

Phosphatidylinositol-3-kinase and AKT1 mutations occur early in breast carcinoma

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Abstract Mutationally activated protein kinases are appealing therapeutic targets in breast carcinoma. Mutations in phosphatidylinositol-3-kinase (*PI3KCA*) have been described in 8–40% of invasive breast carcinomas, and *AKT1* mutations have been characterized in 1–8% of breast carcinomas. However, there is little data on these mutations in breast precursor lesions. To further delineate the molecular evolution of breast tumorigenesis, samples of invasive breast carcinoma with an accompanying in situ component were macro dissected from formalin-fixed paraffin embedded tissue and screened for mutations in *PI3KCA* exons 7, 9, 20, and *AKT1* exon 2. Laser capture micro dissection (LCM) was performed on mutation-positive carcinomas to directly compare the genotypes of separated invasive and in situ tumor cells. Among 81 cases of invasive carcinoma, there were eight mutations in *PI3KCA* exon 20 (7 H1047R, 1 H1047L) and four mutations in exon 9 (2 E545K, 1 E542K, 1 E545G),

totaling 12/81 (14.8%). In 11 cases examined, paired LCM in situ tumor showed the identical *PI3KCA* mutation in invasive and in situ carcinoma. Likewise, 3 of 78 (3.8%) invasive carcinomas showed an *AKT1* E17K mutation, and this mutation was identified in matching in situ carcinoma in both informative cases. Mutational status did not correlate with clinical parameters including hormone receptor status, grade, and lymph node status. The complete concordance of *PI3KCA* and *AKT1* mutations in matched samples of invasive and in situ tumor indicates that these mutations occur early in breast cancer development and has implications with regard to therapeutics targeted to the PI3 kinase pathway.

Keywords Phosphatidylinositol-3-kinase (*PI3KCA*) · *AKT1* · Breast carcinoma · Carcinoma in situ · Laser capture microdissection

Introduction

Dysregulation of pathways regulating cell signaling, growth, proliferation, and apoptosis is central to neoplasia and malignant transformation. It is well established that neoplastic progression involves the acquisition of molecular defects in various signaling pathways in many tumor types [1]. While a stepwise accumulation of genetic defects has been closely correlated with progression from colonic adenoma to invasive carcinoma, the accumulation of specific mutations at various stages in breast carcinogenesis is less well understood [1–5].

Phosphatidylinositol-3-kinase mutations in solid tumors have gained increasing attention since the landmark study of Samuels et al. [6] which revealed somatic mutations in the phosphatidylinositol-3-kinase 110 kDa α catalytic

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subunit (PIK3CA) in one-third of tested colon carcinomas and a smaller percentage of other carcinomas. PIK3CA is activated by transmembrane receptor-tyrosine kinases, including those of the epidermal growth factor receptor (EGFR) family and is also regulated by the Ras/MEK pathway [7–12]. Activated PIK3CA dimerizes with its regulatory subunit, p85, and belongs to a family of lipid kinases that phosphorylate phosphatidylinositol (4, 5) biphosphate (PIP₂) at the 3-position to form phosphatidylinositol (3, 4, 5) triphosphate (PIP₃). The phosphorylated second messenger product, PIP₃, activates plekstrin homology domain containing proteins, primarily Akt family members at the cell membrane [8–10, 12, 13]. Activated Akt in turn activates mammalian target of rapamycin (mTOR) and has far-reaching effects intracellularly, including interaction with transcription factors, metabolic pathways, apoptosis, angiogenesis, and others [8, 9, 14]. Phosphatase and tensin homolog (PTEN) antagonizes PIK3CA signaling by dephosphorylating PIP₃, and some authors have found PIK3CA activation and PTEN dysfunction to be mutually exclusive in breast carcinomas, though data are contradictory [15, 16]. Recently, activating mutations in *AKT1* have also been characterized [17].

PIK3CA mutations most commonly occur in exon 9 (helical domain, E542K, E545K) and exon 20 (kinase domain, H1047R); these two exons account for over 85% of tumor mutations [6, 10, 13, 18, 19]. Studies have documented *PIK3CA* mutations in 8–40% of infiltrating breast carcinomas, [6, 7, 12, 15, 16, 18, 20–31], and this holds across different ethnic groups with varying breast cancer incidence [25, 28]. Some investigators have noted a somewhat higher prevalence of *PIK3CA* mutation in lobular as compared to ductal carcinomas [7, 23, 25, 26, 28]. Several investigators have established the somatic nature of these mutations, as they are absent in adjacent normal breast parenchyma [7, 15, 18, 20–23, 25, 26, 28].

Interestingly, *AKT1* mutations recently identified in breast, ovarian and colorectal carcinoma are situated in the plekstrin homology domain (E17K), rather than in a kinase domain [17, 31–33]. Nevertheless, these *AKT1* mutations are activating; they transform cells and induce leukemia in nude mice [17].

