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Frequent phosphatidylinositol-3-kinase mutations in proliferative breast lesions

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The phosphatidylinositol-3-kinase pathway is one of the most commonly altered molecular pathways in invasive breast carcinoma, with phosphatidylinositol-3-kinase catalytic subunit (*PIK3CA*) mutations in 25% of invasive carcinomas. Ductal carcinoma *in situ* (DCIS), benign papillomas, and small numbers of columnar cell lesions harbor an analogous spectrum of *PIK3CA* and *AKT1* mutations, yet there is little data on usual ductal hyperplasia and atypical ductal and lobular neoplasias. We screened 192 formalin-fixed paraffin-embedded breast lesions from 75 patients for point mutations using a multiplexed panel encompassing 643 point mutations across 53 genes, including 58 *PIK3CA* substitutions. *PIK3CA* point mutations were identified in 31/62 (50%) proliferative lesions (usual ductal hyperplasia and columnar cell change), 10/14 (71%) atypical hyperplasias (atypical ductal hyperplasia and flat epithelial atypia), 7/16 (44%) lobular neoplasias (atypical lobular hyperplasia and lobular carcinoma *in situ*), 10/21 (48%) DCIS, and 13/37 (35%) invasive carcinomas. In genotyping multiple lesions of different stage from the same patient/specimen, we found considerable heterogeneity; most notably, in 12 specimens the proliferative lesion was *PIK3CA* mutant but the concurrent carcinoma was wild type. In 11 additional specimens, proliferative epithelium and cancer contained different point mutations. The frequently discordant genotypes of usual ductal hyperplasia/columnar cell change and concurrent carcinoma support a role for *PIK3CA*-activating point mutations in breast epithelial proliferation, perhaps more so than transformation. Further, these data suggest that proliferative breast lesions are heterogeneous and may represent non-obligate precursors of invasive carcinoma.
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The phosphatidylinositol-3-kinase pathway is one of the most commonly mutated pathways in invasive breast carcinoma. Activating mutations in the phosphatidylinositol-3-kinase catalytic subunit (*PIK3CA)* are present in ~25% of invasive carcinomas and are present in an even higher percentage (on the order of 40%) of low-grade estrogen receptor-positive carcinomas (luminal A intrinsic subtype).[1–10] *PIK3CA* mutations cluster in 'hotspots' in exon 9 (helical domain, E542K and E545K) and exon 20 (kinase domain, H1047R/L).[1–10] In addition, this pathway is activated by mutations in the plekstrin-homology domain of *AKT1* in ~5% of breast carcinomas, by the loss of the phosphatase PTEN (phosphatase and tensin homolog on chromosome 10), or rarely by amplification or alterations in other regulatory subunits, and is a target of active drug development.[8–11] In recent large studies of estrogen receptor-positive tumors, the presence of activating *PIK3CA* hotspot point mutations has been paradoxically associated with more favorable outcome as compared with breast carcinomas with wild-type *PIK3CA*.[3,12,13]

We and other groups have previously shown that the *PIK3CA* genotype is concordant between ductal carcinoma *in situ* (DCIS) and concurrent invasive carcinoma in 66–100% of tested samples.[14–17] However, several small studies suggest that pre-neoplastic or benign breast lesions, such as papillomas, radial scars, or columnar cell lesions, may also very frequently harbor *PIK3CA* mutations.[18–23] The goal of this study was to systematically screen a large number of hyperplastic and putative precursor breast lesions, along with accompanying carcinomas for known activating mutations in *PIK3CA* and other key signaling molecules.

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Materials and methods

After Institutional Review Board approval, computerized pathology files of Oregon Health and Science University (2005–2010) were surveyed for breast specimens containing usual ductal hyperplasia, columnar cell change, atypical hyperplasia, and lobular neoplasia. Usual and atypical ductal hyperplasias were classified using the criteria of Page, Schnitt and others,[24,25] and the columnar cell lesion spectrum classified using the criteria of Schnitt *et al*.[24,26] Slides were reviewed to confirm histologic diagnosis and select lesions for genotyping. Where available, a spectrum of lesions was isolated from the same specimen, including proliferative lesions, atypical lesions, *in situ*, invasive, and/or metastatic carcinoma. Data on invasive tumor size, grade, nodal status, and hormone receptor status were abstracted from pathology reports. There was no known prior chemotherapy or radiation therapy for breast carcinoma, with the exception of two recurrent cases as noted (excluded from totals by histologic lesion Table 1, but included in Figure 2 and Supplementary Table 1). Some patients had recent prior breast excisional biopsy/lumpectomy at other hospitals and presented to our institution for definitive surgery, such that carcinoma was not available for genotyping.

Lesional tissue was isolated by punching the formalin-fixed paraffin-embedded tissue block(s), using 1 or 2 mm coring devices, with a hematoxylin and eosin (H&E) stained slide as the template. Multiple cores of each lesion were collected; cores were re-embedded in paraffin and an H&E slide was prepared from the recipient block in order to confirm and/or re-classify lesional tissue. Unsatisfactory cores were excluded from further testing. This method yielded samples composed of lesional epithelium, surrounding myoepithelium, and stroma, with variable percentage of lesional cells. Diagnoses of usual ductal hyperplasia and columnar cell change were reviewed by two pathologists (DA, MLT). Usual ductal hyperplasia and columnar cell change were frequently intimately associated and were combined for most analyses. A separate subgroup analysis was conducted on 'pure' lesions consisting of $>80\%$ usual ductal hyperplasia or $>80\%$ columnar cell change. Likewise, the analysis of atypical lesions included only specimens composed of $>80\%$ atypical lesional epithelium; there was frequently a small percentage of accompanying columnar cell lesion. Other mixed lesions were excluded from analysis; however, they are illustrated in Figure 2 and annotated as such in Supplementary Table 1A.

