPshot raw data, Supplementary Table S3). In all 3 analyses (either exon or in combination), a gain of mutations in metastases was detected (exon 9, $P = 0.09$; exon 20, $P = 0.3$). In the combined analysis, 21 patients changed genotype from wild type to mutant ($P = 0.08$), compared to 11 patients who lost mutations in the metastases.

A subset of the samples (randomly selected) were also analyzed by DxS to validate the SNaPshot results by another method (Table 2). An agreement of 89% (34/38) between the 2 detection methods was obtained.

Laser capture microdissection (LCM) heterogeneity substudy

In a small subset of primary tumors with mutational analyses from 2 tissue blocks from the same primary tumor, discrepant results lead us to look further into heterogeneity of *PIK3CA* mutations. In 10 primary breast cancer samples, from 5 patients with 2 discrepant primary samples, full sections followed by LCM from 4 different invasive areas (Fig. 1 and Supplementary Table S4) showed heterogeneity. In 4/10, discordance was observed between LCM and core results, with the core analysis in 2 samples detecting a mutation that was not detected by LCM, suggesting that a mutated subclone was located deeper in the core than the

Table 1. *PIK3CA* status, exon 9, exon 20, and combined (exon 9 and/or 20) mutations in 100 patients with paired results available

***PIK3CA* status**		**Asynchronous metastasis**	
Primary tumor	Wild type	Mutant	Total
Exon 9			
Wild type	76	10	86
Mutant	3	11	14
Total	79	21	100
Exon 20			
Wild type	43	21	64
Mutant	15	21	36
Total	58	42	100
Exon 9/20			
Wild type	35	21	56
Mutant	11	33	44
Total	46	54	100

NOTE: Show the number of patients gaining and loosing mutations when comparing primary tumors and matched metastases. The primary tumor mutational status is assessed in primary tumor, nodal status not included.

Table 2. *PIK3CA* analysis by SNaPshot and DxS method in a subset of the samples (randomly selected) to validate the SNaPshot results by another method

Patient	Sample location	*PIK3CA* genotype by DxS	*PIK3CA* genotype by SNaPshot	DxS/SNaPshot agreement	Discordance in DxS between primary tumor and metastasis	Heterogeneity between "same[a]" samples by DxS
1	Primary tumor	H1047R	H1047R	Yes		
1	Metastasis	H1047R	H1047R	Yes		
2	Primary tumor	WT	WT	Yes		
2	Axillary node[b]	WT	WT	Yes		
3	Metastasis	E542K	E542K	Yes		
4	Primary tumor	H1047R	H1047R	Yes		
4	Metastasis	H1047R	H1047R	Yes		
5	Primary tumor	E545K	E545K	Yes		
6	Primary tumor	H1047R	H1047R	Yes		
6	Metastasis	H1047R	H1047R	Yes		
7	Primary tumor	E542K	E542K	Yes		
7	Metastasis	E542K	E542K	Yes		
8	Primary tumor	E545K	E545K	Yes		
8	Metastasis	E545K	E545K	Yes		
9	Primary tumor	WT	WT	Yes	Gained mutation	
9	Metastasis	E545K	E545K	Yes		
10	Primary tumor	H1047R	H1047R	Yes	Lost mutation	
10	Metastasis	WT	E524K	No		
11	Primary tumor	WT	WT	Yes	Gained mutation	
11	Metastasis	E542K	E542K	Yes		
12	Primary tumor	WT	H1047R	No		
12	Metastasis	WT	WT	Yes		
13	Primary tumor	H1047R	H1047R	Yes		
14	Primary tumor	H1047R	H1047R	Yes	Lost mutation	
14	Metastasis	WT	WT	Yes		
15	Primary tumor	WT	WT	Yes		
16	Primary tumor	WT	WT	Yes	Gained mutation	
16	Metastasis	E545K	E545K	Yes		Heterogeneity
16	Metastasis	WT	WT	Yes		
17	Primary tumor	H1047R	H1047R	Yes	Lost mutation	
17	Metastasis	WT	WT	Yes		
18	Primary tumor	WT	WT	Yes		
18	Primary tumor	WT	WT	Yes		
19	Primary tumor	WT	E545K, H1047R	No		Heterogeneity
19	Primary tumor	E545K	E545K	Yes		
19	Axillary node	WT	E545K, H1047R	No		Heterogeneity
19	Axillary node	E545K	E545K	Yes		
20	Primary tumor	WT	WT	Yes		

[a]In a few cases, adjacent tissue blocks from the same primary tumor or metastasis were analyzed. Discordant results indicate heterogeneity.