Molecular studies of the role of kinase mutations in the progression of in situ breast carcinoma to invasive carcinoma are limited. Two *PIK3CA* mutations have been reported among only 45 DCIS cases tested for *PIK3CA* (6.6%) [20, 21, 28]. No *AKT1* mutations were identified in a study of 15 cases of pre-invasive disease [33]. We sought to further characterize mutations in the protein kinases *PIK3CA*, and *AKT1* in breast carcinoma, and specifically to investigate whether such mutations are already present at the stage of in situ carcinoma.

Methods

Patients and tissues

This study was approved by the Institutional Review Board at Oregon Health & Science University (OHSU). The archives of OHSU Department of Pathology were searched for invasive breast carcinoma with an accompanying in situ component. Consecutive cases between 2002 and 2006 were screened; cases were excluded if the invasive tumor was represented on only one tissue block or if the in situ component was present only in foci intimately admixed with the invasive carcinoma, or was too small for study. Eighty-one cases of breast carcinoma with accompanying in situ carcinoma were selected for study (72 ductal, 9 lobular). Tumor characteristics were recorded, including: infiltrating carcinoma type, grade (modified Bloom-Richardson score), size, nodal involvement, and hormone status, and in situ carcinoma type and nuclear grade (see below).

Mutational analysis of invasive tumor

Five-micrometer sections of formalin-fixed paraffin embedded (FFPE) tissue were prepared, and invasive tumor was macro dissected with a clean scalpel blade, using corresponding H & E stained sections as a guide, then de-paraffinized with successive xylene and graded ethanol washes. DNA was extracted using the Qiagen DNA Mini Kit (QIAGEN, Valencia, CA). Custom primers were synthesized by Invitrogen (Carlsbad, CA) or Integrated DNA Technology (IDT, Coralville, IA). *PIK3CA* exons 7, 9, and 20 were amplified by PCR in 81 cases using the primers detailed in Table 1A. The primers for exon 9 did not amplify the known pseudogene using an annealing temperature of 58°C [15]. *AKT1* exon 2 was amplified in 78 cases with the primers listed in Table 1B. Positive and negative control reactions were performed with each batch of PCR. PCR contained 1× buffer, 50 μM dNTPs, 300 nM forward primers, 300 nM reverse primer, and 1.4 U of Taq polymerase (Expand High Fidelity PCR System, Roche, Indianapolis, IN) in a final volume of 20 μl. Nested PCR was performed on selected macro dissected samples (Table 1). Amplicons were screened on a Transgenomic WAVE HPLC system (Transgenomic, Omaha, NE; melting temperature determined empirically for each primer pair); suspected mutations were confirmed by bidirectional sequencing on an ABI 3130 sequencer using the Big-Dye terminator method (same primers as for PCR, Table 1). Normal tissue away from tumor in two mutated cases was also macro dissected and analyzed for *PIK3CA* mutation by PCR and WAVE HPLC as above.

Table 1 Macro dissection primers

	Forward	Reverse
<i>A. PIK3CA primers</i>		
Macro dissection primers		
Exon 7	TGAATTTTCCTTTTGGGGAAG	CAAACCTCCAACCTCTAAGCATGG
Exon 20	CATTTGCTCCAAACTGACCA	GGTCTTTGCCTGCTGAGAGT
Exon 9	TTGAAAATGTATTTGCTTTTTCTGT	CATGTAAATTCTGCTTTATTTATTCCA
Macro dissection nested primers		
Exon 7–1	CCCATTATTATAGAGATGATTG	CAAACCTCCAACCTCTAAGCATGG
Exon 7–2	TGAATTTTCCTTTTGGGGAAG	GTTTCCTAAGAGATGGAAG
Exon 20–1	CATTTGCTCCAAACTGACCA	GGTCTTTGCCTGCTGAGAGT
Exon 20–2	TTCTTATAGGTTTCAGGAGATGTGTT	TGTGGAATCCAGAGTGAGCTT
Exon 9–1	GATTGGTTCTTTCCTGTCTCTG [6]	CCACAAATATCAATTTACAACCATTG [6]
Exon 9–2	TTGAAAATGTATTTGCTTTTTCTGT	CATGTAAATTCTGCTTTATTTATTCCA
Redesigned nested primers for LCM		
Exon 20–1	TTGCATACATTCGAAAGACC [6]	TGTGGAATCCAGAGTGAGCTT
Exon 20–2	AACTGAGCAAGAGGCTTTGG	TGTGGAATCCAGAGTGAGCTT
Exon 9–1	TTGAAAATGTATTTGCTTTTTCTGT	CTGAGATCAGCCAAATTCAGTT
Exon 9–2	CCAGAGGGGAAAAATATGACAA	AGCACTTACCTGTGACTCCA
<i>B. AKT primers</i>		
Macro dissection primers		
Exon 2	AGGCACATCTGTCCTGGCAC	ACCGGAGAGCCCTAAGTCTAA
Nested primers		
Exon 2–1	AGGGTCTGACGGGTAGAGTG	CAAATCTGAATCCCGAGA
Exon 2–2	AGTGTGCGTGGCTCTCACCA	AGCCTCACGTTGGTCCACAT

