PIK3CA Mutation Associates with Improved Outcome in Breast Cancer

Kevin Kalinsky,[1] Lindsay M. Jacks,[2] Adriana Heguy,[4,5] Sujata Patil,[2] Marija Drobnjak,[6] Umeshkumar K. Bhanot,[6] Cyrus V. Hedvat,[3] Tiffany A. Traina,[1] David Solit,[5] William Gerald,[†] and Mary Ellen Moynahan[1]

Abstract

Purpose: In breast cancer, somatic mutations in the *PIK3CA* gene are common. The prognostic implication of these activating mutations remains uncertain as moderately sized studies have yielded variable outcomes. Our aim was to determine the prognostic implications of PIK3CA mutations in breast cancer.

Experimental Design: Archival formalin-fixed paraffin-embedded primary breast tumors, from 590 patients selected for known vital status with a median follow-up of 12.8 years and a tumor >1 cm, were genotyped for PIK3CA mutations. Mutation rates and associations between mutation site and clinicopathologic characteristics were assessed. Progression-free survival, overall survival, and breast cancer–specific survival were examined using Kaplan-Meier or competing risk methodology.

Results: PIK3CA mutation is identified in 32.5% of breast cancers. PIK3CA mutation significantly associates with older age at diagnosis, hormone receptor positivity, HER2 negativity, lower tumor grade and stage, and lymph node negativity. Patients with PIK3CA mutated tumors have significant improvement in overall survival ($P = 0.03$) and breast cancer–specific survival ($P = 0.004$). Analysis for PIK3CA mutation site-specific associations reveals that the H1047R kinase domain mutation highly associates with node negativity ($P = 0.007$), whereas helical domain hotspot mutations associate with older age at diagnosis ($P = 0.004$).

Conclusion: This study defines the positive prognostic significance of PIK3CA mutations. This work is clinically relevant, as it will significantly affect the design of clinical trials planned for phosphatidylinositol 3-kinase–targeted therapy. Future work may define a population of older age breast cancer patients in whom therapy can be minimized. (Clin Cancer Res 2009;15(16):5049–59)

PIK3CA encodes p110α, the predominant isoform of the catalytic subunit of class 1A phosphatidylinositol 3-kinase (PI3K), a lipid phosphokinase. The family of PI3Ks provides signaling for diverse cellular functions, including proliferation, metabolism, migration, translation, apoptosis avoidance, and angiogenesis (see refs. 1, 2 for review). Disruption of this tightly regulated pathway by gene loss (PTEN), mutation (PIK3CA, AKT1, or less commonly PIK3R1), or amplification (PIK3CA) is one of the most common alterations in human cancers (3). PIK3CA mutations result in constitutive activation of p110α, increasing lipid kinase activity and resulting in an increase in activated Akt (4–6). Cultured cells expressing PIK3CA mutations show increased angiogenesis, acquire features of cellular transformation, and are resistant to cell death (1, 4, 5, 7–10). In primary nontransformed cells, Akt activation has an opposite effect through promotion of cellular senescence (11, 12). In animal models, differences in tumorigenic effects have also been described: increased PIK3CA activity induces tumorigenesis in xenografts and chick embryo (7, 10), expression of activated Akt1 is insufficient to induce tumors in mouse mammary epithelium (13, 14), whereas p110α expression induces mouse mammary tumors that are typically microscopic and occur at low frequency (15).

The PIK3CA mutation frequency has ranged from 8% to 40% in human breast cancers (16–21). The majority of mutations in breast cancer occur at three hotspots (HS): E542K and E545K at exon 9, which encode the helical domain (HD), and H1047R at exon 20, which encodes the kinase domain (KD; refs. 18–20).

Authors' Affiliations: [1]Breast Cancer Medicine Service, Departments of [2]Biostatistics and Epidemiology and [3]Pathology, [4]Geoffrey Beene Translational Core Facility, [5]Human Oncology and Pathogenesis Program, and [6]Pathology Core Facility, Memorial Sloan-Kettering Cancer Center, New York, New York

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

†Before completion of this project, our respected colleague and coauthor William Gerald passed away.

Requests for reprints: Mary Ellen Moynahan, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 381, New York, NY 10065. Phone: 646-888-4561; Fax: 646-888-4555; E-mail: moynaham@mskcc.org.

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Translational Relevance

In this analysis of 590 patients with primary invasive breast cancers with a median follow-up of 12.8 years, we define the positive prognostic significance for PIK3CA mutations. Patients with mutated tumors (192 tumors: 32.5%) show an improvement in progression-free, distant progression-free, overall, and breast cancer–specific survival. In accordance, PIK3CA mutations are associated with favorable clinicopathologic features: lower tumor grade, hormone receptor–positive status, HER2 negativity, older age at diagnosis, lower tumor stage, and lymph node negativity. Notably, site-specific analysis associates helical domain hotspot mutations with older age at diagnosis, whereas the H1047R mutation strongly associates with lymph node negativity. The protective effect observed with PIK3CA mutations will affect the clinical trial design of phosphatidylinositol 3-kinase–targeted therapy. Future work may identify PIK3CA mutation as a predictive marker, highlighting a group of older women with breast cancer in whom limited therapy may be appropriate.

When the entire coding region is analyzed, additional missense mutations occur in up to 18% of breast cancers (19, 20). In cell-based assays, these rare mutations confer a gain of function as measured by lipid kinase activity, constitutive activation of Akt, and cellular transformation, with a range of oncogenic potency (4). The frequency of PIK3CA mutation supports the significance of PI3Ks in breast cancer biology.

It is hypothesized that somatic PIK3CA mutations impart a multifaceted growth advantage to cancer cells, including resistance to antiestrogen therapy, stimulation of angiogenesis, and increased invasiveness. These attributes would be expected to contribute to human cancer progression; however, the clinical relevance of cell-based and animal model phenotypes remains speculative until formally assessed in human disease. Associated with a poor prognostic effect in colon cancer (22), the prognostic significance of PIK3CA mutations remains inconclusive in breast cancer as moderately sized studies have shown inconsistent results, from no survival effect (20, 23) to decreased overall survival (OS; ref. 18) to improved progression-free survival (PFS; ref. 19). To identify the prognostic significance and functional attributes that PIK3CA mutations impart to breast tumor biology, we did PIK3CA mutation analysis on 590 archival primary breast tumors with median follow-up of 12.8 years.