Paraffin shavings from the recipient blocks were prepared and DNA was extracted from paraffin using standard protocols (Qiagen Qiamp Mini kit, Valencia, CA). DNA yield was quantitated on a Nanodrop

Table 1 Mutational status by lesion

Breast lesions: histologic type	*Lesions with point mutation (%)*	*Mutations (*PIK3CA *gene unless indicated otherwise)*
Proliferative (usual ductal hyperplasia/columnar cell lesion)	31/62 (50%)	Two exon 7 single mutations: C420R Seven exon 9 single mutations: E542K, E545K,G, Q546R
Pure usual ductal hyperplasia	6/12 (50%)	Twenty exon 20 single mutations: H1047R
Pure columnar cell lesion	11/26 (42%)	Two double mutations exon 9 and 20: H1047R with E542K or E545K
Atypical (atypical ductal hyperplasia/flat epithelial atypia)	10/14 (71%)	Four exon 4 mutations: N345K Three exon 9 mutations: E545K, E542K Three exon 20 mutations: H1047R, H1065L One *KRAS* exon 1 mutation: G12D (in a lesion with N345K mutation)
Lobular neoplasia (atypical lobular hyperplasia/ lobular carcinoma in situ*)*	7/16 (44%)	One exon 4 mutation: N345K Three exon 9 mutations: E542, E545K Three exon 20 mutations: H1047R
Ductal carcinoma in situ	10/21 (48%)	One exon 4 mutations: N345K Five exon 9 mutations: E545K, Q546R Three exon 20 mutations: H1047R One *AKT1* exon 2 mutation: E17K
Invasive carcinoma	13/37 (35%)	Three exon 4 mutations: N345K
Invasive ductal	7/24 (29%)	Five exon 9 mutations: E545K, Q546R
Invasive lobular	6/11 (54%)	Three exon 20 mutations: H1047R, H1047L
Special type	0/2 (0%)	Two *TP53* mutations: exon 5 R175H, exon 8 R273H One *FBXW7* exon 8 mutations: R393stop (in a carcinoma with E545K mutation)
Node metastasis	5/6 (83%)	Three exon 9 mutations: E542K, E545K One exon 20 mutation: H1047R One *TP53* exon 5 mutation: R175H One *FBXW7* exon 8 mutation: R393stop (in a tumor with E545K mutation) One *FGFR2* exon 11 mutation: N550K (in the tumor with H1047R mutation)

(Thermo Scientific, Wilmington DE) or Qubit fluoromenter (Life Technologies, Grand Island, NY). DNA extracts (0.75 μg) were screened for a large panel of activating point mutations using a multiplexed PCR–mass spectroscopy-based technique encompassing 643 point mutations in 53 genes (Sequenom MassArray, Sequenom, San Diego, CA), as previously described.[19,27] In brief, the mutation panel covers point mutations in *AKT1/2/3*, *ALK*, *BRAF*, *CDK4*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERCC6*, *FBX4*, *FBXW7*, *FES*, *FGFR1/2/3/4*, *FOXL2*, *GNA11*, *GNAQ*, *GNAS*, *HRAS*, *IDH1/2*, *IGF1R*, *KDR*, *KIT*, *KRAS*, *MAP2K1/2/7*, *MET*, *MYC*, *NEK9*, *NRAS*, *NTRK1/2/3*, *PDGFRA*, *PIK3CA*, *PIK3R1/4/5*, *PKHD1*, *PRKCB1*, *RAF1*, *RET*, *SMO*, *SOS1*, *STAT1*, *TEC*, and *TP53*. The panel includes 58 substitutions in 34 codons of the *PIK3CA* gene. Results were excluded if >5% of assays failed, unless a point mutation was confirmed. Point mutations identified by mass spectroscopy screening were confirmed by direct sequencing on an ABI3130 sequencer (Applied Biosystems, Carlsbad, CA) using the BigDye terminator method, with or without the use of a locked nucleic acid probe to partially suppress amplification of the wild-type allele, as previously described in detail.[28] The assay sensitivity is ~5–10% mutant allele for mass spectroscopy screening and 0.6–2.5% mutant allele for locked nucleic acid sequencing.[27,28]

Ki-67 immunohistochemical staining was performed on a subset of pure columnar cell lesions using antibody clone 30-9 (pre-dilute, cell conditioning 1, and mild antigen retrieval) on Ventana XT instruments with Ultraview detection (all from Ventana, Tucson, AZ), using standard protocols. Statistical analysis with χ^2 and Fisher's exact test were computed using Excel software.