[b]Axillary node, surgically removed synchronous with primary tumor.

section dissected by laser capture. In one sample, LCM detected a mutation that was not present by the core analysis and in the final LCM and core analysis each detected a different mutation.

Correlations with clinicopathologic variables

No correlations were found between *PIK3CA* mutations in the primary tumors and clinicopathologic parameters (Table 3). By logistic regression analysis, there were no associations between *PIK3CA* mutations as dependent variable and the clinicopathologic variables including nodal status, tumor size, ER, HER2, PTEN, and histological type.

PIK3CA mutations and outcome

Log-rank analysis revealed that *PIK3CA* mutations in primary tumors (combined exon 9 and/or exon 20) were

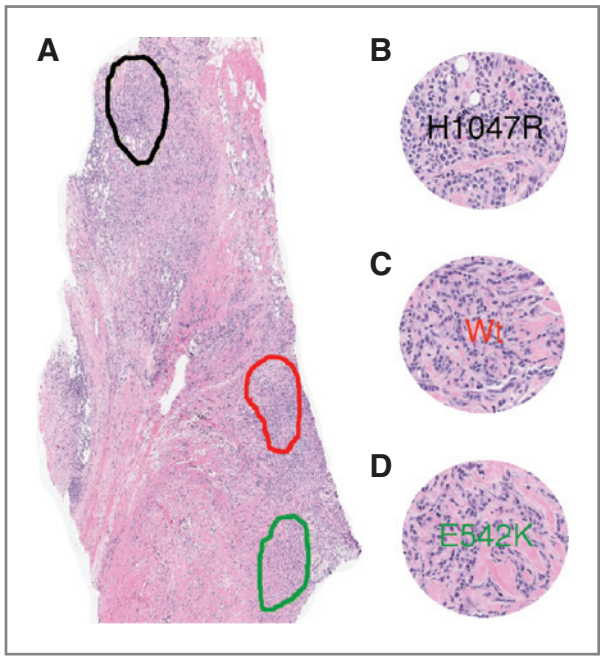

Figure 1. Primary breast cancer, different invasive areas were laser captured and subsequent mutation analysis showed heterogeneity. A, full section. B, area with exon 20 mutation (H1047R). C, area with wild type (Wt). D, area with exon 9 mutation (E542K). Overall, the status was a double mutation exon 9/exon 20 from the core analysis.

associated significantly with a longer TTR than in wild-type tumors (log-rank, $P = 0.03$; Fig. 2). This finding seemed to be driven more strongly by exon 9 mutations ($P = 0.01$), as exon 20 mutations considered alone were not significantly associated with a longer TTR ($P = 0.07$). The median TTR was 49 months (CI: 40–68) for the cohort. Patients harboring an exon 9 and/or exon 20 mutation in their primary tumors had a median TTR of 65 months (CI: 49–102) compared to 37 months (CI: 32–60) in wild-type patients, with a difference of 28 months in the 2 groups. In a multiple regression analysis including *PIK3CA* status in primary tumor (exon 9 and/or exon 20), nodal status, malignancy grade, tumor size, ER status, HER2, and adjuvant treatment (chemotherapy and/or endocrine treatment), the only independent factor reaching statistical significance for longer TTR was ER positivity ($P = 0.005$).