Materials and Methods

Patient database and tissue specimens. The sample population is selected from patients that underwent surgery for primary breast cancer from 1992 to 1996 with pathologic specimens housed at Memorial Sloan-Kettering Cancer Center. Cases were deemed suitable for analysis if a tumor size of >1 cm was noted in the Institutional Database and vital status was known. To reach statistical significance, a sample size of 450 cases was recommended to detect an overall difference in median PFS of 8.4 versus 6 mo with 85% power at a two-sided α level of 5%. Based on an expectation that 75% of pathologic samples would be informative for mutation detection, potential procurement of 600 cases was planned. Sections from 590 formalin-fixed paraffin-embedded (FFPE) breast tumors were procured following confirmation of breast tumor by pathologic review for DNA extraction. Human Tissue Utilization Committee approval and an Institutional Review Board–approved Waiver of Authorization were obtained for the study. All information and samples were recorded to protect patient confidentiality.

Genomic DNA isolation. Genomic DNA was extracted from two 10 μ sections ("curls") using the FormaPure kit (Agencourt), in a 96-well format, using a modified version of the manufacturers' method in a semi-automated fashion. Briefly, 400 μL of FormaPure Lyse Buffer were added per well manually followed by 1-h incubation at 65°C and 15 min at 95°C and addition of 20 μL of 40 mg/mL proteinase K, shaken at 1,400 rpm for 2 min, and then incubated for 72 h at 65°C. This extended heating incubation allows for the DNA-protein cross-linking from formalin fixation to be reversed (24). All subsequent steps of solid-phase reverse immobilization bead binding, isopropanol and ethanol washes, and elution were automated on a Beckman Coulter Biomek NX following the manufacturers' instructions. DNA was eluted in 70 μL of nuclease-free water and dsDNA was quantified by Quant-iT PicoGreen reagent (Invitrogen). The average yield was 373 ng dsDNA.

Mutation detection by Sequenom MassARRAY system. The iPLEX Gold genotyping assay was used. This assay is based on a multiplexed PCR step to amplify a 100- to 150-bp fragment spanning the mutation of interest followed by a single-base primer extension assay. The extension primers are designed immediately adjacent to the mutation site and extended by a single nucleotide at the mutation site, dependent on the template sequence [wild-type (WT) or mutant]. The allelic-specific difference in mass between extended products, as measured by time of flight following laser deionization, allows for the Sequenom software to make a genotype call (Fig. 1B). Multiplexed assays were designed using the Assay Design 3.1 Sequenom software. PCR primers and extension primers for the various mutations are described in Supplementary Table S1. Multiplexed PCR was done in 384-well plates (Supplementary Methods). Mutation status was confirmed for PIK3CA HS and AKT1(E17K) mutations by alternate multiplex MassARRAY analysis.

Confirmation of rare PIK3CA mutations and the AKT1(E17K) mutation by Sanger sequencing was done as described in Supplementary Methods.

Tissue microarray immunohistochemical assessment of HER2 expression. Triplicate tissue cores measuring 0.6 mm in diameter were transferred to a recipient tissue microarray (TMA) paraffin block using the ATA-27 automated arrayer (Beecher Instruments). Five-micron sections were cut from the block and placed on charged slides for staining. Immunohistochemical analysis for HER2 protein expression was done using the HercepTest antibody (K5204, DAKO). Standard protocol was followed according to the manufacturer's instructions. TMA slides were scanned with the Aperio ScanScope XT (Aperio) using a 20× objective. Whole slide images were segmented using TMALab (Aperio). Individual core images were analyzed with the Aperio IHC membrane algorithm, which scores individual cells as 0, 1+, 2+, or 3+ based on both the intensity of staining and membrane completeness. Breast tumors were labeled as 3+ if >30% of cells were identified with 3+ complete membranous staining, 2+ if >10% of cells scored as 2+, and 1+ if >10% of cells scored as 1+. For analysis, $HER2^+$ was defined as 3+ staining and $HER2^-$ as 0, 1+, or 2+ staining. The optimal threshold for intensity and completeness was determined empirically by analyzing a separate series of known breast cancer cases and adjusting the algorithm settings to accurately reflect the manual interpretation and fluorescence *in situ* hybridization amplification status (25).

Statistical methods. Estimates of PIK3CA mutation rates by site with exact binomial confidence intervals (CI) were calculated. Associations between mutation status and clinicopathologic features were assessed using the χ^2 test or *t* test. End points of interest included PFS, the primary end