Redesigned primers yielding shorter amplicons utilized for PCR of LCM specimens (except cases 11, 12, 24 used same primers as macro dissection nested)

Analysis of invasive and in situ tumor after laser capture micro dissection

Laser capture micro dissection (LCM) was performed with an Arcturus PIXCELL II (Molecular Devices, Sunnyvale, CA) on mutation-positive carcinomas to obtain separate populations of invasive and in situ tumor cells. Seven-micrometer sections were prepared from the paraffin block, de-paraffinized, stained with methyl green, and thoroughly dehydrated in xylene. Laser capture was performed with Arcturus CapSure Macro caps (Molecular Devices), according to manufacturer's instructions. An estimated 1,000 cells were collected per cap per extraction. As a control, invasive tumor was separately captured in the majority of cases to demonstrate the efficacy of PCR and sequencing on LCM-derived tissue and to confirm the presence of the mutation detected by macro dissection. In four cases, only in situ tumor cells were captured. Microdissection of CIS was performed on blocks not containing invasive carcinoma wherever possible or from involved ducts and lobules most distant from the infiltrating component. Care was taken to

avoid foci with overlapping atypical ductal hyperplasia or areas suspicious for invasive carcinoma. Florid ductal hyperplasia accompanying two carcinomas with mutations was also separately laser-captured for analysis.

Caps were incubated at 65°C with proteinase K and buffer for approximately 16 h. DNA was extracted using the Arcturus Pico Pure DNA Extraction Kit (Molecular Devices) and then analyzed by nested PCR and sequencing as above, though primers were redesigned to yield shorter amplicons (Table 1). *AKT1* mutations in LCM tissue were confirmed by mass spectroscopy-based genotyping (Sequenom MassARRAY, Sequenom, San Diego, CA) [34]. In brief, *AKT1* was amplified using the Sequenom OncoCarta assay primers. PCR-amplified DNA was cleaned using EXO-SAP (Sequenom) and a primer extension reaction was performed using TYPLEX chemistry. Extended product was desalted using Clean Resin (Sequenom), and spotted onto Spectrochip II matrix chips using a nanodispenser (Sequenom). Chips were run on a Sequenom MassArray MALDI-TOF system. Sequenom Typer Software and visual inspection were used to interpret mass spectra.

Analysis of estrogen receptor, progesterone receptor, Her-2/Neu, and E-cadherin status

Immunohistochemical staining for estrogen receptor (ER) and progesterone receptor (PR) and Her-2/Neu staining was performed for all 81 tumors; in some cases, it was performed on a prior core needle biopsy specimen. Staining was performed on Ventana Benchmark or Benchmark XT instruments (Ventana, Tucson, AZ) with ER clone 6F11, PR clone 16, and Ventana PATHWAY™ Her-2/neu (clone CB11, each purchased from Ventana). In those cases in which Her-2/Neu staining was equivocal, reflex Her-2/Neu fluorescence in situ hybridization (FISH) was performed (Path-Vysion, Abbott-Vysis, Des Plaines, IL) [35]. Characteristics of the overall cohort include 81% ER positive, 52% PR positive, 19% Her-2/neu positive, and 9% triple negative. In order to substantiate a diagnosis of lobular carcinoma, histologic features as well as lack of E-cadherin staining was required; E-cadherin staining was performed as above using clone ECH-6 (Ventana). Only one case with probable lobular morphology was shown to be E-cadherin positive, and thus included in the ductal cohort. Of the cohort overall, 18, 53, and 28% were modified Bloom-Richardson grade I, II, III, respectively.

Statistics

The correlation of *PIK3CA* and/or *AKT1* mutational status to clinical variables was analyzed by Fisher's exact test (two-tailed) or chi-squared analysis using the Statview program (SAS Institute Inc., Cary, NC). A P-value of 0.05 was considered significant.

Results

PIK3CA mutations in invasive carcinoma

Invasive tumor from 81 cases of breast carcinoma was macro dissected from paraffin embedded tissue and screened for *PIK3CA* mutations by PCR and denaturing HPLC, and mutations were confirmed by direct sequencing. The final dataset included analysis of exon 20 for all 81 cases; analysis of exons 7 and 9 was successful in 80 cases each. There were eight exon 20 mutations (seven H1047R, one H1047L), four exon 9 mutations (two E545K, one E542K, one E545G), and no exon 7 mutations. The mutation status of invasive tumor was confirmed with LCM-purified tumor cells in all nine tested cases (Table 2); purified invasive tumor in case 12 showed mutant allele only, without detectable wild-type sequence. In total, 12 out of 81 invasive carcinomas demonstrated *PIK3CA* mutations yielding a mutation frequency of 14.8% (Fig. 1; Table 2).