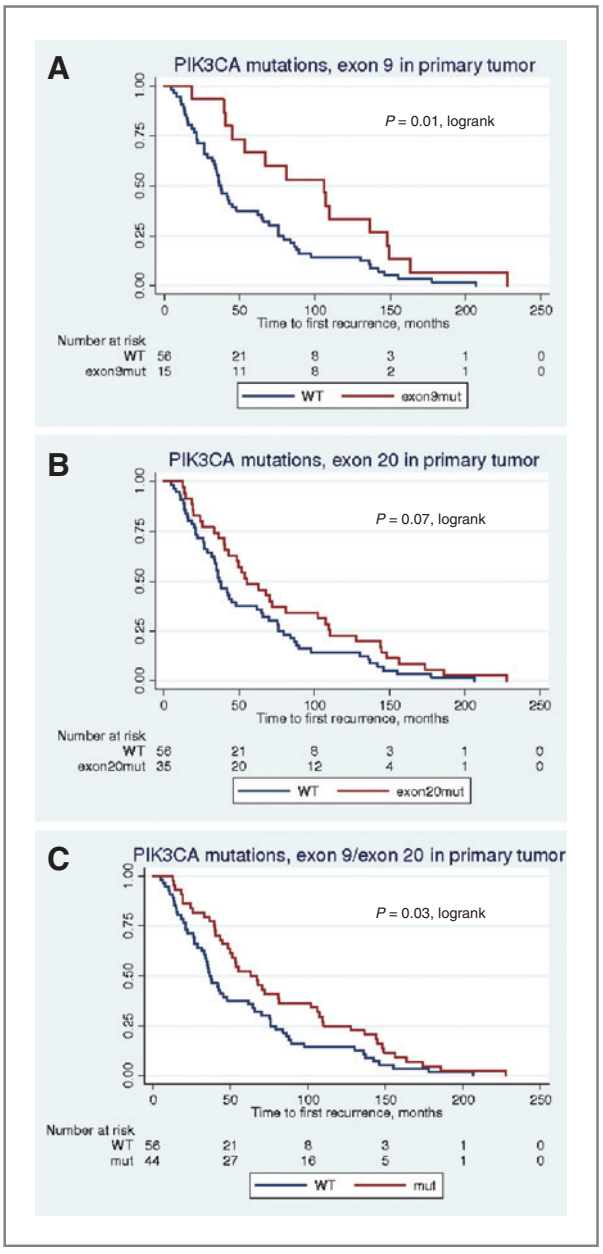

Figure 2. Kaplan–Meier time to recurrence curves. *PIK3CA* mutation status in primary tumor and time to first recurrence. A, *PIK3CA* mutations in exon 9 (exon 20 mutated patients excluded). B, *PIK3CA* mutations in exon 20 (exon 9 mutated patients excluded). C, *PIK3CA* mutations in exon 9 and/or exon 20.

PTEN expression and PI3K/PTEN/AKT pathway activation

PTEN IHC was used as a surrogate for PTEN loss. Using a cut point H-score of ≤ 10, corresponding to the lower quartile, no significant difference was detected with 26% PTEN loss in the primary tumors compared to 20% in the metastases. Analyzing PTEN as a continuous variable, there was a significantly higher H-score in metastases (mean = 104) than in the primary tumors (mean = 82, $P = 0.005$). As a measure of PI3K pathway activation at recurrence, pAKT expression was used as a surrogate marker for pathway activity. The associations of pAKT with combined PTEN/*PIK3CA* status (Fig. 3) at recurrence showed that the PTEN negative and/or *PIK3CA* mutated group had an increased pAKT (mean = 117) compared with the PTEN positive/*PIK3CA* wild-type group (mean = 93, $P = 0.2$), suggesting an increased downstream signaling. Analyzing PTEN and *PIK3CA* status with pAKT separately showed nonsignificant correlations.