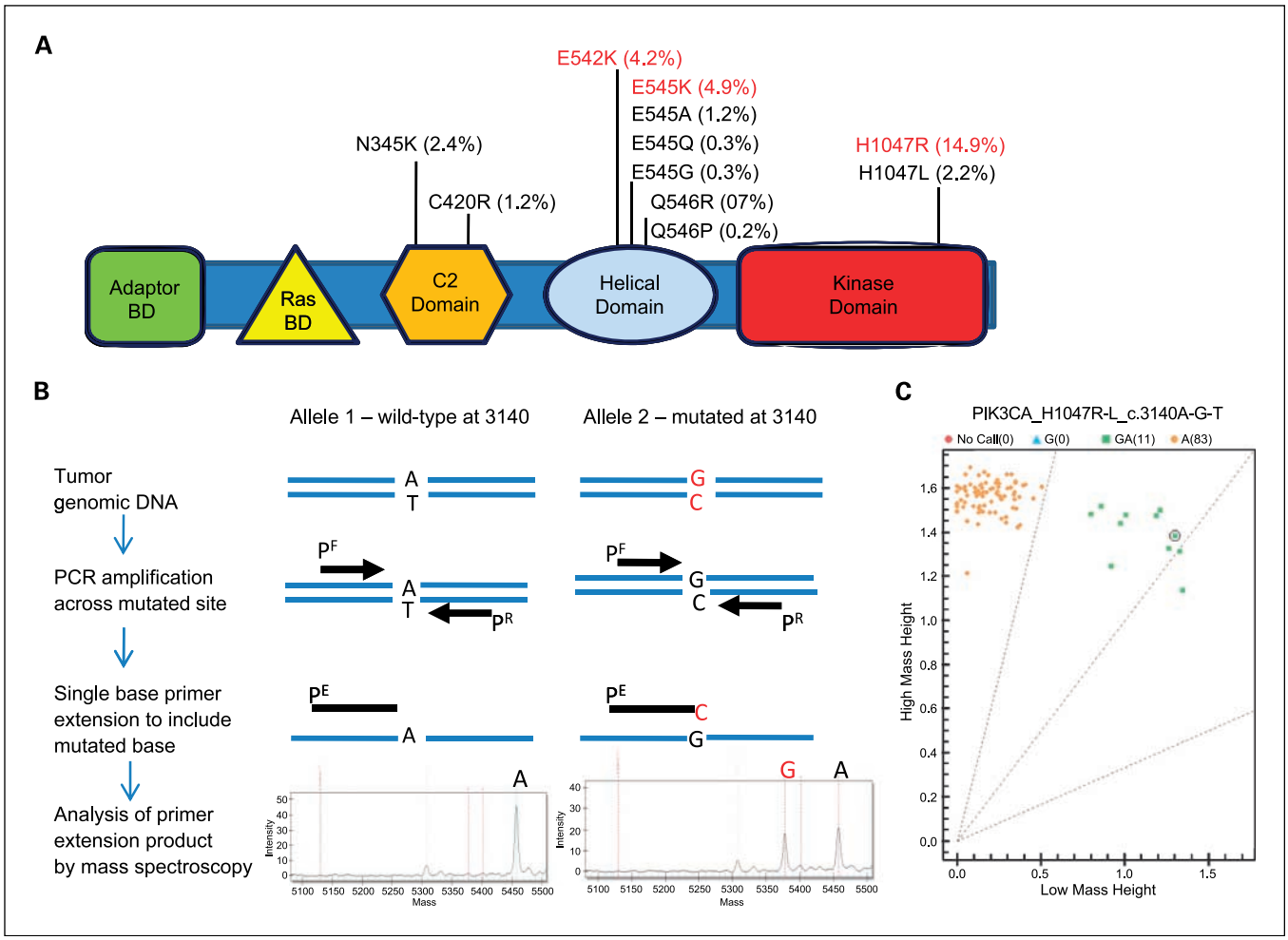

Fig. 1. PIK3CA mutation genotyping and rates. *A*, depiction of the PIK3CA gene with key functional domains identified. The PIK3CA HS mutation terminology is based on their frequency of detection in cancers (E542K, E545K, and H1047R: highlighted in *red*). Other PIK3CA missense mutations are identified less commonly but most cluster at or near the HS mutation sites. The PIK3CA mutation rates (%) are identified for each PIK3CA mutation in the 590 breast tumors. *B*, example of PIK3CA 3140 A > G (H1047R) mutation detection assay by MassARRAY (Sequenom) genotyping. This method is designed to generate a small amplicon at a known mutation site from small amounts of tumor-derived DNA. Specific extension primers include the mutated base. The single nucleotide change in the extension product is detected by mass spectroscopy. Left, analysis of a WT A allele at position 3140; right, analysis of a mutated allele containing a G allele at position 3140. *C*, cluster plot of 94 samples for the H1047R mutation. Homozygosity for the high mass WT adenine (*A*) nucleotide is depicted by orange circles and represents WT tumors, and heterozygous samples (mutated tumors) are depicted by green circles and contain both the WT high mass A allele and the mutant low mass guanine (*G*) nucleotide.

point, distant PFS, OS, and breast cancer–specific survival (BCSS). The PFS, distant PFS, and OS were defined as the time from diagnosis to first recurrence, first distant recurrence, and death from any cause or last follow-up, respectively. The Kaplan-Meier method and Cox proportional hazards models were used in univariate and multivariate analysis to assess the association of mutation status and clinicopathologic characteristics on PFS and OS. For the PFS end point, 23 patients who presented with stage IV disease and 6 patients who developed a contralateral breast cancer and progressed during follow-up were excluded. However, PFS results were similar even with inclusion of these patients. Because ~20% of the cohort died from other or unknown causes, methods of competing risk survival analysis were used to estimate and compare the cumulative incidence of disease-specific death, with death due to other or unknown cause treated as a competing factor (26). To assess the effects of mutation status and clinicopathologic characteristics on disease-specific survival, multivariate competing risk regression analysis was used (27). A P value of ≤0.05 was considered statistically significant in all analyses, including analysis of secondary end points and subgroups. No adjustments for multiple tests were made due to the exploratory nature of the additional analyses. All statistical analyses were done using SAS 9.1 (SAS Institute) or the cmprsk package in R.

Results

PIK3CA mutation detection in archival FFPE tissue. We assessed the PIK3CA genotype for HS and most rare mutations previously identified in breast cancer in 590 primary breast tumors (Fig. 1A). As FFPE tumor tissue is the most common and accessible source of archival tissue, it was important to assess the feasibility of mutation detection in this valuable resource. PIK3CA genotyping was done by multiplex MassARRAY (Sequenom) on native archival DNA and was informative in all cases for the three HS mutations (Fig. 1B and C). A smaller subset of 192 cases underwent Sanger resequencing for all PIK3CA exons, but this method was inferior for mutation detection on the FFPE-derived DNA. Although tumor sections were not specifically assessed for tumor quantity at the time of procurement, the

Table 1. Patient and tumor characteristics by PIK3CA mutation status

Characteristic	Overall cohort (*N* = 590)	Mutation type		*P**
		WT (*n* = 398)	Any PIK3CA (*n* = 192)	
	n (%)	*n* (%)	*n* (%)	
Age (y)				
Median (range)	58 (27-89)	57 (27-89)	61 (33-89)	0.002
<50	160 (27%)	123 (31%)	37 (19%)	0.01
50-69	304 (52%)	195 (49%)	109 (57%)	
≥70	126 (21%)	80 (20%)	46 (24%)	
Menopausal status				
Pre	158 (27%)	118 (30%)	40 (21%)	0.02
Post	426 (72%)	274 (69%)	152 (79%)	
N/A	6 (1%)	6 (2%)	—	
Race				
Caucasian	467 (79%)	315 (79%)	152 (79%)	0.42
Black	54 (9%)	37 (9%)	17 (9%)	
Asian	17 (3%)	9 (2%)	8 (4%)	
Hispanic	21 (4%)	17 (4%)	4 (2%)	
Other	20 (3%)	12 (3%)	8 (4%)	
Unknown	11 (2%)	8 (2%)	3 (2%)	
Tumor size (mm)				
Median (range)	20 (0-150)	20 (1-150)	18 (0-140)	0.08
T stage				
T0	1 (0%)	1 (0%)	—	0.02
T1	332 (56%)	207 (52%)	125 (65%)	
T2	225 (38%)	166 (42%)	59 (31%)	
T3	23 (4%)	16 (4%)	7 (4%)	
T4	9 (2%)	8 (2%)	1 (1%)	
Nodal status				
Negative	300 (51%)	190 (48%)	110 (57%)	0.03
Positive	246 (42%)	177 (44%)	69 (36%)	
Unknown	44 (7%)	31 (8%)	13 (7%)	
N stage				
N0	300 (51%)	190 (48%)	110 (57%)	0.16
N1	129 (22%)	92 (23%)	37 (19%)	
N2	54 (9%)	37 (9%)	17 (9%)	
N3	62 (11%)	47 (12%)	15 (8%)	
Unknown	45 (8%)	32 (8%)	13 (7%)	
Stage†				
I	211 (36%)	128 (32%)	83 (43%)	0.07
II	204 (35%)	144 (36%)	60 (31%)	
III	117 (20%)	83 (21%)	34 (18%)	
IV	23 (4%)	18 (5%)	5 (3%)	
Unknown	34 (6%)	24 (6%)	10 (5%)	
Grade‡				
1	27 (5%)	11 (3%)	16 (8%)	<0.0001
2	204 (35%)	116 (29%)	88 (46%)	
3	288 (49%)	225 (57%)	63 (33%)	
N/A/Unknown	71 (12%)	46 (12%)	25 (13%)	
Histology				
Ductal	474 (80%)	318 (80%)	156 (81%)	0.04
Lobular	60 (10%)	38 (10%)	22 (11%)	
Ductal, lobular	30 (5%)	18 (5%)	12 (6%)	
Special type	25 (4%)	23 (6%)	2 (1%)	
Lymphovascular invasion				
Negative	312 (53%)	205 (52%)	107 (56%)	0.27
Positive	199 (34%)	140 (35%)	59 (31%)	
Unknown	79 (13%)	53 (13%)	26 (14%)	
ER				
Negative	186 (32%)	142 (36%)	44 (23%)	0.0005
Positive	366 (62%)	225 (57%)	141 (73%)	
Unknown	38 (6%)	31 (8%)	7 (4%)	
PR				
Negative	229 (39%)	173 (43%)	56 (29%)	0.0002
Positive	314 (53%)	189 (47%)	125 (65%)	
Unknown	47 (8%)	36 (9%)	11 (6%)	