Results

Patients and Genotyping

We reviewed H&E-stained archival slides of formalin-fixed paraffin-embedded breast excisional specimens (breast reduction, surgical biopsy, lumpectomy, and mastectomy) to select lesions for genotyping, to include benign/proliferative epithelial lesions, atypical lesions, lobular neoplasia, DCIS, invasive carcinoma, and lymph nodes metastasis, where available.[24–26] We focused on specimens with benign proliferative lesions, including usual ductal hyperplasia and columnar cell change, especially those with accompanying carcinomas available for comparative study. Lesions were screened for known hotspot point mutations using a multiplexed mass spectroscopy-based approach with a panel covering 643 common activating point mutations in 53 known cancer genes, including 58 hotspot substitutions in the *PIK3CA* gene, along with coverage of other breast cancer genes such as *AKT1*, *ERBB2*, *PIK3R1*, and *TP53*; identified mutations were sequence confirmed.[19–21,27,28] The final study group included 192 breast lesions successfully genotyped from 75 patients, as follows (Supplementary Table 1A and B): 62 usual ductal hyperplasia/columnar cell lesion, 14 atypical ductal hyperplasia/flat epithelial atypia, 16 lobular neoplasias, 21 DCIS, 37 invasive carcinomas, 6 lymph node metastases, and 36 other lesions (papillomas, mixed histology, and from patients with recurrent cancer).

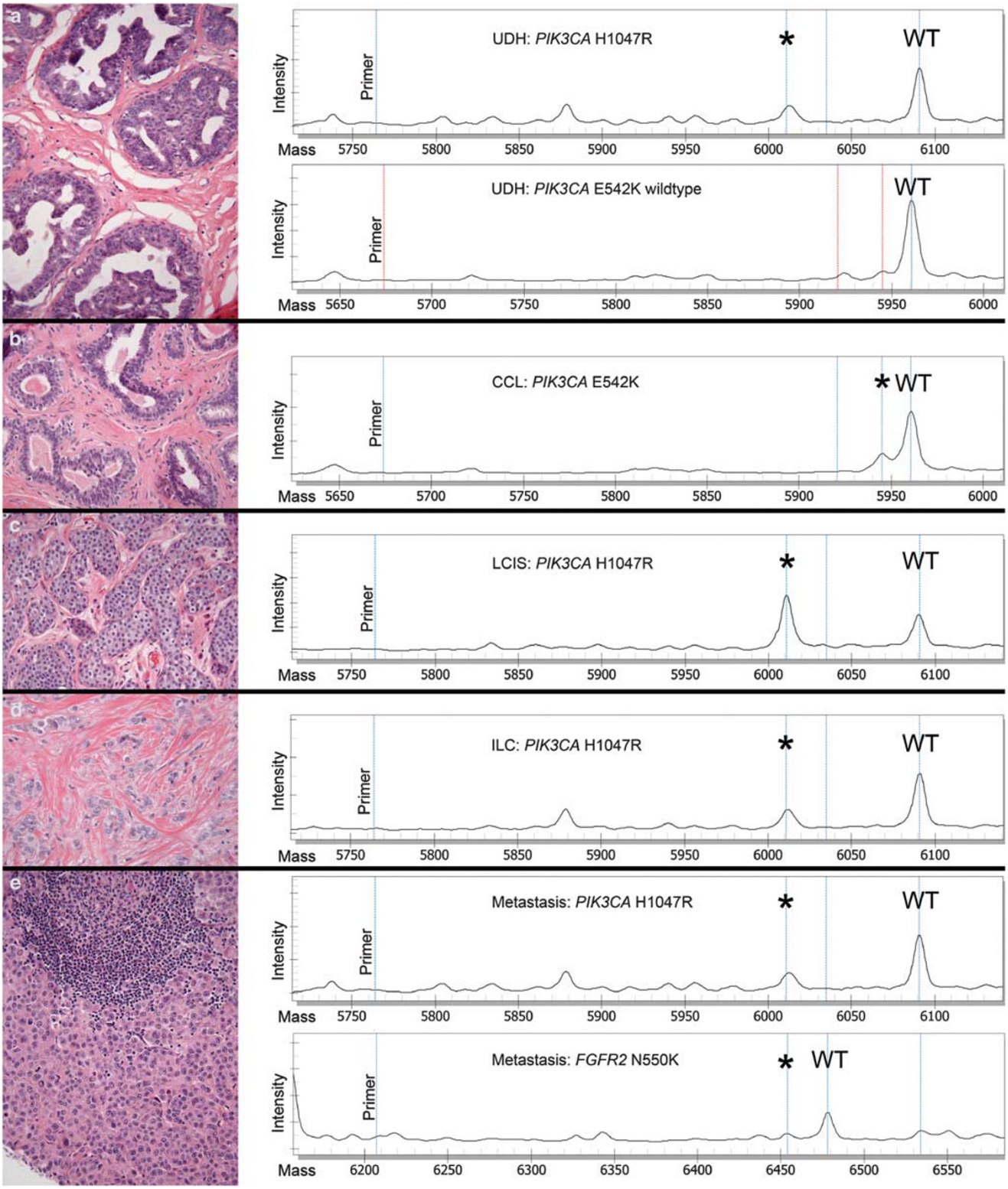
Mutational Profile of Usual Ductal Hyperplasia and Columnar Cell Lesions without Atypia

Usual ductal hyperplasia was often intimately admixed with, or adjacent to, epithelium showing columnar cell change; thus, a combined group of usual ductal hyperplasia and columnar cell lesion was analyzed. We found *PIK3CA*-activating point mutations in half of the proliferative lesions in this category (31/62, 50%; Table 1, Figures 1 and 2, Supplementary Table 1B). No point mutations in other genes of the large screening panel were detected in usual ductal hyperplasia/columnar cell lesions. We separately analyzed smaller subgroups of 'pure' lesions (>80% usual ductal hyperplasia and >80% columnar cell lesion, respectively). Again, nearly half of the lesions in each of these subgroups harbored *PIK3CA* mutations: usual ductal hyperplasia 6/12 (50%) and columnar cell lesion 11/26 (42%; Table 1, Supplementary Table 1B). In many