Table 3. Patient and tumor characteristics by *PIK3CA* mutation status (exon 9 and/or exon 20) in primary breast cancer

Patient and tumor characteristics by *PIK3CA* mutation status in primary tumor

Variable	*N* = 101	Mutant	WT	*P* value
PIK3CA status				
Primary tumor	101	45	56	
Age at diagnosis (years)				
Mean (range)	57 (32–87)	59 (34–86)	56 (32–87)	0.4
Lymph node status (*n* pos. nodes)				
0	31	17	14	0.4
1–3	31	13	18	
>3	37	14	23	
Unknown	2			
Tumor size (mm)				
0–20	44	24	20	0.2
21–50	41	15	26	
>50	8	3	5	
Unknown	8			
Histology				
Lobular carcinoma	9	6	3	0.3
Ductal carcinoma	87	36	51	
Other	5	3	2	
Malignacy grade				
Grade 1	7	2	5	0.6
Grade 2	58	26	32	
Grade 3	23	8	15	
Unknown	13			
Biopsy location				
Locoregional[a]	30	13	17	0.9
Distant[b]	71	32	39	
HER2 status				
Negative	90	40	50	0.7
Positive	9	3	6	
Unknown	2			
ER status				
Negative	19	6	13	0.2
Positive	78	37	41	
Unknown	4			
PTEN status (cutoff, ≤ 10 = neg)				
Negative	25	7	18	0.07
Positive	72	35	37	
Unknown	4			
Ki67 status (cutoff, ≤ 20 = low)				
Low	43	19	24	0.9
High	53	23	30	
Unknown	5			

[a]Locoregional: ipsilateral axillary/collum/supra- or infraclavic lymph nodes, cicatrice, residual mamma.
[b]Distant: CNS, liver, lungs, bones, lymph nodes intrathoracal, abdomen, skin (outside regio mammalis), pleura exudate, contralateral axillary/collum/supra- or infraclavic lymph nodes.

Discussion

This study is the first to report the frequency of *PIK3CA* mutations in primary tumors and corresponding metastases in breast cancer. *PIK3CA* mutations represent one of the most frequent genetic alterations in breast carcinomas, being detected in 45% of the primary tumors in this study. This is higher than previously reported frequencies in the range of 20% to 40% (2, 6–13) and may be due to the selected cohort of patients all having recurrence. In the

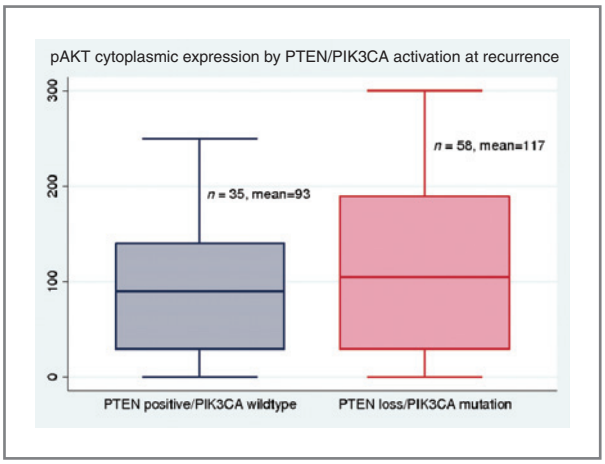

Figure 3. Box plot showing the correlation between expression of pAKT at recurrence as a marker of downstream PI3K pathway signaling and combined PTEN/*PIK3CA* status at recurrence. Box plots in red indicate an active PI3K pathway.

metastases, the frequency of mutations was 53%, indicating that half of the tumors at relapse are addicted to the PI3K pathway. Approximately one third of the patients changed mutational status in the asynchronous metastases and a change to mutant from wild type was strongly favored. This disparity is in line with intertumeral heterogeneity detected between primary tumors and metastases genotypically as well as phenotypically by different methods as IHC, CGH, point mutations, and gene expression profiles (34–37). *PIK3CA* mutations may be acquired during tumor progression, suggesting an increase in the activation of the PI3K pathway. Somatic mutations identified in cancers are either "drivers" or "passengers" (38). The high frequency of *PIK3CA* mutations at recurrence suggests that they are driver mutations as they are biological positively selected for during tumorigenesis.