(*Continued on the following page*)

Table 1. Patient and tumor characteristics by PIK3CA mutation status (Cont'd)

Characteristic	Overall cohort (*N* = 590)	Mutation type		*P**
		WT (*n* = 398)	Any PIK3CA (*n* = 192)	
	n (%)	*n* (%)	*n* (%)	
HR				
Negative	143 (24%)	116 (29%)	27 (14%)	<0.0001
Positive	408 (69%)	251 (63%)	157 (82%)	
Unknown	39 (7%)	31 (8%)	8 (4%)	
HER2				
Negative	440 (75%)	281 (71%)	159 (83%)	<0.0001
Positive	60 (10%)	54 (14%)	6 (3%)	
Unknown	90 (15%)	63 (16%)	27 (14%)	

NOTE: Statistical tests are based on available data; duct carcinoma *in situ*, unknown, and N/A not included in *P* value calculation.
*χ^2 test used for binary/categorical variables; *t* test used for continuous variables.
†Pathology review identified one ductal carcinoma *in situ* (T0) without invasive cancer.
‡Lobular carcinomas and tumors with special type histology were not analyzed for grade and are designated N/A.

pathology was reviewed post hoc on 419 cases to evaluate the percentage of tumor tissue on the whole sections. There was ≥50% tumor in 96.6% of cases, with 85.4% of cases containing >70% tumor.

The overall incidence of PIK3CA mutations is 32.5% (192 tumors; 95% CI, 28.8-36.5%), with 24.1% occurring at the three HS sites (142 tumors; 95% CI, 20.7-27.7%) and a cumulative incidence of 8.5% for rare mutations (50 tumors; 95% CI, 6.4-11.0%; Fig. 1A). As previously reported, the most frequent PIK3CA HS mutation in breast cancer occurs in the KD (H1047R: 14.9%), with the second most common region in the HD (E542K and E545K: 9.2% total, with similar frequencies). No tumors harbor multiple HS mutations. Rare PIK3CA mutations are identified at low frequency, with the H1047L and N345K mutations each occurring in ~2% of cases (Fig. 1A). However, it is likely that the rare PIK3CA mutation rate is slightly underrepresented, as not all rare PIK3CA mutations were assessed and others were not adequately informative by this method (data not shown; Supplementary Data).

PIK3CA mutation associates with an older age at diagnosis. Patient characteristics differentially associate with a breast tumor harboring a PIK3CA mutation compared with WT PIK3CA (Table 1). Compared with WT, PIK3CA mutations are identified in a significantly older patient population ($P = 0.002$) and, in accordance, in postmenopausal women when evaluated by menopausal status ($P = 0.02$). Although data are available on oral contraceptive and postmenopausal hormone therapy use, the patient numbers for these demographic details are too infrequent for analysis. No differences in race are observed between these two cohorts ($P = 0.42$); however, our population is 79% Caucasian with small sample sizes in non-Caucasian groups.

PIK3CA mutation associates with "good" prognosis discriminators. Tumor features that associate with PIK3CA mutation include lymph node negativity ($P = 0.03$) and lower tumor stage ($P = 0.02$), although tumor size is not significant as a continuous variable (Table 1). Notably, PIK3CA mutated breast tumors are highly associated with lower grade scores, hormone receptor (HR) positivity, and HER2 negativity (all variables: $P \leq 0.0001$). Based on historical analysis, HR status is available for the majority of patients, with HR status unknown in 39 patients (7%). No difference is identified in the PIK3CA mutation rate between estrogen receptor–positive (ER^+)/progesterone receptor–positive (PR^+) tumors and ER^+/PR^- tumors (data not shown). Whereas HER2 positivity is defined as tumors with 3+ immunohistochemical staining, PIK3CA mutated tumors associate with HER2 negativity regardless of whether tumors scored as 2+ are included in the $HER2^-$ or $HER2^+$ cohort (data not shown). PIK3CA mutations associate with tumor histology ($P = 0.04$), most likely due to the difference in special type histology tumors that were infrequent in both cohorts.

Improved patient outcome, including OS and BCSS, associates with PIK3CA mutation. The median follow-up for the cohort is 12.8 years. Patients harboring a PIK3CA mutated breast tumor show a marginally significant longer PFS and distant PFS compared with patients with WT tumors ($P = 0.06$ and $P = 0.09$, respectively; Fig. 2A and B). There is a significant improvement in OS for patients with PIK3CA mutated breast tumors ($P = 0.03$; Fig. 2C). Most notably, there is a strong association with improved BCSS in patients with PIK3CA mutation ($P = 0.004$; Fig. 2D). In multivariate analysis, adjusting for mutation status, age, nodal status, tumor stage, grade, HR, and HER2 status, the only predictors that maintain significance are nodal status and tumor stage for all clinical end points analyzed: PFS, OS, and BCSS (Supplementary Table S2; data not shown).