Figure 1 Morphologic and genotypic characterization of breast lesions, patient 11. (**a**) Usual ductal hyperplasia (UDH, left). PCR–mass spectroscopy analysis (right) demonstrates a phosphatidylinositol-3-kinase catalytic subunit (*PIK3CA*) H1047R mutant (*) peak (upper), along with a larger wild-type peak (WT). The H1047R mutation was confirmed by direct sequencing (data not shown). The lower tracing demonstrates wild-type *PIK3CA* E542K assay. (**b**) Columnar cell lesion (CCL, left). PCR–mass spectroscopy indicates a *PIK3CA* E542K mutant peak (*), along with a larger WT peak. The E542K mutation was confirmed by direct sequencing (data not shown). (**c**) Lobular carcinoma *in situ* (LCIS, left). PCR–mass spectroscopy analysis (right) shows a *PIK3CA* H1047R mutant (*) peak, along with a larger WT peak. The H1047R mutation was confirmed by direct sequencing (data not shown). All other screened codons were WT. (**d**) Invasive lobular carcinoma (ILC, left). PCR–mass spectroscopy analysis (right) demonstrates a *PIK3CA* H1047R mutant (*) peak, along with a larger WT peak. The H1047R mutation was confirmed by direct sequencing (data not shown). All other screened codons were WT. (**e**) Lobular carcinoma metastatic to lymph node (left). PCR–mass spectroscopy analysis (right) demonstrates a *PIK3CA* H1047R mutant (*) peak, along with a larger WT peak in the upper panel. In addition, a *FGFR2* N550K mutant peak (*) was seen, shown together with its WT sequence in the lower tracing. Both mutations were confirmed by direct sequencing (data not shown). All other screened codons were WT. The small peaks in the middle of the *PIK3CA* H1047R PCR–mass spectroscopy tracings represent WT peaks from an unrelated, multiplexed assay. Original magnifications for hematoxylin and eosin stained sections: ×200.

cases, multiple non-atypical epithelial lesions from the same specimen/patient were screened and often had discordant genotypes, as discussed in detail below and illustrated in Figures 1 and 2. In this group of proliferative lesions, *PIK3CA* exon 20 kinase domain mutations were most common (22 exon 20 mutations and 9 exon 9 mutations), unlike in invasive and *in situ* carcinomas (see below and Table 1).

Retrospective review revealed no qualitative difference in morphology of usual ductal hyperplasia/columnar cell lesion with *PIK3CA* mutant or



wild-type genotype. The 'pure' columnar cell lesion group was further stratified as columnar cell change (no hyperplasia, 3 of 8 with mutations), columnar cell hyperplasia (3 of 6 with mutations), or both (5 of 12 with mutations). We also used Ki-67 immunohistochemical stating to assess proliferative fraction of the 'pure' columnar cell lesion group. The mean Ki-67 staining in the columnar cell lesions with *PIK3CA* mutations was 2% (1.4% s.d., $n=8$ with tissue available for analysis), whereas the mean Ki-67 in the wild-type columnar cell lesions was 3.7% (2.4% s.d., $n=15$).

Mutational Profile of Atypical Ductal and Lobular Lesions

Atypical ductal hyperplasia and flat epithelial atypia comprise the group of atypical ductal lesions.[24,26] Seventy-one percent of atypical ductal lesions contained *PIK3CA*-activating point mutations (10/14, 71%; Table 1, Supplementary Table 1B). However, this includes three samples of atypical ductal epithelium from the same patient/specimen (all with *PIK3CA* exon 4 N345K substitution) and two atypical lesions from another patient (both wild type). Combining lesions by patient nevertheless yields 8/11 (72%) of atypical specimens with activating point mutations. One atypical hyperplasia had a *PIK3CA* N345K mutation, co-occurring with a *KRA*S G12D mutation.

Sixteen lesions of atypical lobular hyperplasia and/or lobular carcinoma *in situ* were screened, yielding 7/16 (44%) of lesions with mutations, all in the *PIK3CA* gene (Table 1, Supplementary Table 1B).

Mutational Profile of *In Situ*, Invasive, and Metastatic Carcinoma

We conducted mutational screening on 21 samples of DCIS, resulting in 10/21 (48%) lesions with activating point mutations in the PI3K-AKT pathway (Table 1, Supplementary Table 1B). The invasive carcinoma subgroup included 24 cases of invasive ductal carcinoma, 11 invasive lobular carcinoma, 1 invasive papillary carcinoma, and 1 metaplastic carcinoma. Overall, 13 of 37 (35%) of invasive carcinomas harbored mutations, again with the vast majority in the *PIK3CA* gene. For the *in situ* and invasive carcinomas combined, there were 10 *PIK3CA* exon 9 mutations and only 6 exon 20 mutations; the frequency of exon 20 mutations was different in UDH/CCL as compared with carcinoma (exon 20 mutations UDH/CCL = 22/62; carcinoma = 6/57, $P=0.001$), whereas exon 9 mutations were not significantly different in these groups (exon 9 mutations UDH/CCL = 9/62; carcinoma = 10/57, $P=0.2$). We encountered TP53 mutations (R175H and R273H) in two invasive ductal carcinomas and an *FBXW7* mutation was seen along with a *PIK3CA* exon 9 mutation in an invasive lobular carcinoma. The invasive ductal subgroup included 7/24 (29%) of carcinomas with mutations, whereas the invasive lobular subgroup had 6/11 (54%) point mutations (Table 1, Supplementary Table 1B).

