PIK3CA Mutations and PTEN Loss Correlate with Similar Prognostic Factors and Are Not Mutually Exclusive in Breast Cancer

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Abstract **Purpose:** The phosphatidylinositol 3′-kinase/Akt pathway is frequently altered in breast cancer. PTEN, a phosphatase that opposes the effect of phosphatidylinositol 3′-kinase, can be mutated or lost, whereas the *PIK3CA* gene is mutated. These have been proposed as alternative mechanisms, and their clinicalpathology significance is under discussion. In this study, we aimed to explore whether PIK3CA mutations and PTEN loss are mutually exclusive mechanisms, correlate with other known clinicopathologic markers, or have clinical implication in breast cancer.

Experimental Design: Exons 9 and 20 of the *PIK3CA* gene were analyzed in 270 breast tumors, and mutations were detected by single-stranded conformational analysis followed by sequencing. The expression of PTEN was evaluated by immunohistochemistry in 201 tumors.

Results: PIK3CA mutations were found in 24% of the tumors and associated with estrogen receptor$^+$ status, small size, negative HER2 status, high Akt1, and high cyclin D1 protein expression. PTEN was negative in 37% of the cases and PTEN loss was associated with PIK3CA mutations ($P = 0.0024$). Tumors presenting PTEN loss or both alterations were often estrogen receptor$^+$, small in size, and HER2$^-$. PIK3CA mutations predicted for longer local recurrence-free survival. Moreover, PTEN loss by itself or combined with mutated PIK3CA tended to confer radiosensitivity. In addition, the patients with high S-phase fraction had longer recurrence-free survival if they carried mutations in the *PIK3CA* gene and/or had lost PTEN, whereas the same alterations were associated with shorter recurrence-free survival among patients with low S-phase fraction.

Conclusions: PIK3CA mutations and PTEN loss were not mutually exclusive events and associated with similar prognostic factors.

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The phosphatidylinositol 3′-kinase (PI3K) pathway plays a central role in regulating cell proliferation, growth, apoptosis, and motility. The class I PI3K enzyme is a heterodimer composed by a regulatory (p85) and a catalytic subunit (p110) that can be activated by tyrosine kinase receptors, such as HER2. On activation, the enzyme generates phosphatidylinositol 3,4,5-trisphosphate, which acts as a second messenger to activate PKB/Akt and other downstream proteins involved in the aforementioned cellular processes (1, 2).

Under normal physiologic conditions, the levels of phosphatidylinositol 3,4,5-trisphosphate are tightly regulated in the cells by phosphatidylinositol 3,4,5-trisphosphate phosphatases, such as PTEN (phosphatase and tensin homologue deleted on chromosome 10). Evidence exists that deregulation of the PI3K/Akt pathway by loss, overexpression, or genetic changes of its members leads to malignant transformation (2).

Recently, the gene encoding the PI3K catalytic subunit p110α (*PIK3CA*) has been found mutated in several cancers from colon (3–5), lung (4, 6), ovaries (3, 7), liver (6), brain, stomach (4), and breast (4, 7–9). Most of the mutations have been localized to hotspots in exons 9 and 20 of the *PIK3CA* gene, and their nature seems to be oncogenic (10) due to promotion of cell growth and invasion (11).

Moreover, PTEN and Akt are frequently altered in breast cancer. Loss of expression but not mutations is the most common alteration of PTEN (12–14), although Akt can be affected by increased expression or activation (15–17). Taken together, these changes are proposed to activate the PI3K/Akt pathway and there are reasons to believe that this possibly leads to therapy resistance (18–20).

In this study, we aimed to explore the clinical significance of PIK3CA mutations in breast cancer by correlating this variable with the estrogen receptor (ER) α status and other well-defined markers and members of its pathway [PTEN, HER2, phosphorylated Akt (pAkt), and cyclin D1 expression]. We also intended to decipher the role of PIK3CA mutations in relation to therapy response among patients treated with or without tamoxifen and with radiotherapy or chemotherapy.

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Received 7/5/06; revised 2/7/07; accepted 3/28/07.

Grant support: Swedish Cancer Foundation.

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doi:10.1158/1078-0432.CCR-06-1609

Materials and Methods

In 1976, the Stockholm Breast Cancer Group initiated a trial to compare postoperative radiotherapy with adjuvant chemotherapy (21). The trial included premenopausal and postmenopausal patients with a unilateral, operable breast cancer. Using a 2 × 2 factorial study design, the postmenopausal patients were also randomized to tamoxifen treatment or no endocrine treatment. There were thus a total of four treatment groups: adjuvant chemotherapy, adjuvant chemotherapy plus tamoxifen, radiotherapy, and radiotherapy plus tamoxifen.

Tamoxifen was given postoperatively at a dose of 40 mg daily for 2 or 5 years. Surgery consisted of modified radical mastectomy. The patients were required to have either histologically verified lymph node metastases or a tumor diameter, measured on the surgical specimen, exceeding 30 mm. Patient accrual started in November 1976 and ended in April 1990. The current study included a subset consisting of 280 postmenopausal patients for whom frozen tumor tissue was still available after hormone receptor assays had been done in routine practice. This subset showed no bias in comparison with all the 679 postmenopausal patients in the trial in terms of tumor characteristics and treatment.