PIK3CA HS helical and KD site-specific mutations differentially associate with patient- and tumor-specific classifiers. The PIK3CA HS mutation terminology is based on their frequency of detection in cancers and on their higher oncogenic potency as determined in cell-based assays. Recent research notes differences in the protein partners required for site-specific HS PIK3CA mutation activity and different tumorigenic potential in animal models (7, 28). As these findings underscore the potential that site-specific mutations impart clinical differences in tumor biology, we did analyses by mutation site stratification. Older age at diagnosis is solely attributed to patients with a breast tumor harboring a HD HS mutation. The median age at diagnosis is 57 years for patients with a WT tumor or H1047R KD mutated tumor and 63 years for patients with a HD HS mutated tumor (Table 2). Supporting this finding, patients with a HD mutated tumor are significantly more likely to be postmenopausal at presentation ($P = 0.04$). Most notably, lymph node–negative status associates specifically with the H1047R KD mutation, in which

64% of patients are lymph node negative compared with 48% of patients with WT and HD mutated tumors ($P = 0.007$). Lower nodal stage, lower overall stage, and smaller tumor size significantly associate with the H1047R mutation. Compared with WT tumors, all HS mutations highly associate with lower grade scores, HR positivity, and HER2 negativity. The associations with any HS PIK3CA mutation are similar to those with any PIK3CA mutation, except that tumor size as a continuous variable became significant ($P = 0.01$; Table 2). Rare PIK3CA mutated tumors comprise a small cohort and significantly associate with lower grade, HER2 negativity, and absent lymphovascular invasion compared with WT (data not shown).

PIK3CA H1047R mutations are significantly associated with improved survival. Patients with any PIK3CA HS mutated breast tumor show a marginally significant improvement in OS compared with WT ($P = 0.06$; Table 3). Notably, patients with a H1047R KD mutated tumor display a statistically significant improvement in OS when compared with WT (Table 3; Fig. 3A). This improvement in OS is not observed in patients with a HD HS mutated tumor ($P = 0.54$; Table 3; Fig. 3A), showing that the OS benefit observed in patients with PIK3CA mutated tumors is a result of the H1047R KD mutation.

As observed with any PIK3CA mutation, patients with any HS mutation display a significant improvement in BCSS when compared with WT ($P = 0.01$; Table 3). However, in Fig. 3B, the BCSS curves for KD HS and HD HS mutations overlap and are significantly different from WT tumors, suggesting a difference in outcome benefit. This favorable end point, when analyzed separately, reaches significance only for patients with H1047R mutated tumors ($P = 0.03$); however, the survival rates for the HS HD mutation are similar, and the lack of observed significance may be due to smaller cohort size (Table 3). In patients harboring a H1047R mutated tumor, the probability of death due to breast cancer at 5 and 10 years decreases 2.8- and 2-fold, respectively. For any PIK3CA mutation, there is a 2-fold decrease in death due to breast cancer at both 5 and 10 years. Patients with

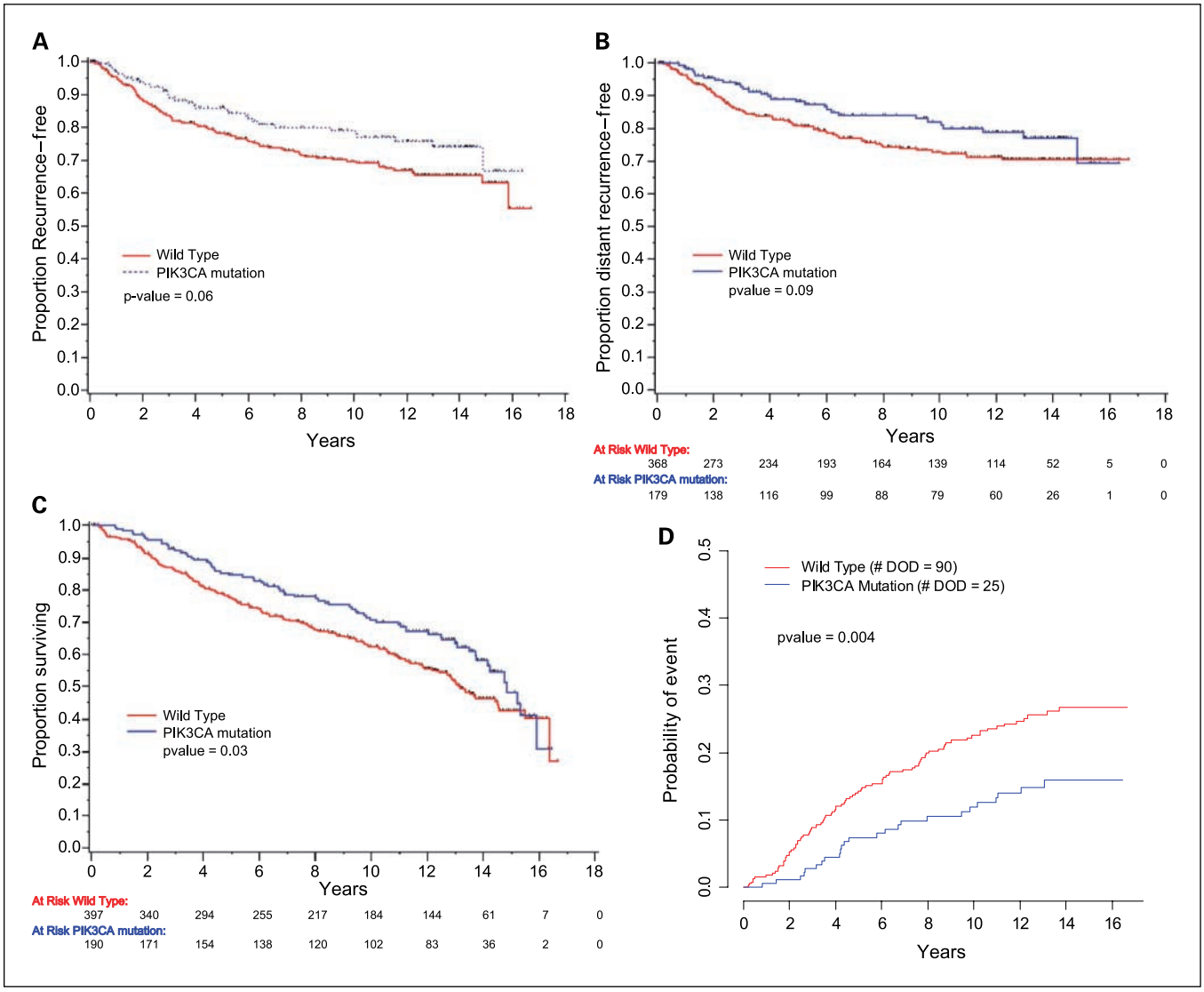

Fig. 2. PIK3CA mutation status and clinical outcome. Patients with PIK3CA mutated tumors are marginally associated with a longer PFS (*A*) and distant PFS (*B*) and are significantly associated with an improved OS (*C*) and BCSS (*D*) compared with WT. DOD, dead of disease (breast cancer).