In correlating genotype with other biomarkers, the overwhelming majority of invasive carcinomas in the study were estrogen receptor-positive, precluding meaningful analysis of hormone receptor profile. The five estrogen receptor-negative tumors were wild type for *PIK3CA*; both *TP53*-mutated tumors were estrogen receptor- and Her-2/neu-negative. There were no differences in genotype by carcinoma grade (data not shown).

We analyzed axillary lymph node metastases from a small number of patients, yielding 5/6 (82%) of metastasis with point mutations. Four cases had *PIK3CA* mutations; two of these had coincident mutations in other genes: *PIK3CA* E545K and *FBXW7**, as was also seen in the primary breast carcinoma; and the other had *PIK3CA* H1047R and *FGFR2* N550K, with only the *PIK3CA* mutation in the primary breast sample (Table 1, Supplementary Table 1B).

Comparison of the Mutation Spectrum of Proliferative, Atypical, and Malignant Breast Lesions in Individual Patients

Multiple concurrent epithelial lesions with different histologic features were available for mutation screening in 58 patients/specimens, including 51 with invasive or *in situ* carcinoma. Figure 2 illustrates the mutation profile over the spectrum of breast lesions by specimen/patient (also see Supplementary Table 1 for further details). In six specimens, point mutations were identified in usual ductal hyperplasia/columnar cell lesions, and the same mutations were found in concurrent carcinomas (concordant with point mutations), as depicted at the top of Figure 2 (patients 1–6). Likewise, in a subset of 13 patients the proliferative lesions and concurrent carcinomas each screened as wild type for the tested panel (concordant wild type, Figure 2, patients 49–60). However, the majority of specimens contained breast proliferative lesions and carcinoma with discordant genotypes. Eight patients had proliferative lesions screened as wild type, with an apparent 'gain' of activating point mutations in carcinoma ('discordant: carcinoma mutant,' Figure 2 patients 40–47). Interestingly, there were 12 breast specimens harboring usual ductal hyperplasia/columnar cell lesion with activating point mutations, accompanied by wild-type invasive or *in situ* carcinoma ('discordant: carcinoma wild-type,' Figure 2, patients 19–30). The vast majority of these proliferative lesions had *PIK3CA* exon 20 H1047R mutations[10]. Further, 11 other patients had a spectrum of lesions with multiple different *PIK3CA* point mutations ('discordant multiple,' Figure 2, patients 7–16,18).

