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Phosphatidylinositol-3-kinase pathway mutations are common in breast columnar cell lesions

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The phosphatidylinositol-3-kinase pathway is one of the most commonly mutated pathways in invasive breast carcinoma, with *PIK3CA* mutations in ~25% of invasive carcinomas, and *AKT1* mutations in up to 5%. Ductal carcinoma *in situ*, and benign papillomas harbor similar mutations. However, activating point mutations in breast columnar cell lesions have been infrequently studied. Twenty-three breast resection specimens containing columnar cell lesions were identified; 14 with associated invasive carcinoma or carcinoma *in situ*. DNA extracts were prepared from formalin-fixed paraffin-embedded tissue and screened for a panel of point mutations (321 mutations in 30 genes) using a multiplex PCR panel with mass-spectroscopy readout. *PIK3CA* mutations were identified in 13/24 columnar cell lesions (54%) and 3/8 invasive carcinomas (37%). The mutation status of columnar cell lesions and associated carcinoma was frequently discordant. Of the 14 cases, only 5 demonstrated the same genotype in matched samples of columnar cell lesions and carcinoma (4 wild type, 1 *PIK3CA* H1047R). Interestingly, five patients had mutations in columnar cell lesions with wild-type carcinoma; two patients had different point mutations in columnar cell lesions and carcinoma. Only three cases had wild-type columnar cell lesion and mutated carcinoma. The 50% *PIK3CA* mutation prevalence in columnar cell lesions is greater than reported in most studies of invasive breast cancer. Further, columnar cell lesions and carcinoma were frequently discordant for *PIK3CA/AKT1* mutation status. These findings raise interesting questions about the role of *PIK3CA/AKT* pathway in breast carcinogenesis, and the biologic/precursor potential of columnar cell lesions.
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The phosphatidylinositol-3-kinase pathway is activated in numerous cancer types and 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, with mutations clustering in 'hotspots' in exon 9 (helical domain) and exon 20 (kinase domain).[1–10] In addition, this pathway is activated by mutations in the plekstrin-homology domain of *AKT1* in up to 5% of breast carcinomas, or by the loss of the phosphatase PTEN (phosphatase and tensin homolog) in nearly half of breast cancers.[2,11] Several groups have demonstrated a similar frequency of mutations in breast carcinoma *in situ*, with paired invasive and *in situ* carcinoma from the same patient concordant for *PIK3CA* mutation status in 66–100% of tested cases.[12–15] However, other breast proliferative or putative precursor lesions have been little studied. Li *et al*[13] found *PIK3CA* hotspot mutations in only 6% of 52 tested cases of Ductal Intraepithelial Neoplasia 1A–B lesions (DIN1A–B, atypical ductal hyperplasia and flat epithelial atypia, also known as columnar cell change with atypia). Our group previously identified *PIK3CA/*

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AKT1 mutations in a substantial proportion of benign papillary lesions (65%), neoplasms that are generally not considered to represent precursors of breast cancer.[16]

Columnar cell lesions of the breast have been recognized historically under a wide variety of different names, and were recently renamed and studied in detail. Columnar cell lesions frequently coexist with atypical hyperplasias and low-grade ductal carcinoma *in situ*, lobular carcinoma *in situ*, or invasive breast carcinomas.[17–20] Columnar cell lesions are characterized by variably dilated acini lined by one to several layers of cells with ovoid to elongated nuclei, often with apical snouts (termed columnar cell change or columnar cell hyperplasia). According to the criteria proposed by Schnitt *et al*,[17,21] columnar cell lesions with cytologic atypia have rounded nuclei, may have nucleoli, and/or cytologic features overlapping low-grade carcinoma; the term flat epithelial atypia encompasses both columnar cell change with atypia, and columnar cell hyperplasia with atypia. The role, if any, of columnar cell lesions along the spectrum of morphologic and pathogenetic progression from normal breast, to *in situ* or invasive breast carcinoma remains speculative. Recent loss of heterozygosity, comparative genomic hybridization, and immunohistochemical studies have suggested that columnar cell lesions, might represent the 'missing link' in low-grade breast cancer progression, as the earliest identifiable non-obligate precursor.[18,22–28] In order to further address the pathogenetics of columnar cell lesions, we screened a cohort of columnar cell lesions, most with matched normal breast tissue and/or concurrent carcinoma, for a large panel of known activating point mutations, including *PIK3CA* and *AKT1* mutations.