Table 2. Patient and tumor characteristics by PIK3CA mutation type

Characteristic	WT (*N* = 398)	Mutation type					
		HS (*n* = 142)		HS kinase (*n* = 88)		HS helical (*n* = 54)	
	n (%)	*n* (%)	*P**	*n* (%)	*P**	*n* (%)	*P**
Age (y)							
Median (range)	57 (27-89)	61 (33-89)	0.004	57 (27-89)	0.1	63 (35-88)	0.004
<50	123 (31%)	28 (20%)	0.03	21 (24%)	0.42	7 (13%)	0.02
50-69	195 (49%)	78 (55%)		47 (53%)		31 (57%)	
≥70	80 (20%)	36 (25%)		20 (23%)		16 (30%)	
Menopausal status							
Pre	118 (30%)	29 (20%)	0.03	20 (23%)	0.17	9 (17%)	0.04
Post	274 (69%)	113 (80%)		68 (77%)		45 (83%)	
N/A	6 (2%)	—		—		—	
Race							
Caucasian	315 (79%)	113 (80%)	0.24	68 (77%)	0.48	45 (83%)	0.26
Black	37 (9%)	12 (8%)		10 (11%)		2 (4%)	
Asian	9 (2%)	7 (5%)		4 (5%)		3 (6%)	
Hispanic	17 (4%)	2 (1%)		1 (1%)		1 (2%)	
Other	12 (3%)	6 (4%)		3 (3%)		3 (6%)	
Unknown	8 (2%)	2 (1%)		2 (2%)		—	
Tumor size (mm)							
Median (range)	20 (1-150)	18 (0-80)	0.01	18 (0-70)	0.02	19.5 (1-80)	0.16
T stage							
T0	1 (0%)	—	0.06	—	0.10	—	0.52
T1	207 (52%)	92 (65%)		58 (66%)		34 (63%)	
T2	166 (42%)	45 (32%)		27 (31%)		18 (33%)	
T3	16 (4%)	4 (3%)		3 (3%)		1 (2%)	
T4	8 (2%)	1 (1%)		—		1 (2%)	
Nodal status							
Negative	190 (48%)	82 (58%)	0.03	56 (64%)	0.007	26 (48%)	0.87
Positive	177 (44%)	49 (35%)		26 (30%)		23 (43%)	
Unknown	31 (8%)	11 (8%)		6 (7%)		5 (9%)	
N stage							
N0	190 (48%)	82 (58%)	0.17	56 (64%)	0.05	26 (48%)	0.95
N1	92 (23%)	24 (17%)		13 (15%)		11 (20%)	
N2	37 (9%)	13 (9%)		7 (8%)		6 (11%)	
N3	47 (12%)	12 (8%)		6 (7%)		6 (11%)	
Unknown	32 (8%)	11 (8%)		6 (7%)		5 (9%)	
Stage[†]							
I	128 (32%)	63 (44%)	0.06	44 (50%)	0.02	19 (35%)	0.78
II	144 (36%)	40 (28%)		22 (25%)		18 (33%)	
III	83 (21%)	26 (18%)		14 (16%)		12 (22%)	
IV	18 (5%)	4 (3%)		3 (3%)		1 (2%)	
Unknown	24 (6%)	9 (6%)		5 (6%)		4 (7%)	
Grade[‡]							
1	11 (3%)	12 (8%)	<0.0001	6 (7%)	0.0004	6 (11%)	<0.0001
2	116 (29%)	69 (49%)		41 (47%)		28 (52%)	
3	225 (57%)	45 (32%)		32 (36%)		13 (24%)	
N/A/Unknown	46 (12%)	16 (11%)		9 (10%)		7 (13%)	
Histology							
Ductal	318 (80%)	117 (82%)	0.17	72 (82%)	0.16	45 (83%)	0.73
Lobular	38 (10%)	14 (10%)		8 (9%)		6 (11%)	
Ductal, lobular	18 (5%)	9 (6%)		7 (8%)		2 (4%)	
Special type	23 (6%)	2 (1%)		1 (1%)		1 (2%)	
Lymphovascular invasion							
Negative	205 (52%)	76 (54%)	0.79	48 (55%)	0.64	28 (52%)	0.89
Positive	140 (35%)	49 (35%)		29 (33%)		20 (37%)	
Unknown	53 (13%)	17 (12%)		11 (13%)		6 (11%)	
ER							
Negative	142 (36%)	30 (21%)	0.0003	19 (22%)	0.004	11 (20%)	0.01
Positive	225 (57%)	108 (76%)		67 (76%)		41 (76%)	
Unknown	31 (8%)	4 (3%)		2 (2%)		2 (4%)	
PR							
Negative	173 (43%)	40 (28%)	0.0003	22 (25%)	0.0003	18 (33%)	0.12
Positive	189 (47%)	94 (66%)		62 (70%)		32 (59%)	
Unknown	36 (9%)	8 (6%)		4 (5%)		4 (7%)	

(*Continued on the following page*)

Table 2. Patient and tumor characteristics by PIK3CA mutation type (Cont'd)

Characteristic	WT (*N* = 398)	Mutation type					
		HS (*n* = 142)		HS kinase (*n* = 88)		HS helical (*n* = 54)	
	n (%)	*n* (%)	*P**	*n* (%)	*P**	*n* (%)	*P**
HR							
Negative	116 (29%)	17 (12%)	<0.0001	10 (11%)	0.0002	7 (13%)	0.007
Positive	251 (63%)	120 (85%)		75 (85%)		45 (83%)	
Unknown	31 (8%)	5 (4%)		3 (3%)		2 (4%)	
HER2							
Negative	281 (71%)	120 (85%)	0.0005	73 (83%)	0.01	47 (87%)	0.01
Positive	54 (14%)	5 (4%)		4 (5%)		1 (2%)	
Unknown	63 (16%)	17 (12%)		11 (13%)		6 (11%)	

NOTE: Statistical tests are based on available data; duct carcinoma *in situ*, unknown, and N/A not included in *P* value calculation.
*χ^2 test used for binary/categorical variables; *t* test used for continuous variables.
†Pathology review identified one ductal carcinoma *in situ* (T0) without invasive cancer.
‡Lobular carcinomas and tumors with special type histology were not analyzed for grade and are designated N/A.

rare mutations show a trend toward a significant improvement in PFS, distant PFS, OS, and BCSS; with a larger cohort of patients, this observation may have reached significance (Supplementary Fig. S1).

BCSS outcomes are maintained when analyzed by HR subgroups. An exploratory analysis was done to evaluate whether the beneficial effect of PIK3CA mutation status on clinical outcome was independent of endocrine receptor status. Patients with HR^+, PIK3CA mutated tumors show a longer BCSS compared with patients with HR^+, PIK3CA WT tumors ($P = 0.03$; Fig. 3C). Women with ER^-, PIK3CA mutated tumors also display a longer BCSS compared with women with ER^-, PIK3CA WT tumors ($P = 0.04$; Fig. 3D).