PIK3CA mutation detection methodology

PIK3CA mutations were detected by primer extension methodology with the SNaPshot assay. It is a rapid and inexpensive method, which combines multiplex PCR amplification with a multiplex primer extension assay enabling detection of all 4 mutations in one reaction. The method was evaluated by Hurst and colleagues and 100% concordant results were obtained with existing PCR-based screening methodology and no false positive results were obtained (39). Primer extension SNaPshot assays have been developed for several genes with common mutations, for example *BRCA1/2* (40). It has a high analytical sensitivity as it detects mutant DNA composing 5% to 10% of the total DNA present (39). False negative results can occur due to sparse amount of tumor tissue below the detection limit. Incorrectly detected genotypes are rare (<1%) with this method which produces rarely (<2%) different genotypes in separate runs (41). The method has numerous advantages; it is fast, sensitive—tolerating a mix of normal and small quantities of tumor tissue, and can be assessed on routine paraffin sections evading the need for frozen tumor specimens and microdissection. Several methods for point mutation genotyping exist; though no methods have become a "golden standard." A subset of samples were analyzed by both DxS and SNaPshot methods and the results were largely concordant, the SNaPshot method detecting a few more mutations than the DxS. Importantly, in line with SNaPshot data the DxS data confirmed changed mutation status between primary tumors and metastases; finding cases with both gains and losses of *PIK3CA* mutations. In addition, the DxS results also indicate intratumoral heterogeneity.

We report high frequencies of mutations in primary breast carcinomas and metastases, and the primer extension method is likely to have superior detection rates compared to conventional sequencing with its lower sensitivity (42), because it tolerates higher levels of normal DNA contamination.

Mutational events in the tumor pathogenesis

It has been implied that *PIK3CA* mutations arise late in tumorigenesis as a stepwise accumulation of genetic defects resulting in progression from precursor lesions to invasive carcinoma (14, 43). In contrast, mutations have been reported to occur in preinvasive lesions in the breast (15, 16). Our LCM substudy indicated that primary tumors exhibit prominent microheterogeneity in *PIK3CA* mutation. Intratumoral genotypic and phenotypic heterogeneity in primary breast is well known, reflecting subclonal diversity (44–46).

Our data show that overall the frequency of *PIK3CA* mutations in the metastases tended to increase, as more patients gained mutations. This might be due to heterogeneity in the primary tumors with small undetectable mutated subclones, which our LCM substudy supported. These subclones may in some patients grow up during tumor progression due to a selective advantage. Also although *PIK3CA* mutation might, for example, provide for increased survival it might actually slow the rate of metastasis by reducing invasive signaling (47). A *PIK3CA* mutational acquisition during tumorigenesis is also a possibility, as neoplastic progression in many tumor types involves the acquisition of molecular defect in various signaling pathways. At the same time, we observed a number of cases where a mutant primary was followed by a wild-type metastasis; it is possible that a selective advantage offered by *PIK3CA* mutation may be substituted for by other wild-type subclones containing other aberrations inducing a more aggressive potential hence resulting in outgrowth of wild-type metastases. A study in colorectal cancer revealed, in agreement with our data, intratumoral heterogeneity as well as heterogeneity between primary tumors, lymph nodes, and metastases (48). We conclude that *PIK3CA* mutations are early

events, that intratumoral heterogeneity exists, and that additional events during disease progression are possible.