Radiotherapy was given with a high-voltage technique. The dose was 46 Gy with 2 Gy per fraction 5 days a week for a total treatment time of ~4.5 weeks. The target volume included the chest wall, the axilla, the supraclavicular fossa, and the internal mammary nodes. For most of the patients randomized to chemotherapy, the treatment consisted of 12 courses of chemotherapy according to the original Milan protocol (100 mg/m^2 cyclophosphamide orally at days 1-14, 40 mg/m^2 methotrexate i.v. on days 1 and 8, and 600 mg/m^2 5-fluorouracil i.v. on days 1 and 8). During the first 18 months of the trial, however, cyclophosphamide was replaced by 10 to 15 mg chlorambucil orally on days 1 to 8, and up to 18 months was allowed for the 12 courses to avoid dose reductions. The patients were followed for a median period of 11 years. A distant recurrence was registered in 132 patients and a local recurrence in 46 patients. This study was approved by the local ethical committee at Karolinska Hospital.

Genomic DNA preparation. The tumor tissue was digested in a proteinase K solution at 55°C during 36 h. The DNA was extracted with phenol, phenol-chloroform (1:1), and chloroform. The nucleic acids were precipitated in 95% ethanol/0.1 mol/L sodium acetate for 1 h at 70°C and then pelleted by centrifugation at 12,000 × g. The DNA was washed with 70% ethanol, dried at vacuum, and dissolved in sterile water. The concentration of DNA was estimated using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

PCR. Exons 9 and 20 of PIK3CA were individually amplified from genomic DNA by two-step PCR. The sequence for PIK3CA was the same used by Samuels et al. (accession no. NM_006218; ref. 4). The primers were designed using the web-free software Primer3,[4] and the primer specificity was controlled by blasting the primers to other sequences.[5] The designed primers were purchased from Invitrogen.

Because exon 9 has a nearly identical sequence to that found at 22q11.2, we chose the optimal PCR conditions to avoid contamination. A "touchdown" PCR program (2.5 mmol/L $MgCl_2$ and three cycles of amplification at annealing temperatures of 64°C to 63°C followed by 30 cycles at 62°C and a final extension cycle of 72°C for 4 min) was done followed by sequencing of the resulting product (from three of the tumors). In this way, we discarded the presence of mutations known to be found at the 22q11.2 area [e.g., a C instead of A at position 1634 of PIK3CA coding sequence (where 1 is the first base of the starting codon) and deletion of G1658 and C instead of T at position 1659]. The following primers were used in the first PCR: exon 9, 5′-CCAAATAAATTACTGGATTTGTTCT-3′ (forward) and 5′-GATTTTCCACAAATATCAATTTACAA-3′ (reverse). For exon 20, the PCR conditions were as follows: 2 mmol/L $MgCl_2$ and 38 cycles of amplification at annealing temperature of 55°C. The primers were the following: 5′-TGGGGTAAAGGGAATCAAAAG-3′ (forward) and 5′-CCTATGCAATCGGTCTTTGC-3′ (reverse). The products from the first PCR were diluted 1:20 to 1:10 for a nested PCR. The nested primers were the following: exon 9, 5′-AATCCAGAGGGGAAAAATATGA-3′ (forward) and 5′-TGAGATCAGCCAAATTCAGTTA-3′ (reverse). The PCR conditions were as follows: 2 mmol/L $MgCl_2$ and 15 cycles with annealing temperature of 61°C. Exon 20 was spliced into two fragments to get a more suitable product size for the single-stranded conformational analysis. The primers were the following: exon 20, 5′-TTTGCTCCAAACTGACCAAAC-3′ (forward 1), 5′-ACTCCAAAGCCTCTTGCTCA-3′ (reverse 1), 5′-TGCATACATTCGAAAGACC-3′ (forward 2), and reverse (reverse 2). The PCR conditions were similar to those used for exon 9 but annealing temperature was 62°C. A negative control consisting of H_2O was included in each step.

Single-stranded conformational analysis and sequence analysis. The single-stranded conformational analysis was used to screen all the samples for possible mutations. The nested PCR products were labeled by inclusion of [α-^{32}P]dATP (Amersham Biosciences AB) through 9 (exon 9) and 12 (exon 20) cycles of PCR under the same conditions as above. Electrophoresis was done on a nondenaturing 6% polyacrylamide gel and a mutation detection enhancement gel and run for 16 h at 3 W. The dried gels were exposed to an X-ray film at -70°C for 24 h (Cronex 4, DuPont) and detected by autoradiography. Shifted bands were cut from the gel and reamplified with 25 cycles before sequencing the DNA bands. The fragments were purified by ExoSAP-IT (USB Corp.) and sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing kit on a MegaBACE 500 DNA Analysis System (GE Healthcare). All mutations identified were confirmed by a second analysis of the original DNA sample.

Immunohistochemistry. PTEN immunostaining was done using the same method published for cyclin D1 (22). The primary antibodies were a mouse monoclonal anti-PTEN Ab-4 (clone 17.A; LabVision) diluted 1:40 and a mouse IgM (Dako) as negative control. The antibodies were applied overnight at 4°C. As secondary reagent, we used the anti-mouse DakoCytomation EnVision+ System labeled with horseradish peroxidase (Dako) for 30 min. The color was developed with 3,3′-diaminobenzidine/H_2O_2 for 10 min at room temperature, and cell nuclei were counterstained with hematoxylin. Staining of MCF-7 cells was used to control the day to day variations of the technique. Three other cell lines, T47D, MDA-MB-231 (with intact PTEN), and MDA-MB-468 (with PTEN heterozygous deletion and mutation), were also included to control the primary antibody. These cells were a kind gift from Dr. Göran Landberg (Lund University, Malmö, Sweden). Immunostaining of the other proteins comprised in this study (HER2, Akt1, Akt2, and pAkt) has been previously reported (16).