AKT1(E17K) mutation is identified in breast cancer. A somatic missense mutation in the pleckstrin homology domain of AKT1 has been reported and confirmed to occur at low frequency in a variety of tumors (29–31). The AKT1(E17K) mutation results in increased localization of AKT1 to the plasma membrane, increased activation of the kinase, and transformation in cultured cells (30). Mutually exclusive with respect to PIK3CA mutations, the AKT1(E17K) mutation occurs with an incidence rate of 3.6% (21 tumors; 95% CI, 2.2-5.4%) in our cohort (data not shown). Similar to PIK3CA mutations, and in agreement with a recent report, the AKT1(E17K) mutation commonly associates with HR^+ tumors (32). However, due to the low mutation rate, the prognostic significance of the AKT1(E17K) mutation will not be determined in single studies and will likely require a meta-analysis.

Discussion

This study shows the feasibility and utility of archival FFPE human tumor samples procured by routine pathologic sectioning for the assessment of somatic missense mutations. MassARRAY genotyping generates small amplicons, and the single-base extension primer methodology tolerates a mix of normal and tumor tissue, alleviating the requirement for frozen tumor specimens and tumor microdissection. In this study, we identify PIK3CA mutations in 32.5% of invasive breast primary tumors, 24.1% occurring at the three HS sites and 8.5% for the combined rare PIK3CA mutations. The frequency of HS mutations correlates well with those previously reported, and this is the first large assessment of rare PIK3CA mutations in breast cancer (4). In breast cancer, the mutation rate in the PI3K/Akt pathway as determined by either PIK3CA mutation or AKT1(E17K) mutation is 36%.

PIK3CA mutation in breast tumors associates with classic good prognostic determinants, which translate into improved

Table 3. OS and BCSS by PIK3CA mutation type

Mutation type	OS (95% CI)			BCSS (95% CI)		
	Survival rates			Probabilities of death		
	5 y	10 y	*P*	5 y	10 y	*P*
Wild-type	77% (73-81)	62% (57-67)		14% (11-18)	23% (18-27)	
vs						
Any PIK3CA mutation	85% (78-89)	71% (63-77)	0.03	7% (4-11)	12% (7-17)	0.004
HS mutation	86% (79-91)	70% (61-78)	0.06	5% (1-9)	11% (6-17)	0.01
HS kinase	89% (80-94)	76% (65-84)	0.005	5% (0-10)	11% (4-19)	0.03
HS helical	82% (68-90)	59% (43-72)	0.54	6% (0-13)	11% (2-20)	0.13
Rare PIK3CA mutation	80% (64-89)	72% (56-83)	0.19	14% (3-24)	—	0.10

clinical outcome. A prototype good mutation in cancer exists with fibroblast growth factor receptor 3 gene mutations, which associate with low grade and superficial urinary bladder tumors (33, 34). The "protective" role that PIK3CA somatic activating mutation plays in breast cancer is unexpected, especially because its function is central to the PI3K/Akt signaling pathway. Other genetic/epigenetic aberrations associated with the pathway, most notably HER2 amplification and PTEN loss, impart a poor prognosis in breast cancer. Prior studies describe a fairly consistent association with PIK3CA mutation and HR^+ breast cancer but reported inconsistent clinical outcome associations (18–20, 23). Given the natural history of HR^+ breast cancer, in which only half of all recurrences will occur within 5 years of follow-up (35), long clinical follow-up data were paramount to the study design and determining the prognostic effect of PIK3CA mutation. As our cohort consists of primary invasive breast tumors from 1992 to 1996, we used the historical analysis of endocrine receptor status; however, during this time period, HER2/neu status was not routinely done at diagnosis. We did immunohistochemical assessment of HER2 expression on TMAs and identified an inverse correlation with PIK3CA mutation and HER2 overexpression. This finding agrees with most prior studies, which note an inverse correlation with PIK3CA mutation and HER2 overexpression (16, 23) or no correlation (18, 19, 36). One study reports an association with HER2 overexpression as determined by 2+ or 3+ positivity and PIK3CA mutation status (20). In our cohort, PIK3CA WT tumors are associated with HER2 negativity regardless of whether tumors with 2+ staining are included in the $HER2^-$ or $HER2^+$ cohort. The association with HER2 negativity and PIK3CA mutations is consistent with