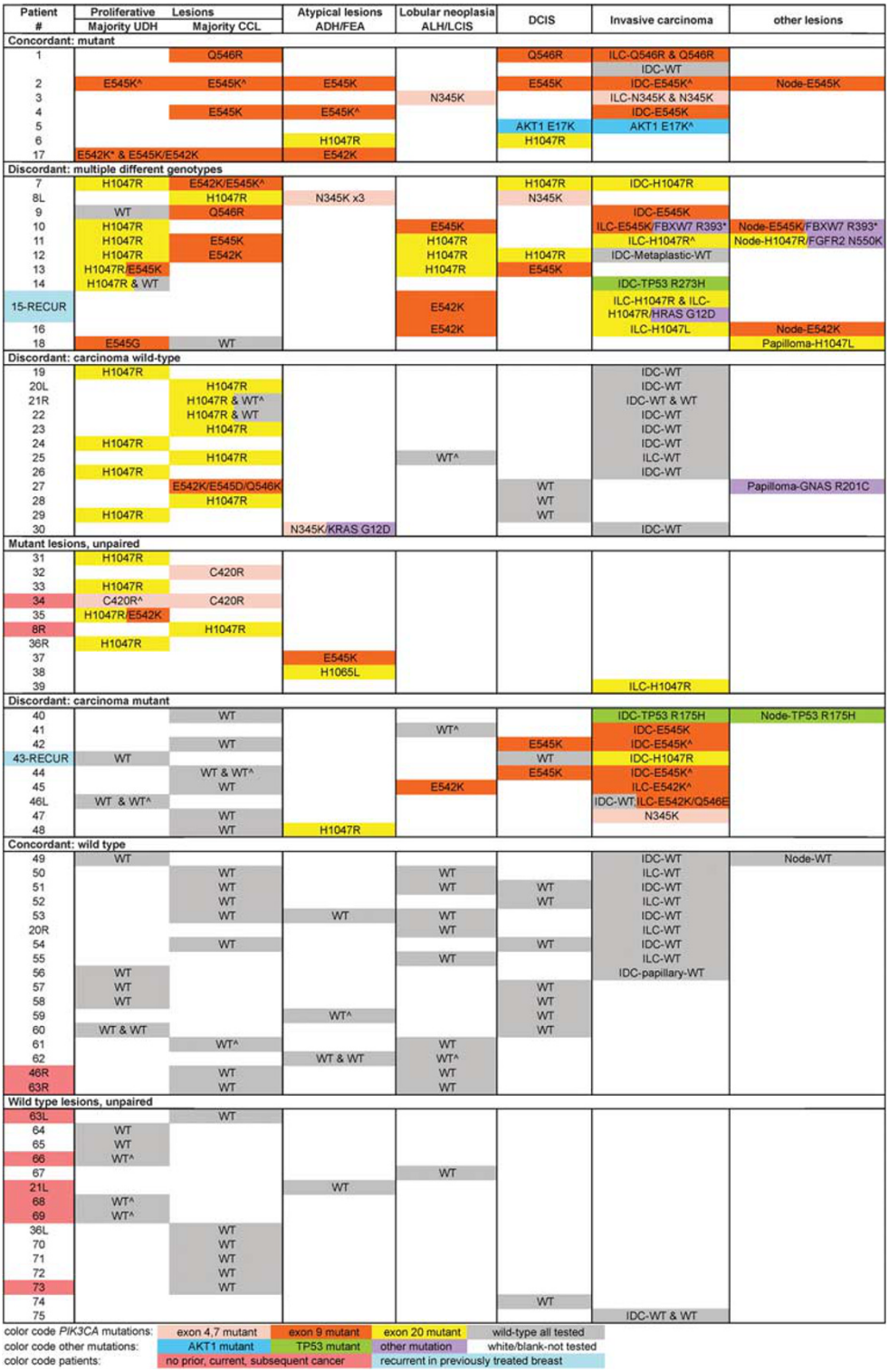


Figure 2 Mutational status by patient. Each row indicates samples from a single patient; mutations are color-coded. Blank boxes indicated no lesion/not tested. '&' Two separate lesions tested. '/' Two mutations identified within the same lesional sample. '^'Mixed with other histologies. These lesions were excluded from Table 1. See Supplementary Table for further detail. ADH, atypical ductal hyperplasia; ALH, atypical lobular hyperplasia; CCL, columnar cell lesion; DCIS, ductal carcinoma *in situ*; FEA, flat epithelial atypia; LCIS, lobular carcinoma *in situ*; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; UDH, usual ductal hyperplasia; WT, wild type for all point mutations screened.

Of the proliferative lesions from patients with no known prior, concurrent, or subsequent ipsilateral invasive or *in situ* carcinoma, 8/10 were wild type, whereas only 2 had *PIK3CA* mutations (H1047R, C420R; see Figure 2 and Supplementary Table 1, patients 8R, 21L, 34, 46R, 63R&L, 66, 68, 69, 73; 3 had contralateral cancer).

Although the study was focused on proliferative lesions, we also analyzed carcinoma *in situ* as compared with concurrent carcinoma where possible, yielding good concordance despite small numbers (Supplementary Table 1C). For DCIS and paired invasive ductal carcinoma, five out of seven specimens had concordant genotypes (concordant mutant or concordant wild type). One specimen had wild-type DCIS but *PIK3CA* H1047R mutation in a recurrent invasive ductal carcinoma (discordant: carcinoma mutant, Figure 2, Supplementary Table 1C, patient 43), whereas another had mutant DCIS with wild-type invasive metaplastic carcinoma (discordant: carcinoma wild type, Figure 2, Supplementary Table 1C, patient 12). Interestingly, concordance was also good for lobular neoplasia (atypical lobular hyperplasia/lobular carcinoma *in situ*) and concurrent invasive lobular carcinoma. Six specimens containing both lesions had concordant *PIK3CA* genotypes (four concordant wild-type and two concordant mutant). Two patients had a *PIK3CA* exon 9 E542K mutation in lobular neoplasia, but the invasive carcinomas each harbored a *PIK3CA* codon 1047 mutation; one of these was a recurrent carcinoma with an additional *HRAS* mutation in one of two tested invasive carcinomas (discordant multiple, Figure 2, Supplementary Table 1C, patient 15).

Discussion

Activating *PIK3CA* point mutations are among the most common genetic aberrations in invasive breast cancer,[1–10] and several studies have shown that these mutations exist at nearly the same frequency in DCIS.[14–17] Accumulating evidence suggests that *PIK3CA* point mutations may also be common in other proliferative breast lesions such as benign papillomas and columnar cell lesions.[18–23] Prior studies have failed to identify *PIK3CA* mutations in histologically normal breast tissue, even with the same sensitive methods employed here.[1,8,16,19] In this study, we demonstrated a 50% frequency of *PIK3CA* point mutations in usual ductal hyperplasia and columnar cell lesions across 62 lesions, representing the largest series to date. We also found similarly a high frequency of *PIK3CA* mutations in smaller cohorts of atypical ductal lesions (71%) and lobular neoplasia (44%), and a near-lack of other 'hotspot' activating mutations in genes such as *AKT1*. Table 2 represents a summary of our laboratory's experience with activating point mutations in proliferative and malignant breast lesions in this and prior studies. Our data on *PIK3CA* mutations in DCIS (48% in this study and 45% combined) and invasive mammary carcinoma (35% in this study and 34% combined) matches the literature.[1–8] We identified point mutations in other genes in invasive carcinomas, but not in 'earlier' lesions, including *TP53*, *FBXW7*, *FGFR2*, and *HRAS*.

In analyzing multiple breast lesions from the same specimen/patient, spanning the morphologic spectrum of non-atypical epithelial lesions (usual ductal hyperplasia and columnar cell change), atypical ductal lesions, lobular neoplasia, *in situ*, invasive, and metastatic carcinoma, we found a wide diversity of genotypes. These observations provide some insight as to the role of activating *PIK3CA* mutations in breast carcinogenesis, as well as the precursor status of these early breast lesions.