Scoring. PTEN immunoreactivity was examined by two independent observers who were blinded to the clinical data. The staining was mainly visible in the cytoplasm of tumor cells. The tumor was graded as PTEN$^-$ when there was none or weakly immunostaining of the invasive cells compared with the histologically normal cells, conforming the stroma or a duct and the intraductal component (if present). Likewise, tumors were considered PTEN$^+$ when the invasive cells were equally or strongly stained compared with aforementioned structures. A light microscopy Leica DM LS (Leica Microsystems) was used to evaluate the slides.

Statistics. The relationships between different variables were assessed by the χ^2 test or χ^2 test for trend when required. The product-limit method was used for estimation of cumulative probabilities of recurrence-free survival. Differences in recurrence-free survival between groups were tested with the log-rank test. Multivariate analysis of recurrence-free survival was done with Cox proportional hazard regression. This was also true for interaction analysis of different factors and treatment by including the variables × (potential predictive factor), treatment, and the interaction variables × treatment. All the procedures

[4] http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
[5] http://www.ncbi.nlm.nih.gov/BLAST

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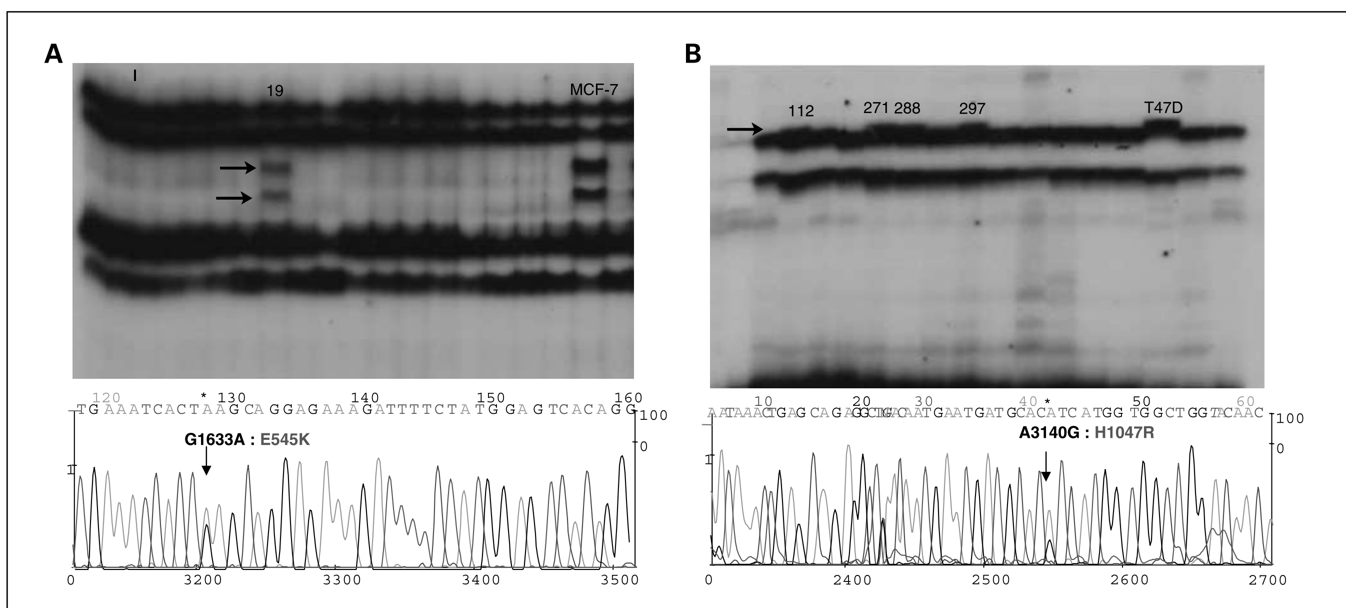

Fig. 1. Example of the most common somatic mutations found in exon 9 (*A*) and 20 (*B*) of the *PIK3CA* gene. The autoradiograms show the shifted bands (*arrows*) in some of the mutated samples, whereas the sequences of tumor 19 (mutated in exon 9) and tumor 271 (mutated in exon 20) are represented in the chromatograms below. Arrow, mutations; asterisk, base that is changed.

are comprised in the statistical package ''Statistica'' (1999 Statistica for Windows; StatSoft, Inc.). The criterion for statistical significance was $P < 0.05$.

Results

With single-stranded conformational analysis and sequence analysis, we identified tumors with aberrant bands as well as the nature of the mutation (Fig. 1A and B). The screening of 270 tumors revealed that 30 tumors (11%) carried missense mutations in exon 9 and 36 tumors (13%) were mutated in exon 20. One tumor presented mutations in both exons. The most common mutation found in exon 9 was G1633A:E545K (18 tumors), whereas the commonest mutation in exon 20 was A3140G:H1047R and occurred in 33 tumors. In the helical region, we found a new mutation (A1637C:Q546P) only present in one tumor. In the catalytic domain, we report three new mutations (Table 1). Analysis of the six breast cell lines screened in this study is also summarized in Table 1. MCF-10A, a non-tumorigenic epithelial breast cell line, did not present mutations.

PIK3CA mutations in relation to other clinical variables. PIK3CA mutations were often found in ER^+ ($P = 0.052$), small-sized ($P = 0.057$), low HER2 expressing tumors ($P = 0.013$) and tended to be present more frequently among tumors without HER2 amplification ($P = 0.083$). On the other hand, the PIK3CA mutations were associated with high Akt1 ($P = 0.032$) and cyclin D1 protein expression ($P = 0.031$). PIK3CA mutations did not significantly associate with node status, Akt2 expression, or pAkt (Table 2). On the other hand, $pAkt^+$ ($P = 0.0033$), total Akt^+ ($tAkt^+$; $P = 0.0019$), and cyclin $D1^{++}$ ($P = 0.031$) phenotypes were significantly associated with mutated PIK3CA (PIK3CA mut) and/or $HER2^+$ in a combined variable.