Materials and methods

This project was approved by the Institutional Review Boards of Stanford University and Oregon Health & Science University. Cases of columnar cell lesions were identified from the files of Stanford University Pathology, with two cases from Oregon Health & Science University. Slides were reviewed to identify columnar cell lesions, including columnar cell change, columnar cell hyperplasia, and flat epithelial atypia (columnar cell change with atypia and columnar cell hyperplasia with atypia) using the criteria of Schnitt *et al*.[17,21] In addition, representative blocks of normal breast tissue, and breast carcinoma from the same specimen/patient, where available, were also selected. Parameters of size, stage, grade, and hormone receptor status for carcinomas were obtained from the pathology reports.

Lesions of interest were marked on slides, and the corresponding area of the formalin-fixed, paraffin-embedded tissue block was isolated by punching the block with 1 or 2 mm core device. Multiple cores of each lesion were collected, some from different tissue blocks, and the cores were re-embedded. Paraffin shavings from the recipient block were collected, and slides were prepared from central depth in the recipient block, in order to allow re-evaluation of the lesional tissue for diagnostic confirmation (RBW and MLT). This method yielded samples comprised of lesional epithelium, surrounding myoepithelium, and stroma. As columnar cell lesions without hyperplasia often line dilated spaces and are associated with hypercellular stroma, the percentage of lesional cells was quite variable. Atypia in columnar cell lesions was assessed, and discrepant cases were resolved by consensus review; controversial or heterogeneous cases were further noted as borderline for atypia.

DNA was extracted from the paraffin shavings using standard protocols (RecoverAll Total Nucleic Acid Isolation Kit #AM1975, Ambion/Applied Biosystems, Austin, TX, USA). DNA extracts were screened for a panel of point mutations using a multiplex PCR panel as previously described.[16,29] In brief, sequences of interest were amplified by PCR; PCR products were then annealed to primers directly adjacent to targeted mutation sites. A primer extension reaction adds one base pair, and the resultant product is analyzed by mass spectroscopy (Sequenom MassARRAY), which allows identification of the added base pair by molecular weight. The mutation panel covers 321 mutations in 30 genes, including *ABL*, *AKT1/2/3*, *BRAF*, *CDK4*, *CTNNB1*, *EGFR1*, *ERBB2*, *FBX4*, *FBXW7*, *FGFR1/2/3*, *FLT3*, *GNAQ*, *HRAS*, *JAK2*, *KIT*, *KRAS*, *MAPK2K1/2*, *MET*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTPN11*, *RET*, *SOS1*, and *TP53*. The panel includes 41 substitutions in 23 codons of the *PIK3CA* gene.[29] Assays were previously validated in the laboratory using known controls, and 17 of the 20 mutations were independently confirmed by direct sequencing of the sample DNA on an ABI3130 sequencer using the BigDye Terminator method, with or without the use of a locked nucleic acid probe to suppress amplification of the wild-type allele (Ang *et al*, in preparation). For the remaining cases, sequence reactions were unsuccessful, or insufficient DNA remained. Patient identity across specimens in several of these cases was confirmed by the identification of a common single-nucleotide polymorphism in multiple samples, or by testing a panel of single-nucleotide polymorphisms for an unrelated gene.

Results

Mutational Status of Columnar Cell Lesions, Carcinoma, and Normal Breast Tissue

Twenty-four columnar cell lesions from 22 patients were identified for study, including columnar cell change, columnar cell hyperplasia, and flat

epithelial atypia (columnar cell change with atypia). Normal breast tissue from the same specimen was available in 19 patients. Fourteen cases also had concurrent carcinoma, including ductal carcinoma *in situ*, lobular carcinoma *in situ*, invasive ductal carcinoma, and metastatic carcinoma (Table 1; Supplementary Information online).