PIK3CA correlations with clinicopathologic parameters and outcome

In this study *PIK3CA* mutation was neither in univariate nor in multivariate analyses associated with clinicopathologic parameters, including ER, HER2, PTEN, lymph node, tumor size, and histological status. This may be due to the small size of the study, making it difficult to evaluate associations between *PIK3CA* mutations and clinicopathologic variables. Also, our patient material was highly selected, as all the patients had recurrence, making this kind of analyses difficult. Others have reported associations with favorable clinicopathologic features (6, 7, 13). The presence of *PIK3CA* mutation has been suggested as a positive prognostic factor with improved outcome (9, 13, 47). Consistently we report that in the univariate analyses, *PIK3CA* mutation (exon 9 and in the combined analysis) was associated with a significantly longer TTR, indicating a positive prognostic significance of *PIK3CA* mutations in breast cancer. In the multivariate analysis, *PIK3CA* mutation did not reach significance as an independent positive prognostic factor. These clinical findings indicating a protective effect of the somatic mutations are surprising given the clear relationship between mutation and PI3K pathway activation in preclinical studies. It is possible that pathway activation driven by *PIK3CA* mutation itself, in contrast to, for example, PTEN loss or upstream receptor activation, might favorably affect tumor cell survival while reducing aggressiveness. The impact of the *PIK3CA* mutations on clinical outcome is however still unknown, as prior studies report inconsistent results. The further elucidation of the precise role of these highly frequent mutations in breast cancer is warranted.

PTEN and PI3K pathway activation

The frequency of PTEN deficiency dropped from 26% in the primary to 20% in the metastases. As metastatic samples tend to be smaller and better fixed this may reflect better epitope preservation. In any event, in contrast to *PIK3CA*, it does not appear that the frequency of PTEN loss increases in metastases. Pathway activation assessment in archival samples is challenging due to the strong dependence of phosphoepitope preservation on fixation. Thus, minor differences in fixation may superimpose a great deal of noise on the assay. Nonetheless, as this sample series showed relatively high levels of pAKT overall relative to other archival sets examined with the same protocol (Gardner unpublished data), some speculations from the data are warranted. There was a modest association between *PIK3CA* mutation or PTEN loss and pAKT expression in the metastatic samples, consistent with Ellis and colleagues (47) and prior literature reporting correlation with *PIK3CA* mutations/PTEN loss and activated pAKT, but this did not reach statistical significance in our study. High pAKT in general has been associated with worse outcome in breast cancer, which would thus appear to be inconsistent with the improvement in outcome associated with *PIK3CA* mutation. However, alterations in other pathways may be responsible for the activation of pAKT, including HER2 amplification and PTEN loss, which themselves impart a poor prognosis in breast cancer.

Targeting PI3K

Numerous inhibitors targeting the PI3K–AKT pathway for the treatment of cancer is in clinical development or in early clinical trials. These include dual PI3K–mTOR inhibitors, PI3K inhibitors, AKT inhibitors, and mTOR inhibitors (49, 50). These inhibitors might also be effective in reversing resistance to therapies. The most effective type of therapeutic used to inhibit this pathway is likely to depend on the particular mechanism of PI3K–AKT activation. Tailored treatment based on somatic gene alterations have become standard practice, for example trastuzumab in HER2 amplified breast cancer, and anti EGFR therapy confined to KRAS wt colon cancer. *PIK3CA* mutation is predictive of response to *PIK3CA* inhibitors *ex vivo*, and may be predictive in the clinic. Our results emphasize the attractiveness of *PIK3CA* inhibitory therapy in breast cancer, with the high frequency of mutation in the primaries and even higher 53% frequency in the metastases.

Elucidating the prognostic and predictive significance of these mutations is warranted as PI3K inhibitors become available, making clinicians able to choose and optimize appropriate tailored therapy for patients with recurrent breast cancer, and hence hopefully improve prognosis. Importantly, our findings demonstrate that when such inhibitors are to be used in the metastatic setting, the presence of mutation should be assessed in the target lesion.

In conclusion, our findings indicate that *PIK3CA* mutations occur at high frequency in primary and metastatic breast cancer. Genetic as well as protein expression changes in the PI3K–AKT pathway occur at recurrence. We report a high occurrence of discrepancy in *PIK3CA* mutations between primary tumors and metastases, with a trend toward gain of mutations in metastatic breast cancer, and speculate that *PIK3CA* mutational status can be acquired during disease progression. Whether this is due to heterogeneity related clonal selection or late stage acquisition of mutations during the process of metastasis is unknown, but may have an impact when using PI3K targeted therapy in the metastatic setting and if confirmed, emphasizes the necessity of determining the *PIK3CA* mutational status in the metastatic disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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