No cases showed multiple *PIK3CA* mutations. Among the 12 mutation positive cases, there was 1 lobular carcinoma (of 9 screened) and 11 ductal carcinomas (of 72 screened).

PIK3CA mutations in paired in situ carcinoma

To determine whether associated in situ carcinoma harbored the same *PIK3CA* mutation as seen in the invasive component, LCM was used to isolate separate populations of invasive and in situ tumor (Fig. 2). Of eight cases with exon 20 mutations, all eight showed an identical exon 20 mutation in the in situ tumor (Fig. 1; Table 2). In three cases with exon 9 mutations, LCM confirmed a matching exon 9 mutation in the invasive and in situ elements. Analysis of micro dissected material was unsuccessful for the fourth case. Overall, there was perfect concordance (11/11) between *PIK3CA* mutations in paired samples of in situ and invasive ductal carcinoma (Table 2), indicating that *PIK3CA* mutations are an early event in breast carcinogenesis.

PIK3CA mutations in normal breast tissue and hyperplastic lesions

In two *PIK3CA* mutation-positive cases, sufficient normal tissue was available for macro dissection and analysis. In both of these cases, the normal tissue was negative for *PIK3CA* mutation, as shown in prior studies [7, 15, 18, 20–23, 25, 28]. Further, we analyzed epithelial hyperplasia in cases with available material. Two mutation positive invasive carcinomas had accompanying non-atypical florid hyperplasia suitable for LCM (Fig. 2). For case 51, micro dissected florid hyperplasia showed an identical H1047R mutation as seen in the CIS and invasive carcinoma. In contrast, hyperplasia associated with case 24 was wild-type for *PIK3CA* exon 20.

AKT1 mutations in invasive carcinoma

Extracted DNA from 78 cases of macro dissected invasive carcinoma was successfully screened for *AKT1* exon 2 mutations by PCR amplification and direct DNA sequencing. Three *AKT1* mutations were identified, all E17K (3/78, 3.8%). These mutations were present in tumors without identified *PIK3CA* mutations. The overall frequency of *PIK3CA* and *AKT1* mutations was 15/81 (18.5%).

AKT1 mutations in paired in situ carcinoma

As described above, LCM was used to isolate pure populations of CIS from cases harboring *AKT1* mutations in the invasive component. In two cases, the identical E17K was identified in the DCIS component by mass spectroscopy-based genotyping. The DCIS in case 29 demonstrated

Table 2 *PIK3CA* and *AKT1* mutant invasive and in situ carcinoma, with clinical parameters

Case	Macro-dissected	LCM invasive	LCM in situ	Size (cm)	Nodal status	Grade (type)	ER	PR	HER2
<i>PIK3CA</i> exon 9									
6	E545K	E545K	E545K	1.4	+(1/30)	II (D)	+	+	–
20	E545K	E545K	E545K	1.5	ND	I (D)	+	+	–
42	E542K	E542K	E542K	1.0	–(0/4)	II (D)	+	–	+
79	E545G	NA	NA	2.0	+(7/20)	I (L)	+	+	–
<i>PIK3CA</i> exon 20									
11	H1047R	H1047R	H1047R	1.5	–(0/3s)	III (D)	+	–	Eq
12	H1047R	H1047R	H1047R	8.1	+(3/7)	III (D)	+	–	–
14	H1047R	H1047R	H1047R	2.5	–(0/11)	II (D)	+	+	–
24	H1047R	H1047R	H1047R	2.5	+(2/15)	II (D)	+	–	–
25	H1047L	H1047L	H1047L	2.0	–(0/6)	II (D)	+	+	–
51	H1047R	H1047R	H1047R	2.6	+(8/18)	I (D)	+	+	–
63	H1047R	ND	H1047R	4.8	+(2/15)	I (D)	+	+	–
77	H1047R	ND	H1047R	1.4	–(0/17)	II (D)	+	+	Eq
<i>AKT1</i> exon 2									
5	E17K	ND	E17K	1.6	–(0/1s)	III (D)	+	–	–
29	E17K	ND	E17K	2.0	+(1/10)	II (D)	+	+	–
76	E17K	E17K	NA	1.8	ND	III (D)	+	+	–

s Sentinel lymph node

Size refers to greatest dimension of invasive component

ND not done, NA no amplification (PCR and sequencing/Sequenom unsuccessful on LCM material)