Table 2 Activating point mutations in breast proliferative lesions and carcinoma: our experience

	PIK3CA-AKT1 mutations (%)					
Study	*CCL/UDH*	*With atypia*	*Lobular neoplasia*	*DCIS*	*Invasive carcinoma*	*Discordant mutational status among spectrum*
Present study	31/62 (50%)	10/14 (71%)	7/16 (44%)	10/21 (48%)	13/37 (35%)	31/51
Troxell *et al*[19]	13/24 Columnar cell (54%)		0/2 (0%)	4/8 (50%)	3/8 (37.5%)	9/14
Kehr *et al*[20]	3/8 (37.5%)	2/3 (66%)		3/14 (21%)	0/38 (0%) Mucinous	6/16
Flatley *et al*[21]	3/8 (37.5%) and other proliferative		0/1	4/11 (36%)	6/20 (30%) Micropapillary	3/7
Total above	*62/120 (52%)*		*7/19 (37%)*	*18/40 (45%) Excluding mucinous*	*22/65 (34%) Excluding mucinous*	*49/88*
Troxell *et al*[18] different method	40/61 (66%)	8/11 (72%)	0/1 (0%)	3/6 (50%)	1/10 (Papillary CA) 10%	Not applicable
Wolters *et al*[22] radial scar	9/16 (56%)	4/5 (80%)		1/1 (100%)	0/1 (0%)	Not applicable

Abbreviations: CCL, columnar cell lesion; DCIS, Ductal carcinoma *in situ*; UDH, usual ductal hyperplasia.

If *PIK3CA* mutations are an early or late 'driver' of breast carcinogenesis, these mutations would be expected to appear in committed precursor lesions, and persist through the development of carcinoma as further genetic changes evolve, perhaps as reflected in the 'concordant mutant' and 'discordant: carcinoma mutant' groups of specimens in our study, respectively (Figure 2). However, we identified a relatively large group of specimens/patients in which activating *PIK3CA* mutations were present in usual ductal hyperplasia and/or columnar cell change, but the accompanying carcinomas were wild type for these mutations (Figure 2, see 'discordant: carcinoma wild type'). This result is not likely to be biased by assay sensitivity, as the mass spectrometry screening method was previously demonstrated to have a sensitivity of 5–10% mutant allele,[27] and the carcinomas isolated generally had greater lesional cellularity as compared with the non-atypical epithelial lesions. Further, these findings have implications for clinical and research genotyping of breast carcinomas, emphasizing the need for prudent isolation of lesional tissue in order to avoid 'false positives,' as could occur if a wild-type invasive carcinoma is accompanied by proliferative epithelium harboring *PIK3CA* mutation.

Many studies over the past 20 years have applied genomic assays in an attempt to map breast cancer progression and to identify early breast precursor lesions (Supplementary Table 2).[24,29–34] Nevertheless, defining the role of usual ductal hyperplasia and columnar cell lesions as precursors, non-obligate precursors, or unrelated bystanders in the sequence of breast carcinogenesis remains a challenge. It has recently been proposed that columnar cell lesion may represent the first histologically distinct, but non-obligate precursor of breast carcinoma based on genetic and immunohistochemical data as recently summarized.[24,19,35,36] Genomic findings in usual ductal hyperplasia have varied widely based on tissue substrate (fresh/frozen/formalin fixed), method of lesion dissection, method of genomic analysis, including karyotypic analysis, loss of heterozygosity/allelic imbalance, and, most recently, comparative genomic hybridization (Supplementary Table 2).[24,29–34] These prior studies and our data support a general theme of fewer yet more heterogeneous genomic alterations in hyperplasia as compared with carcinoma (Supplementary Table 2).[24,29–34]

The few studies of point mutations in breast hyperplasias have yielded a paucity of mutations in specific genes such as *TP53* and *PTEN* (Table 3).[37–41] However, Fuqua *et al*[42] reported the K303R mutation in *ESR1* (encoding estrogen receptor-α) in 20/59 (34%) of breast hyperplasias, a mutation that confers estrogen hypersensitivity and has been shown to activate the PI3K pathway *in vitro*.(43) Li *et al*[16] studied *PIK3CA* mutations in usual ductal hyperplasia and found no mutations in 16 lesions, in contrast to our results; the differences may be due to sample size and methodologic sensitivity, as they used less-sensitive direct Sanger sequencing.[16]

The activating *PIK3CA* hotspot mutations seen frequently in breast cancers and proliferative lesions, most notably H1047R, E542K and, E545K, have transforming potential when overexpressed in a variety of cell culture models,[8–10] which arise before the most common ancestor in the genomic history of several breast carcinomas[23,44] and have thus been logically viewed as drivers of carcinogenesis. Interestingly, expression of the *PIK3CA* H1047R mutation at physiologic or inducible supraphysiologic levels in the mouse mammary gland leads to increased branching, duct dilation, and luminal epithelial hyperplasia.[45–47] Expressing the *PIK3CA* H1047R mutant at physiologic levels throughout mammary development leads to mouse mammary tumors after very long latency (median tumor free survival of 484 days or 492 days nulliparous, as reported by two independent groups).[46,47] The resulting tumors are heterogeneous in morphology (fibroadenoma, adenocarcinoma, adenosquamous carcinoma, and spindle cell carcinoma) and hormone expression.[46,47] Activation of downstream elements of the *PIK3CA* signaling cascade such as pAKT and pS6 was demonstrated in tumors, but not at time points with early mammary hyperplastic lesions, suggesting crosstalk or additional hits in tumors.[46,47] Further, genetic profiling of mammary tumors in these mouse model systems revealed that they contained the engineered *PIK3CA* mutation, but had also gained other genomic changes such as p53, MET, c-Myc, and other oncogenes, even becoming independent of the *PIK3CA* mutation, as was seen in two-thirds of the tumors in the inducible model.[45,47] These animal models, together with our findings in usual ductal hyperplasia and columnar cell lesions from patient specimens, support the hypothesis that activated *PIK3CA* may have a role in breast epithelial proliferation, more so than as a driver of breast oncogenesis, and alone is insufficient for carcinogenesis.[45–47]