Survival analysis. Mutations in the *PIK3CA* gene were not a marker for distant recurrence-free or breast cancer–free survival

Table 1. Summary of PIK3CA mutations

Exon	Nucleotide	Codon	Domain	No. patients	Breast cancer cell lines*
9	G1624A	E542K	Helical	11	
9	G1633A	E545K	Helical	18	BT 483 †, MDA-MB-231 †, MCF-7 †
9	A1637C	Q546P	Helical	1	
Subtotal				30 (11%)	
20	A3140G	H1047R	Catalytic	33	T-47D †
20	A3062G	Y1021C	Catalytic	1	
20	G3145C	G1049R	Catalytic	1	
20	A3140C	H1047P	Catalytic	1	
Subtotal				36 (13%)	
Total				65/270 (24%) ‡	4/6 (67%)

*SK-BR-3 and BT-474 (high HER2 according to ref. 45) were not mutated.
†Low or negative HER2 according to ref. 45.
‡One tumor had mutations in both exons 9 and 20.

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Table 2. PIK3CA mutations and PTEN expression related to other variables

	PIK3CA wt, *n* (%)	PIK3CA mutant, *n* (%)	PTEN⁻, *n* (%)	PTEN⁺, *n* (%)	PTEN⁻ or PIK3CA mut, *n* (%)	PTEN⁺ and PIK3CA wt, *n* (%)
ER*						
-	66 (84)	13 (16)	11 (20)	44 (80)	17 (31)	38 (69)
+	136 (72)	52 (28)	64 (44)	81 (56)	78 (56)	62 (44)
		P = 0.052		*P* = 0.0015		*P* = 0.0017
Lymph node status						
-	25 (83)	5 (17)	7 (30)	16 (70)	7 (32)	15 (68)
+	180 (75)	60 (25)	68 (38)	110 (62)	88 (51)	86 (49)
		P = NS		*P* = NS		*P* = 0.098
Tumor size						
≤20	79 (69)	35 (31)	40 (48)	43 (52)	49 (60)	32 (40)
21-30	66 (81)	15 (19)	16 (28)	41 (72)	23 (41)	33 (59)
>30	60 (80)	15 (20)	19 (31)	42 (69)	23 (39)	36 (61)
		P = 0.057		*P* = 0.022		*P* = 0.0075
HER2 (gene)[†]						
-	138 (73)	50 (27)	55 (36)	97 (64)	71 (48)	77 (52)
+	41 (85)	7 (15)	8 (25)	24 (75)	9 (29)	22 (71)
		P = 0.083		*P* = NS		*P* = 0.054
HER2 (protein)						
-	140 (72)	54 (28)	64 (41)	91 (59)	82 (54)	69 (46)
+	60 (87)	9 (13)	9 (20)	35 (80)	11 (26)	32 (74)
		P = 0.013		*P* = 0.011		*P* = 0.00080
Akt1[‡]						
-	147 (79)	39 (21)	56 (38)	92 (62)	71 (49)	75 (51)
+	10 (71)	4 (29)	3 (33)	6 (67)	4 (44)	5 (56)
++	40 (66)	21 (34)	14 (34)	27 (66)	18 (47)	20 (53)
		P = 0.032		*P* = NS		*P* = NS
Akt2[‡]						
-	136 (76)	43 (24)	59 (42)	83 (58)	71 (51)	67 (49)
+	53 (76)	17 (24)	13 (26)	37 (74)	18 (37)	31 (63)
++	6 (60)	4 (40)	1 (17)	5 (83)	4 (67)	2 (33)
		P = NS		*P* = 0.027		*P* = NS
pAkt[‡]						
-	97 (76)	31 (24)	38 (40)	58 (60)	51 (54)	43 (46)
+ (1-10%)	47 (77)	14 (23)	19 (40)	29 (60)	21 (46)	25 (54)
++ (>10%)	55 (74)	19 (26)	15 (28)	39 (72)	20 (38)	33 (62)
		P = NS		*P* = NS		*P* = 0.052
Total Akt[§]						
-	117 (78)	33 (22)	48 (41)	70 (59)	59 (51)	57 (49)
+	76 (71)	31 (29)	23 (29)	55 (71)	32 (43)	43 (57)
		P = NS		*P* = NS		*P* = NS
Cyclin D1 (protein)						
-	56 (82)	12 (18)	21 (42)	29 (58)	23 (46)	27 (54)
+	79 (77)	24 (23)	31 (39)	49 (61)	37 (48)	40 (52)
++	33 (65)	18 (35)	12 (27)	32 (73)	20 (47)	23 (53)
		P = 0.031		*P* = NS		*P* = NS
PTEN						
-	47 (64)	27 (36)				
+	101 (83)	21 (17)				
		P = 0.0024				
SPF[‖]						
<5%	45 (71)	18 (29)	20 (44)	25 (56)	28 (65)	15 (35)
5-10%	58 (73)	21 (27)	25 (42)	34 (58)	30 (51)	29 (49)
>10%	75 (77)	23 (23)	24 (32)	52 (68)	30 (41)	43 (59)
		P = NS		*P* = NS		*P* = 0.014

NOTE: SPF was assessed as in ref. 47.
Abbreviation: NS, nonsignificant (when *P* > 0.1).
*Three cases missing.
[†]HER2 gene amplification was determined as in ref. 46.
[‡]Akt1, Akt2, and pAkt are defined as follows: -, no or weak staining; +, strong staining in 1-10% cells; and, ++, strong staining in >10% cells.
[§]Total Akt⁺ (Akt1 >10% or Akt2 >10% or pAkt strong in >10%).
[‖]Thirty cases missing.