Grade is modified Bloom-Richardson grade of invasive carcinoma

Type: D-infiltrating ductal; L-infiltrating lobular

ER (estrogen receptor) and PR (progesterone receptor) immunohistochemical studies scored as positive (+) if greater than 10% of invasive tumor cells showed nuclear staining

HER2 scored as (–) negative with immunohistochemical score of 0, 1+ per Ventana manufacturer's instructions, or 2+ IHC with FISH assay negative for amplification. HER2 scored as (+) positive if 2+ immunohistochemistry and FISH positive for amplification. No cases showed 3+ immunohistochemical staining

HER2 scored as (Eq) equivocal with 2+ immunohistochemistry and FISH HER2: CEP17 ratio of 2.2 (case 11), [35] or immunohistochemistry 2+ and FISH data not available (case 77)

mutant *AKT1* only; the wild-type allele was not detected. In the third case, we were unable to successfully assay DNA from the LCM material.

PIK3CA and *AKT1* mutations and clinicopathologic variables

We compared clinicopathologic parameters of *PIK3CA* and *AKT1* mutant and wild-type tumors. Histologic comparison of the mutation positive and negative invasive cases did not reveal any morphologic distinctions. Further, there was no association between architectural pattern, nuclear grade, or presence of comedonecrosis in carcinoma in situ (Tables 2, 3 and data not shown). ER, PR, and Her-2/neu analysis was performed on all 81 cases. Interestingly, all of the *PIK3CA* mutant cases were ER positive. However, due to the size of the sample, this did not reach statistical significance. Likewise, *AKT1* mutant cases were ER positive (one weakly). Further, there was no statistically significant association of

PIK3CA-mutated tumors with PR expression, Her-2/Neu status, histologic grade, or nodal metastasis (Table 3). Statistics were not calculated for *AKT1* mutations.

Discussion

Cancer is caused by the accumulation of mutations in tumor suppressor genes and oncogenes; this has been shown to occur in a stepwise manner resulting in the progression of pre-neoplastic lesions to frankly invasive carcinoma. We sought to better characterize evolution of tumorigenesis in breast carcinoma by identifying *PIK3CA* and *AKT1* mutations in invasive breast carcinoma and then determining whether these mutations were also present in the accompanying in situ carcinoma.

The most striking result of our study is the concordance between *PIK3CA* and *AKT1* mutations in infiltrating carcinoma and accompanying in situ carcinoma. All the 11