The discordance in *PIK3CA* mutation status between breast proliferative lesions and concurrent carcinoma bears some similarities to that of *FGFR3* mutation and overexpression in early bladder cancers. *FGFR3* mutations are seen in $\sim$60% of *in situ* bladder lesions, but in $<$20% of T2-stage invasive carcinomas.[48,49] Further, genotyping of multiple regions from the same bladder cancer specimen revealed *FGFR3* mutations in the non-invasive component with paired wild-type invasive carcinoma from eight discordant specimens, and different point mutations in one sample (of 43 specimens with multiple areas tested).[48,49] With regard to these findings, the authors suggest that *FGFR3* contributes early in the process of tumor development, and either the *FGFR3* mutant allele was lost in the high stage lesion or it is a small subpopulation of *FGFR3* wild-type cells in the non-invasive lesion that progresses to invasive carcinoma.[48,49]

Table 3 Point mutations in breast hyperplasia: literature review

Reference and method	*Lesions*	*Findings*	*Comments*
TP53			
Millikan et al[37] SSCA and sequencing *TP53* exons 4–8 with FFPE, scalpel dissection.	Typical ductal hyperplasia $N=10$	0/10 With *TP53* point mutation	Cases without cancer *TP53* point mutations: 1/1 Intraductal papillomatous hyperplasia 2/22 Non-proliferative fibrocystic change 2/8 Fibroadenoma
Done et al[38] SSCP and sequence *TP53* exons 4–8 with FFPE needle dissection	Epithelial hyperplasia $N=20$ from 10 specimens with TP53-mutated cancer	0/20 With *TP53* point mutation	27/27 DCIS from 8 cases with IMC have identical TP53 mutation
Kandel et al[39] SSCP and sequence *TP53* exons 4–10 with FFPE dissected	Hyperplasia $N=8$	2/8 With *TP53* mutation	Specimens without cancer Fibrocystic change: 11/22 *TP53* mutation Adenosis: 2/8 with *TP53* mutation
Mao et al[40] HRM and sequencing *TP53* exons 5–8 with frozen tissue	Usual ductal hyperplasia $N=40$ without cancer	0/40 *TP53* mutations	ADH: 12.7% *TP53* mutations DCIS: 21.6%
ESR1 estrogen receptor			
Fuqua et al[42] Partial sequence *ESR1* with FFPE manual dissection	Hyperplasia $N=55$	20/59 (34%) With K303R alteration; conferring hypersensitivity to estrogen	Some normal and fewer distant normal samples with same mutation
Zhang et al[50] Partial sequence *ESR1* with FFPE needle dissection	Hyperplasia $N=7$	0/7 With K303R alteration	0/26 DCIS with K303R alteration 0/215 IMC with K303R alteration in Japanese cohort
Tebbit et al[51] Partial sequence *ESR1* with FFPE or frozen, LCM	Typical epithelial hyperplasia $N=11$	0/11 With K303R alteration	No point mutations in IMC, DCIS, atypical epithelial hyperplasia either
PIK3CA			
Li et al[16] Sequence exon 9, 20 with FFPE LCM	Usual ductal hyperplasia $N=16$	0/16 *PIK3CA* mutations	DIN 1A: 1/20 *PIK3CA* mutations DIN 1B: 2/32 *PIK3CA* mutations DCIS: 14/57 *PIK3CA* mutations IMC: 29/108 *PIK3CA* mutations
PTEN			
Yang et al[41] *PTEN* direct sequencing with frozen tissue	Ductal hyperplasia $N=17$	0/17 *PTEN* mutations	ADH: 4/33 PTEN mutations (in common with accompanying cancer) IMC: 11/50 PTEN mutations Specimens with cancer

Abbreviations: ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma *in situ*; DIN, ductal intraepithelial neoplasia; FFPE, formalin-fixed paraffin-embedded; HRM, high-resolution melting; IMC, invasive mammary carcinoma; LCM, laser capture microdissection; LOH, loss of heterozygosity; SSCA, single-strand conformation analysis; SSCP, single-strand conformational polymorphism.

In conclusion, we have screened a spectrum of 192 breast lesions with emphasis on proliferative lesions, and found that *PIK3CA* mutations were very frequent and were heterogeneous between multiple lesions in the same specimen. Importantly, point mutations in proliferative lesions were often paired with wild-type carcinomas. These findings support the concept of usual ductal hyperplasia and/or columnar cell lesion as heterogenous non-obligate precursors of breast cancer, and to the notion that *PIK3CA* 'hotspot' mutations are important early in breast epithelial proliferation/neoplasia, but may not contribute a selective advantage at later stages of tumor progression. Further studies of functional pathway activation, as well as more detailed genomic and epigenomic studies will be necessary to more fully characterize the putative precursor role of usual ductal hyperplasia and columnar cell lesions, and the molecular evolution of breast carcinogenesis.

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A subset of this data was presented in abstract form at the San Antonio Breast Conference 6 December 2012 (cases with lobular neoplasia and lobular carcinoma). Mass spectroscopy and sequence data from 60 lesions comprise the validation cohort for a novel locked nucleic acid sequencing method, but were not further analyzed by any histologic or clinical parameter.[28] The *PIK3CA* mutation frequency in 23 invasive carcinomas was cited as a comparator group in prior studies of special types of

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)