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(data not shown). However, the risk to relapse with a local recurrence was significantly lower among the patients with PIK3CA mut in comparison with those who carried wild-type PIK3CA (PIK3CA wt; P = 0.023; Fig. 2A). Mutations in the *PIK3CA* gene were associated with approximately half the risk for local recurrence in comparison with the wild-type phenotype (P = 0.07) when adjusting for other factors, such as ER (P = 0.43), node status (P = 0.0029), tumor size (P = 0.000018), and treatment (tamoxifen versus no tamoxifen, P = 0.35, or radiotherapy versus chemotherapy, P = 0.008). We also explored the significance of the PIK3CA mutations for local recurrence-free survival under treatment with radiotherapy or chemotherapy. We found that the patients showed more benefit with radiotherapy than with chemotherapy independently of the PIK3CA wt (P = 0.02) or PIK3CA mut status (P = 0.04).

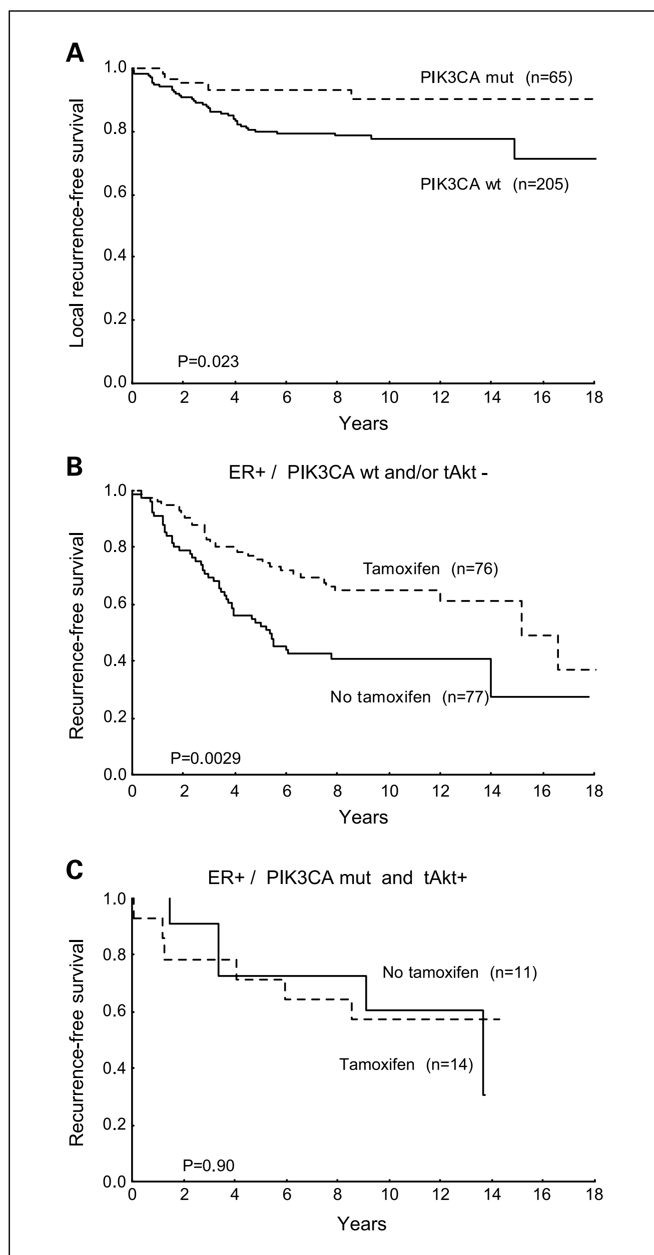

Fig. 2. Local recurrence-free survival for patients with PIK3CA mutations in comparison with those with PIK3CA wt (*A*) and recurrence free-survival for patients with ER^+ tumors treated with or without tamoxifen in relation to PIK3CA wt and/or $tAkt^-$ (*B*) and PIK3CA mut and $tAkt^+$ status (*C*).

Within the group of patients with ER^+ tumors treated with or without tamoxifen, the PIK3CA status was not a predictor of therapy response. Given that Akt might be involved in tamoxifen resistance and the fact that PIK3CA mutations were only weakly associated with Akt, we next analyzed the interaction between the combined variable PIK3CA/tAkt and treatment with tamoxifen. The difference between the hazard ratios for PIK3CA mut/$tAkt^+$ patients [hazard ratio, 1.1 (0.32-3.6); P = 0.90] and PIK3CA wt and/or $tAkt^-$ patients [hazard ratio, 0.49 (0.31-0.79); P = 0.0032] indicates that the absence of PIK3CA mutations or Akt activation is coupled to better recurrence-free survival under tamoxifen treatment (Fig. 2B and C), but the difference was not significant according to Cox analysis (P = 0.27).

PTEN status. Immunohistochemistry was used to detect the expression of PTEN protein in 219 breast tumors. Only those tumors that contained invasive cells together with ductal structures or histologically normal cells were evaluated. Thus, PTEN could be assessed in 201 samples. PTEN was mainly located in the cytoplasm of tumor cells and in some cases in the nucleus. Only the intensity of the staining was taken into consideration. The tumors were divided into four groups: no, weakly, equally, or strongly stained. Further on, the groups no/weak were considered $PTEN^-$ (Fig. 3A and B), whereas equal/strong became $PTEN^+$ (Fig. 3C and D). PTEN was considered negative in 75 tumors (37%) and positive in 126 tumors (63%). PTEN also resulted positive in the cell lines T47D, MDA-MB-231, and MCF-7 (Fig. 3E-G), whereas the MDA-MB-468 cell line was weakly stained (Fig. 3H).