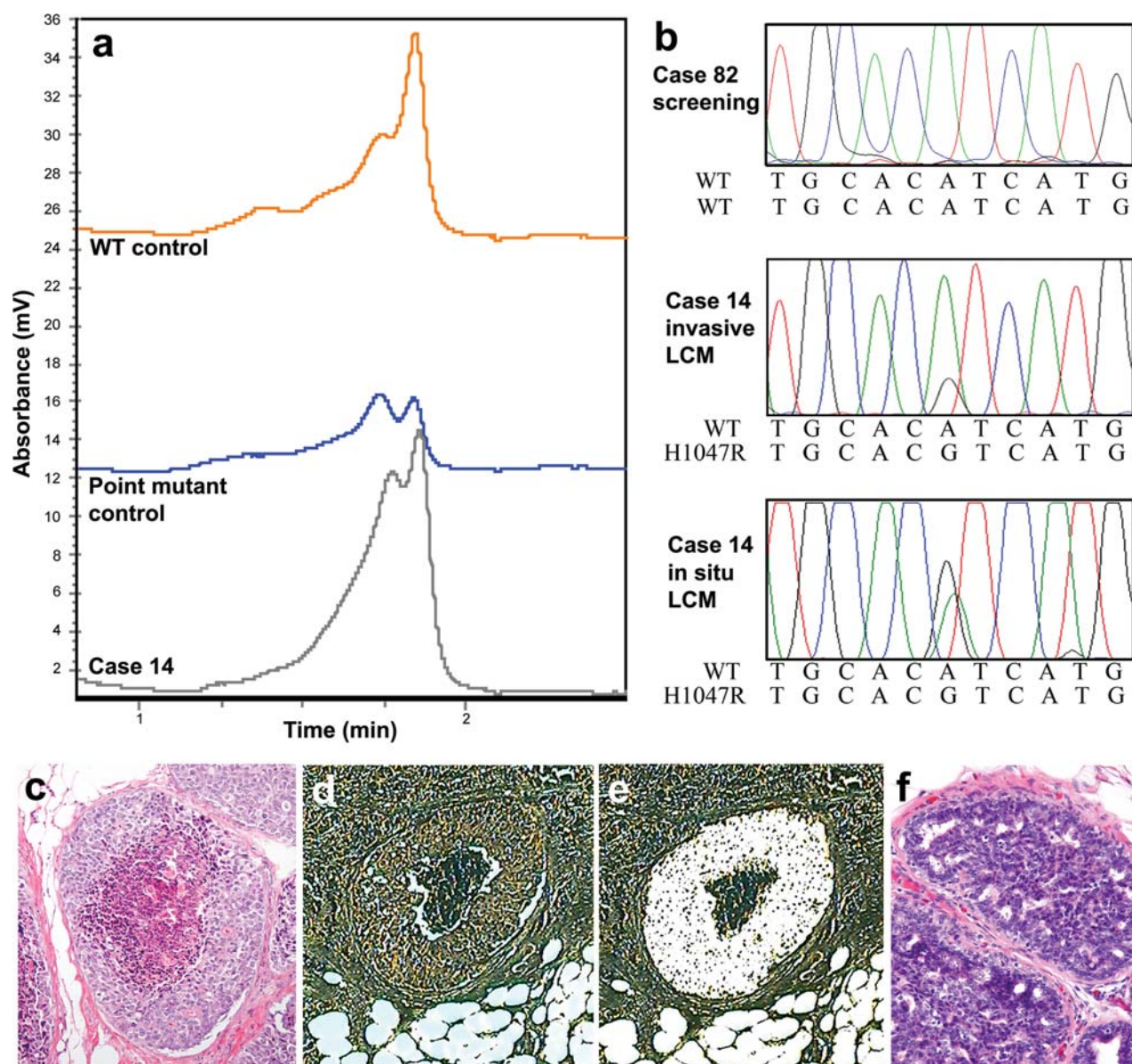


Fig. 1 *PIK3CA* mutations. **a** HPLC WAVE profile of case 14, macro dissection screening. Tracings show HPLC profile run at 57.7° with exon 20 PCR products. Note case 14 profile matches point mutation control specimen. **b** Representative sequence data. *Top*: wild type sequence, case 82, macro dissection screening. *Middle*: H1047R mutant sequence, case 14 micro dissected invasive carcinoma, mutant and wild type allele sequences are seen. *Bottom*: H1047R mutant sequence, case 14 micro dissected in situ carcinoma, mutant and wild type allele sequences are seen. **c** Representative H & E stained section of ductal carcinoma in situ with comedonecrosis, case 12. **d** Methyl

green stained section before laser capture micro dissection, case 12. **e** Methyl green stained section showing same slide and field as (**d**), after laser capture micro dissection of ductal carcinoma in situ. **f** Representative section of florid hyperplasia without atypia from case 51, with H1047R mutation (H & E images captured with Leica DM2000 microscope, and Leica DFC320 camera, 10× and 20× objective for (**c**, **f**), respectively. Methyl green images captured on Arcturus Pixcell II with 10× objective during LCM for (**d**) and (**e**), as described in “[Methods](#)”)

(100%) cases with paired data from invasive and in situ carcinoma showed concordant *PIK3CA* mutations, and 2 of 2 cases showed concordant *AKT1* mutations. Our findings provide strong evidence that *PIK3CA* mutations occur early in the pathogenesis of breast carcinoma, consistent with the observation by Saal and colleagues [15] that the frequency of *PIK3CA* mutations is approximately equal across stage I

through IV tumors. While the numbers are small, our data also suggest that *AKT1* mutations occur early in breast carcinoma.

Previous studies have yielded two *PIK3CA* mutations in 45 cases of DCIS, and no *AKT1* mutations in 15 cases of DCIS. Of note, these were unselected cases of DCIS [20, 21, 28]. Given the different screening strategies, our results