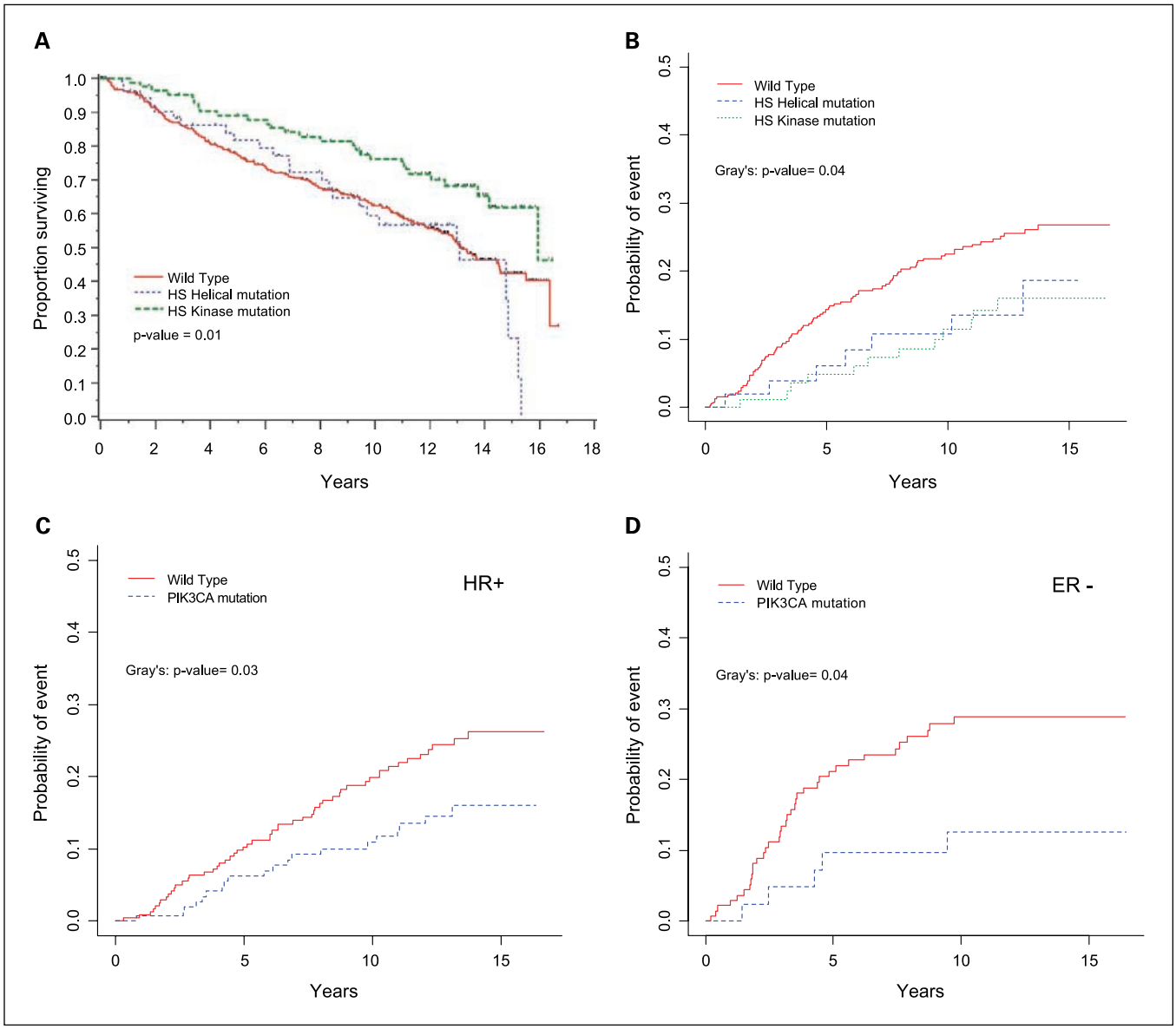

Fig. 3. Patients with PIK3CA mutated tumors show significantly improved OS with an H1047R KD mutation (*A*) and improved BCSS with any PIK3CA HS mutation (*B*) compared with WT. *C* and *D*, BCSS outcomes are maintained in HR^+ and ER^- subgroups.

the favorable prognostic tumor features that correlate with PIK3CA mutations.

The mechanisms of the PIK3CA mutation-associated protection may be different according to mutation type. Whereas both HS mutation sites are associated with lower grade and HR^+ tumors, the strong association with lymph node negativity in H1047R mutants and older age at diagnosis in HD HS mutants suggests key separation of function attributes to the two mutation sites. Mutational activation for the HD requires Ras binding, and HD HS mutations impart a conformational change alleviating interaction with the regulatory PI3K subunit p85 (37). This is opposite to the H1047R mutation, where only p85 binding is required. Interestingly, despite appearing stronger in both oncogenic potency and pathway activation in preclinical models (7, 38), the H1047R mutation has the strongest association with clinical benefit. This finding, along with the minimal effect seen with rare PIK3CA mutations, strengthens the argument that the observed improvement is a direct effect of mutation activation.

Whether the protective effect of PIK3CA mutations is the result of p110α activation, an alternate PIP3 target, or Akt pathway activation is not known and warrants further study. There are many Akt activation phenotypes in cancer and normal cells that potentially explain this protective effect, including oncogene-induced senescence, maintenance of differentiation, and anti-invasive/antimetastatic phenotypes (11, 12, 14). Observed in experimental animal models and in human tissues, oncogene-induced senescence is an endogenous tumor suppressor mechanism triggered by replication stress (39). In hyperproliferative states, such as oncogene activation, biphasic growth is observed with an expansion of mutant cells followed by a senescence program of limited growth (40, 41). Although the duration for growth expansion and suppression is not determined, this program offers a reasonable premise for the long disease-free intervals often observed in breast cancer.

A novel finding for the HD HS mutations is the association of older age at diagnosis. Benign skin lesions have been shown to harbor PIK3CA mutations. In epidermal nevi, congenital lesions that occur early in life, a single rare PIK3CA mutation has been identified (42). However, in seborrheic keratoses, benign dermatologic lesions that commonly develop in older patients, the identified PIK3CA mutations are predominantly HD HS mutations (42). These observations in seborrheic keratoses and breast cancer may underscore an age-associated link in the pathogenesis of PIK3CA HD HS mutations. Women with breast cancer diagnosed at 70 years of age or older have a 36.5% likelihood of having a PIK3CA mutated tumor. In our study, women over the age of 70 comprise 21% of the cohort. The novel finding of HD HS mutations associating with older age at diagnosis is unlikely to be identified in other studies that do not include an older population and are, as a result, less reflective of the natural history of breast cancer. The majority of these cancers will be HR^+ and of low to moderate grade. One death from breast cancer was observed in the node-negative cohort. Although this finding needs further exploration, its significance should not be dismissed. Identifying older age patients in whom breast cancer treatments could be minimized would improve patient quality of life and decrease medical costs.

PIK3CA H1047R mutations significantly associate with lymph node–negative tumors as well as improvement in clinical outcome. Activated Akt has been shown to enhance the early stages of tumorigenesis while showing a suppressive effect on tumor invasion and metastatic potential (14, 15). In bitransgenic mice that express both activated Akt and ErbB2 in the mammary epithelium, accelerated mammary tumor formation and a marked decrease in lung metastasis are observed compared with transgenic mice expressing activated ErbB2 alone (14). The tumors also retain a differentiated histology similar to the lower grade seen in PIK3CA mutated human breast tumors.

In agreement with other studies, we report a highly significant relationship between PIK3CA mutations and HR positivity (18–20, 23, 32). This association is also observed with the AKT1(E17K) mutation. In our cohort, HR^+ tumors have a 43.5% likelihood of harboring a PIK3CA or AKT1(E17K) mutation. Akt-dependent ER activation has been reported, suggesting a preferential growth of ER^+ tumors (43). Mammary tumors derived from transgenic mice that express a constitutively active p110α show high levels of ERα phosphorylation, an uncommon finding in murine mammary tumors. High levels of ERα phosphorylation identified in preneoplastic epithelium were reduced with PI3K inhibitor therapy (15). The cross-talk between nongenomic ER activities and the PI3K/Akt pathway may provide a differential target not observed in other PIK3CA mutated tumor types. Whether breast cancer is unique regarding the protective role for PIK3CA mutation is not known; however, patients with endocrine-resistant PIK3CA mutated breast tumors may be a uniquely sensitive population in which to evaluate novel PI3K inhibitors. Maintenance of endocrine sensitivity is a high priority research goal in the treatment of breast cancer. Further biomarker assessment of tumor tissue for changes in signaling pathway activation at the time of endocrine resistance may identify potential strategies for combined pathway inhibition to restore endocrine sensitivity.

Occurring in approximately one third of invasive breast primary tumors, PIK3CA mutations offer a protective effect for patients with breast cancer. Additional efforts to formally identify the mechanism of protection imparted by PIK3CA mutation are key as PI3K/Akt pathway inhibitors proceed through clinical development for targeted cancer therapy. Tailored treatments based on somatic gene mutations have become the standard of care in other malignancies, such as colon cancer, in which mutant KRAS is a powerful negative predictor of epidermal growth factor receptor response (44). Future treatment approaches might not only be based on the presence or absence of PIK3CA mutations but also tailored to site-specific mutation status. The protective role imparted by a PIK3CA mutation will significantly affect future clinical trial design for PI3K-targeted therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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