PTEN in relation to other clinical markers and survival. Loss of PTEN correlated with PIK3CA mut phenotype (P = 0.0024), ER^+ status (P = 0.0015), small tumor size (P = 0.022), and low HER2 expression (P = 0.011; Table 2). PTEN status alone was not associated with distant, local, or breast cancer recurrence-free survival (data not shown).

About local recurrences, the patients in the $PTEN^-$ group tended to benefit more from radiotherapy than from chemotherapy (P = 0.02) compared with those in the $PTEN^+$ group (P = 0.29). The test for interaction was borderline significant according to the Cox model (P = 0.077).

PIK3CA/PTEN as a combined variable. Due to the association between PTEN loss and mutations in the *PIK3CA* gene, we decided to do a subanalysis combining these variables. Tumors of the type PIK3CA mut and/or $PTEN^-$ were also frequently ER^+ (P = 0.0017), small in size (P = 0.0075), and had nonamplified HER2 (P = 0.054), low HER2 protein expression (P = 0.00080), and low S-phase fraction [(SPF); P = 0.014; Table 2].

Exploring the PIK3CA/PTEN variable in different subgroups (Fig. 4A-C), we found that, among the tumors with low SPF (<5%), the PIK3CA mut and/or $PTEN^-$ type predicted for worse recurrence-free survival (P = 0.020), whereas it indicated better survival among the group with higher S-phase (SPF >10%; P = 0.0073). A test for interaction between SPF and the prognostic value of PIK3CA/PTEN was significant (P = 0.0014). In this Cox model, including the variables PIK3CA/PTEN, SPF, and their interaction, all three variables were significant.

The patients with the PIK3CA mut and/or $PTEN^-$ type tended to benefit more from radiotherapy than from chemotherapy

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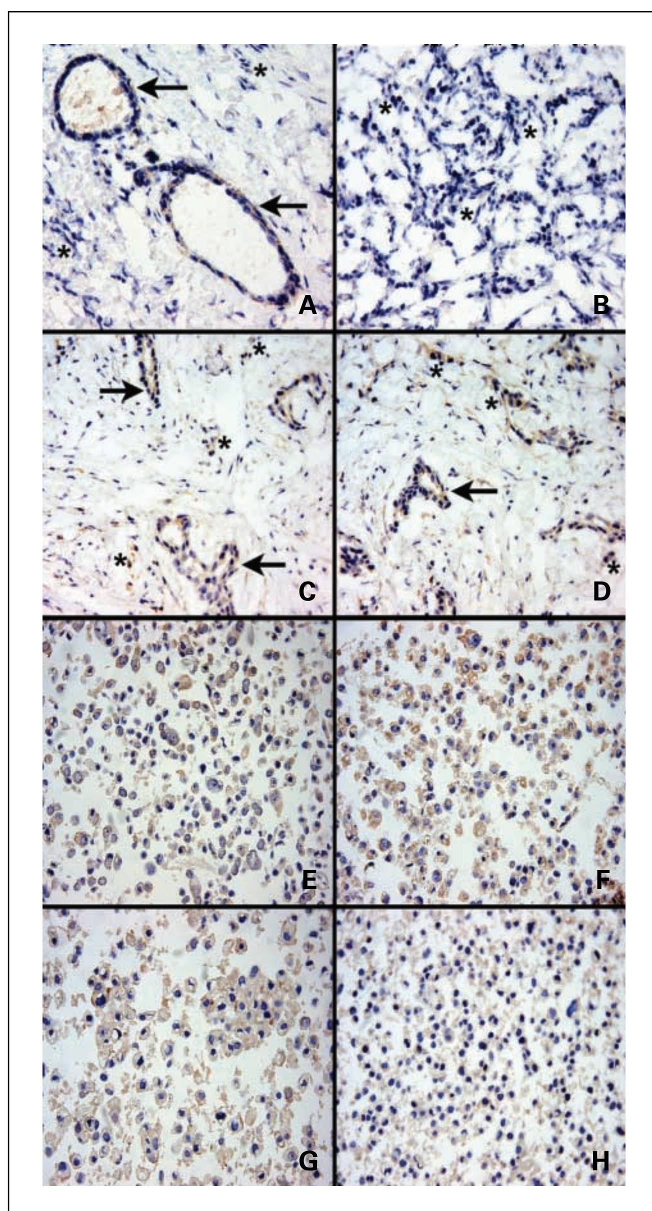

Fig. 3. Immunostaining of PTEN representing two areas of a negative (*A* and *B*) and a positive (*C* and *D*) stained tumors. Arrows, positive ductal structures; asterisk, invasive cells. T47D (*E*), MDA-MB-231 (*F*), and MCF-7 (*G*) breast cancer cell lines with intact PTEN show strong staining, whereas the MDA-MB-468 cells (*H*), with altered PTEN, are weakly stained. Magnification, ×400.

(P = 0.0015) compared with patients with PIK3CA wt and PTEN$^+$ (P = 0.33). The test for interaction was borderline significant (P = 0.065).

Discussion

The PI3K/Akt pathway is relevant for breast cancer development and progression, and each member of this pathway could be a potential predictive or prognostic marker for the disease. Previously, we reported that activated Akt indicated worse outcome among endocrine-treated patients (15) and less response to radiotherapy (16). In this study, we looked for mutations in the *PIK3CA* gene and examined PTEN protein expression in 270 and 201 breast tumors, respectively. We could find associations between these factors and other clinicopathologic markers and also explored the clinical significance of PIK3CA and PTEN status in the largest breast cancer material from a single trial.