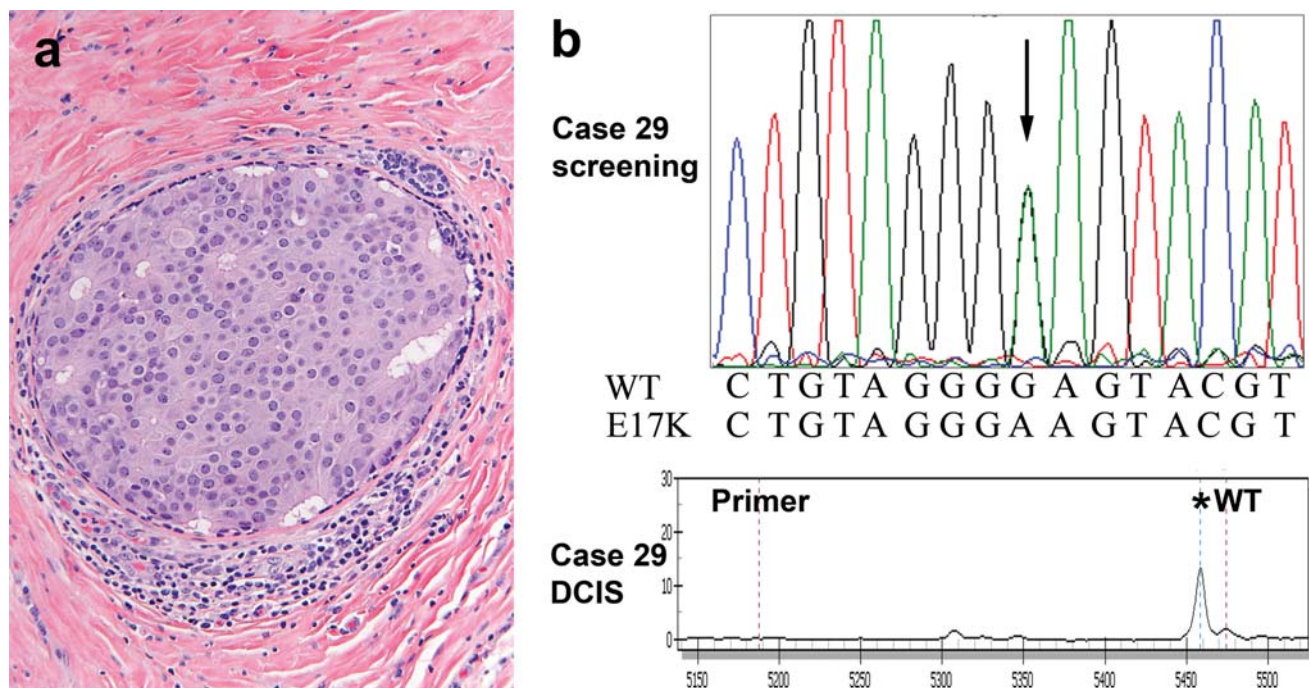


Fig. 2 *AKT1* mutations. **a** Representative H & E stained section of ductal carcinoma in situ, case 29. **b** Top: Sequence data for case 29 macro dissected invasive carcinoma, with E17K mutation, mutant and wild type allele sequences are seen (arrow). **c** Sanger sequencing analysis, case 29 DCIS. Dotted line at far right shows expected location of wild type sequence (WT) which is completely absent; peak corresponds to

mutation (*). Dotted line at far left shows location of unextended primer signal, which is absent with successful PCR reaction (Primer) X-axis indicates molecular mass (Daltons) and Y axis intensity (H & E image captured with Leica DM2000 microscope, and Leica DFC320 camera, 20× objective)

Table 3 Correlation of *PIK3CA* mutation status with clinicopathologic parameters

Clinical variable	<i>PIK3CA</i> mutant (N = 12)	<i>PIK3CA</i> wild type (N = 69)	P value*
LN metastasis+	6/11 = 54.5%	35/65 = 53.8%	>0.99
ER+	12/12 = 100%	54/69 = 78.2%	0.11
PR+	8/12 = 75%	34/69 = 49.2%	0.35
HER2+	1/10 = 10%	14/67 = 20.8%	0.67
Grade			0.30
1	4/12 = 33.3%	11/69 = 15.9%	
2	6/12 = 50%	37/69 = 53.6%	
3	2/12 = 16.6%	21/69 = 30.4%	
Mean tumor size (standard deviation)	2.6 (±2.0) cm	2.1 (±1.1) cm	

LN metastasis+: breast carcinoma metastatic to axillary lymph nodes (lymph node data available on 11 mutant positive cases and 65 wild type cases)

ER, PR scoring and abbreviations as for Table 2

HER2 equivocal cases excluded for statistical analysis (two mutant and two wild type cases)

* Fisher's exact test; except Grade calculated by Chi-square

are not directly comparable to these previous studies [20, 21, 28]. Further, we found an identical *PIK3CA* mutation in one case of florid hyperplasia without atypia accompanying

mutation-positive infiltrating carcinoma. This preliminary finding raises the intriguing possibility that the *PIK3CA* mutations contribute to ductal neoplasia prior to the development of morphologically recognizable in situ carcinoma. Investigation of other breast precursor lesions, including usual and atypical hyperplasia will be of interest in this regard.

We identified *PIK3CA* mutations in exon 20 (8/81) and exon 9 (4/80), and *AKT1* exon 2 E17K mutations (3/78), for a total of 15/81 mutations in invasive breast carcinomas; these mutations were mutually exclusive. Interestingly, the in situ tumor in case 12 was heterozygous for a *PIK3CA* mutation, but in the invasive element the wild-type allele was no longer detectable. In gastrointestinal stromal tumors, hemi/homozygosity for mutant *KIT* due to loss of the wild-type allele is associated with clinically more aggressive disease [36]. Loss of the wild-type allele may be advantageous for *PIK3CA*-mutant cells, as well. Alternatively, there may have been amplification of the mutant *PIK3CA* in case 12, which has been previously observed by Wu et al. [22] in occasional cases of breast carcinoma, and is associated with tumor aggressiveness in endometrial carcinoma [37]. The same considerations also apply to the in situ carcinoma in case 29, in which an *AKT1* E17K mutation was detected in the absence of detectable wild-type *AKT*. To our knowledge, this is the first such

observation for mutant *AKT1*, and it will be interesting to pursue this in future studies.