The analysis of the *PIK3CA* gene was restricted to exons 9 and 20 because >85% of the mutations are clustered in these areas (9). Missense mutations were detected in 24% of the tumors and 67% of the cell lines. Synonymous mutations were not

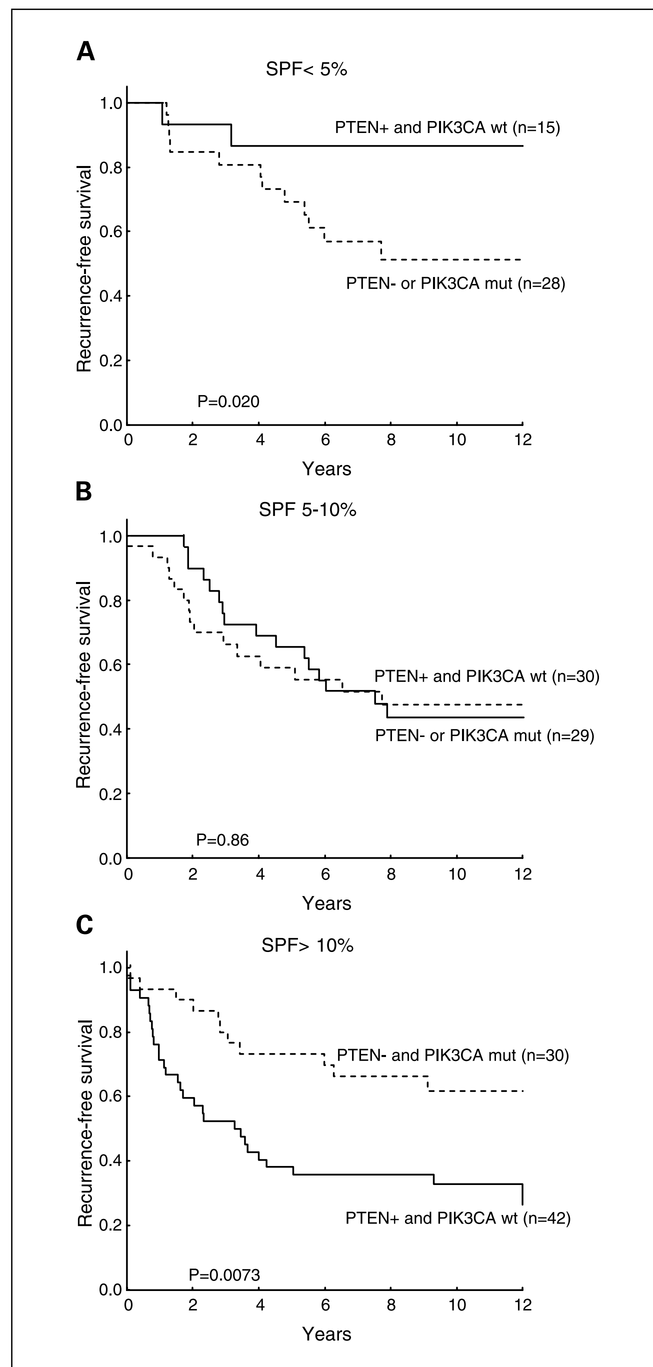

Fig. 4. Recurrence-free survival for patients with the PIK3CA mut and/or PTEN$^-$ type versus PTEN$^+$/PIK3CA wt restricted to low S-phase fraction (SPF, <5%; *A*), SPF (5-10%; *B*), and high SPF (>10%; *C*).

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considered. Because this analysis was limited to exons 9 and 20, the frequency of mutations in the *PIK3CA* gene in breast cancer could have been underestimated. Nevertheless, our results are not in disagreement with others that screened additional exons and found mutations in 26.4% (9) and 21.4% (8) of the tumors. The total frequency of PIK3CA mutations reported thus far in primary breast cancer has been 23.5% (23, 24). Among the cell lines, we found a higher frequency of mutations (67%) compared with other authors (8, 9, 25). This can be explained in part because we only screened 6 cell lines in comparison with those that looked at up to 50 cell lines. However, we observed the same mutations reported by others, except for BT483, where we found the E545K in exon 9 instead of E542K (8, 9). About the distribution of mutations, in our study, exon 20 carried 13% of the mutations whereas exon 9 presented 11%.

The biological significance of these mutations is not well known but they could affect the protein-protein or other intermolecular interactions as well as the lipid substrate specificity of the kinase (7). Other authors have attributed an oncogenic nature to these mutations. *In vitro* and *in vivo* studies with exogenous overexpressed mutants have shown that alterations in the p110α helical or catalytic domains result in a more active PI3K pathway and could raise tumors in animal models (26, 27). However, these results might not be comparable with others using natural mutant cells. Those authors suggested that PIK3CA mutations in the helical domain may even lead to reduced rate of PI3K activation (28).