Previous studies have shown that a substantial proportion of *PIK3CA* point mutations are located in ‘hotspots’ in exon 9 (E542, E545) and exon 20 (H1047), with exon 7 reported as an additional mutation cluster [6, 7, 15, 16, 18, 20–25, 28]. Based on in vitro studies, exon 9 and 20 *PIK3CA* hotspot mutations increase the lipid kinase activity of PIK3CA and activate downstream signaling. In breast epithelial cell culture models, they promote anchorage-independent growth and proliferation, but remain sensitive to pharmacologic inhibition [6, 13, 38–42]. Less common mutations such as H1047L and E542G have also been shown to increase PIK3CA signaling activity, but to a lesser degree than E545K and H1047R [43]. *AKT1* mutations occurring in the plekstrin homology domain (exon 2, E17K) are activating mutations resulting in recruitment of AKT1 to the cell membrane, independent of PI3K.

Based on data from colon and endometrium and limited data for breast, Oda and others have suggested that *PIK3CA* pathway mutations typically arise during or after carcinomas become invasive [44]. Our data provide strong evidence that *PIK3CA* mutations occur in preinvasive lesions in the breast. It is possible that *PIK3CA* mutations play a different role in breast tumorigenesis as compared to colon and endometrium. Samuel and colleagues identified only two *PIK3CA* mutations among 76 colonic adenomas, while the mutation frequency in invasive colonic adenocarcinomas was 32% [6]. Similarly, Hayes et al. [45] found only rare *PIK3CA* mutations in endometrial complex atypical hyperplasia (2/29, 7%), as compared to endometrial carcinoma (17/44, 39%) [46]. It is likely that *PIK3CA* mutations occur relatively later in endometrial and colonic neoplasia when compared with breast carcinogenesis.

Most prior studies have found no correlation between the *PIK3CA* genotype and ER/PR hormone receptor status of infiltrating breast carcinoma; however, four studies did report a correlation of ER positivity with *PIK3CA* mutation [15, 16, 24, 28]. We found that all 12 invasive carcinoma cases harboring a *PIK3CA* mutation were positive for ER (100%), although this sample size was too small to yield statistical significance. Our *AKT1* mutation cases were also ER positive, as reported in the larger study by Stemke-Hale [31]. Buttitta and Perez-Tenorio et al. [23] demonstrated that *PIK3CA* mutation positive breast carcinomas tend to be Her-2/neu negative, while Saal et al. found the opposite [15, 16]. While we did not investigate *PIK3CA* or *AKT1* mutation status and patient outcome, data in the literature are mixed [15, 24, 28]. Despite the fact that *PIK3CA* exon 9 and exon 20 mutations show essentially equivalent cellular transforming ability in vitro, [40] several more recent studies suggested differential outcome based on mutation.

One study showed that exon 20 mutations (kinase domain) confer a more favorable outcome, and exon 9 mutations (helical domain) a relatively poor prognosis [26]. However, this is contradicted in two other studies in which there was an apparently less favorable outcome of breast cancers harboring *PIK3CA* exon 20 mutations [27, 29]. Further, Liedtke et al. [30] found a significant association of *PIK3CA* exon 9 mutation with node-negative status in ER positive cancers. Nevertheless, it should be noted that these studies were conducted retrospectively on patients who had been subjected to a variety of treatments.

The presence of *PIK3CA* and *AKT1* mutations in both invasive and in situ carcinoma suggests an attractive target for treatment and chemo preventative therapy. There are a large number of PIK3CA pathway inhibitors currently in clinical trials [47]. Promising compounds include SF1126, NVP-BEZ235, XL147, and XL765, all of which have shown anti-tumor efficacy in a variety of animal models [48, 49]. AKT inhibitors include Tricarbazine, the lipid-based perifosine, and phosphatidylinositol ether lipid analogs (PIAs) [47, 49]. Other strategies include inhibition of the downstream signaling pathway, including PDK1 and mTOR. PI-103 and NVP-BEZ235 are dual PIK3CA/mTOR inhibitors that may prove particularly efficacious [8, 31, 49, 50].

In summary, we demonstrate that *PIK3CA* and *AKT1* mutations are present at the stage of breast carcinoma in situ, an observation with implications for mechanisms of tumor pathogenesis, as well as treatment and chemoprevention. Further studies are necessary to determine whether and how *PIK3CA* and *AKT1* mutations play a role in the development of hyperplasia, atypia, carcinoma in situ, or invasion in the breast.

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