We attempted to define the nature of the PIK3CA mut by looking at the associations with other clinical markers. In tumors, PIK3CA mut was often related to ER expression and negative HER2 status, which can also be observed among our panel of cell lines often sharing an ER^+/$HER2^-$ phenotype when mutated. In addition, the PIK3CA mutations were related to small tumor size but also associated to other factors, such as PTEN loss, $Akt1^+$, and high expression of cyclin D1, which could indicate a bad course for the disease. Whereas some authors have failed to find clinical useful correlations (3, 7, 8), others have reported common mutations among ER^+ tumors (9). In contrast with our results, these authors also found correlations between PIK3CA mutations, intact PTEN, and high HER2 expression, allowing them to conclude that PIK3CA and PTEN alterations were redundant and other inputs might be needed to activate the PI3K/Akt pathway. However, the same authors found some cases that were PIK3CA/PTEN mutants and $HER2^-$ and PIK3CA and PTEN mutations have been found to frequently coexist in endometrial carcinoma (29). Our results indicate that HER2 overexpression might segregate from PIK3CA mutations and PTEN loss, and in fact, we found significant correlation between Akt activation and the combined variable HER2/PIK3CA. These results allow us to speculate that other alterations in addition to PIK3CA mutation might strengthen the activation of the PI3K/Akt pathway.

Due to the associations with good and bad clinical factors, it is difficult to decipher the significance of this mutation for the patient's survival or their response to treatment.

According to our results, PIK3CA mutations did lower the risk to relapse with local recurrence in comparison with the wild-type phenotype. Assuming the activating nature of these mutations together with the fact that we could only find a weak association with pAkt, this effect might be explained by an Akt-independent mechanism. Besides its survival function, PI3K has been proposed to cause cell death mediated by hypoxia (30), glucose deprivation (31), or serum withdrawal (32). The assumption that the small size of the tumor is due to hypoxic conditions or undernourishment allows us to speculate that, under these conditions, the PI3K induces cell death in the absence of HER2 activation. But PI3K activation has also been shown to induce extracellular signal-regulated kinase 1/2 activation through a pathway that involves Raf1 and PAK1 (33) and high Raf1 activity can induce growth arrest (34), whereas extracellular signal-regulated kinase 1/2 has been associated with ERα phosphorylation at Ser^{118} and better disease outcome in women treated with tamoxifen (35, 36).

Recently, other authors have found PIK3CA mutations in association with ER^+/progesterone $receptor^+$ status, larger tumor size, and poor survival in breast cancer (37). The mutations were detected by single-stranded conformational analysis but the shifted bands were not further sequenced. The risk with this procedure is the difficulty to exclude synonymous mutations or false mutations coming from chromosome 22q11.2, which has an almost identical sequence to that of exon 9.

We also explored the significance of the PIK3CA mutations among ER^+ cases that received tamoxifen or not. We found that ER^+ patients benefit from tamoxifen independently of the PIK3CA status. However, the presence of PIK3CA mutations together with a positive Akt status tended to be coupled to poor effect with tamoxifen treatment.

About PTEN expression, this protein has been found absent or decreased in 27% to 50% of invasive cancers (12, 38–42). In one study comprising 18 *in situ* breast carcinomas, PTEN was lost in 11% of the cases (38). PTEN loss of expression has been correlated to node metastasis, shorter disease-free survival, tumor grade, or aneuploidy in some of these studies but also has been found irrelevant to the clinical outcome in others (43).

When we investigated PTEN protein expression, we found that 37% of the tumors were $PTEN^-$, which agrees with the previous findings. PTEN loss correlated with ER^+ status, small tumor size, and low HER2 expression. PTEN alone did not provide clinical information about distant, local, or breast cancer survival; however, PTEN loss tended to indicate better local disease-free survival for those patients who received radiotherapy in comparison with chemotherapy. Because we observed that those tumors with PTEN loss shared a similar phenotype as the PIK3CA mutants and neither PTEN loss nor PIK3CA mutations correlated significantly with pAkt, we looked at a new combined variable (PIK3CA/PTEN).

Tumors with PIK3CA mutations and/or PTEN loss were also commonly ER^+, small in size, had neither amplified HER2 nor HER2 overexpression, and tended to have lower S phase. This variable had also a clinical value. PIK3CA mutations and/or PTEN loss indicated a shorter recurrence-free survival for those patients with a low SPF but not for patients with a high SPF. These results raise the possibility that, under a high proliferative pressure with deficiency of oxygen or nutrients, the activation of PI3K might lead to cell death through an Akt-independent mechanism.

Finally, we also found that the PIK3CA mut/$PTEN^-$ phenotype tended to confer radiosensitivity and this is in accordance with some *in vitro* and *in vivo* results, indicating that, in the absence of PTEN, ionizing radiation leads to Akt activation, which leads to increased cell size and radiosensitization of cells with PIK3CA mutations/$PTEN^{-/-}$ phenotype (44).

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In conclusion, we have found that PIK3CA mutations and PTEN loss are common alterations in breast cancer. PIK3CA mutations correlated with high Akt1 and cyclin D1 expression. However, PIK3CA mutations, PTEN loss, or the combination of these alterations were associated with similar good clinical markers. These results might indicate that PIK3CA mutations coexist with PTEN loss in ER^{+}/small tumors and that this phenotype might differ from that of HER2 overexpression. The reason for why two so close alterations in the same pathway may coexist is not clear to us. Because we could not find an obvious correlation between PIK3CA mutations and/or PTEN loss to Akt activation, we speculate that in those cases the PI3K may be operating through an Akt-independent mechanism. PIK3CA mut and/or PTEN loss was a predictor of better recurrence-free survival among patients with high proliferating tumors (defined by high SPF), whereas among those with slowly proliferating disease (defined by low SPF) it indicated a poor outcome. This is the first time that the clinical relevance of PIK3CA and PTEN alterations has been studied together in a large breast cancer material from a single trial. Our results support the notion that the PIK3CA and PTEN alterations indeed have clinical significance in breast cancer and that they should be regarded in a tumor context together with other factors before taking a therapeutic decision.

Acknowledgments

We thank Anette Molbaek for sequencing, Agneta Jansson for advice, and Patiyan Andersson for advice and help with primer design.

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