DNA was prepared from formalin-fixed paraffin-embedded tissue, including epithelial lesional tissue and surrounding myoepithelial cells and stroma. DNA extracts were screened for a panel of 321 'hotspot' point mutations in 30 genes; in total, extracts from 65 lesions were analyzed. Point mutations in the *PIK3CA* gene were identified in 13/24 (54%) columnar cell lesions (patients 1–12; 8 exon 20-H1047R; 3 exon 9-E542K; 1 exon 9-E545K; 1 exon 7-C420R; Table 1; Figures 1 and 2). In one case, columnar cell lesions from different areas of the specimen were analyzed separately, and the same mutation was demonstrated (patient 11L, both E542K; contralateral columnar cell lesion was wild type, 11R). Nine of the columnar cell lesions had atypia (flat epithelial atypia), including five cases with *PIK3CA* mutations, and four wild-type cases, not different than the mutation frequency of non-atypical lesions ($P = 0.9156$ χ^2, not significant). Other than a single-nucleotide polymorphism (*MET* T992I) in each of the specimens from one patient, no other panel mutations were found in columnar cell lesions.

In accompanying normal tissue from 19 patients, 18 samples were wild type. One mostly normal sample had an exon 9-E542K mutation (patient 9), like the patient's columnar cell lesion; slide review demonstrated a small amount of contaminating columnar cell lesion (10%).

PIK3CA activating point mutations were identified in 4/8 (50%) of ductal carcinoma *in situ* tested (1 each exon 20-H1047R; exon 9-E542K; exon 9-E545K; exon 4-N345K; Table 1). Of the eight tested invasive ductal carcinomas, mutations were identified in three cases ($3/8 = 37\%$), including an *AKT1* mutation (*AKT1* exon 2-E17K; 2 *PIK3CA* exon 20-H1047R). The size, grade, stage, and hormone receptor status of the invasive carcinomas are listed in Supplementary Information online. No activating hotspot mutations were identified in three nodal metastases, or two examples of lobular neoplasia (atypical lobular hyperplasia, lobular carcinoma *in situ*). Again, no other point mutations were identified with this large screening panel.

Mutational Status of Paired Breast Lesions

In 14 specimens, mutation status of columnar cell lesions could be compared with mutation status of concurrent *in situ* or invasive carcinoma. Interestingly, only five demonstrated the same genotype in matched samples of columnar cell change and

Table 1 Mutation status in columnar cell lesions and concurrent breast lesions

Patient#	*Normal*	*Columnar cell lesion*	*Ductal carcinoma* in situ	*Invasive ductal carcinoma*	*Other*
1	WT	H1047R			
2	WT[a]	H1047R			
3	WT	H1047R			
4	WT	H1047R		H1047R	
5	WT	H1047R	WT		
6	WT	H1047R	WT		
7	WT	H1047R		WT	
8	WT	H1047R	E545K		MET: WT
9	E542K[a]	E542K			
10		E545K			
11L	WT	E542K × 2	H1047R	WT	
12	WT	C420R		WT	MET: WT
13	WT	WT		H1047R	
14	WT[a]	WT	E542K	AKT1 E17K	
15	WT	WT	N345K		
16		WT			
17		WT			
18	WT	WT			
19	WT	WT			
20	WT	WT	WT		
21	WT	WT	WT	WT	ALH:WT
11R	WT[a]	WT			LCIS: WT
22		WT		WT	MET: WT

Abbreviations: ALH: atypical lobular hyperplasia; LCIS: lobular carcinoma *in situ*; MET: lymph node metastasis.

[a]Predominantly normal with low % columnar cell lesion (CCL): patient 2—20% CCL, patients 9 and 14—10% CCL, patient 11R—5% CCL (see Supplementary Table).

Patient 11 with bilateral specimens (11L, 11R).

11L: 2 columnar cell lesions from distant blocks analyzed, both with E542K mutation.

Shading indicates mutation: light shading—*PIK3CA* exon 20; medium shading—*PIK3CA* exon 4, 7, 9; black—*AKT1* exon 2.

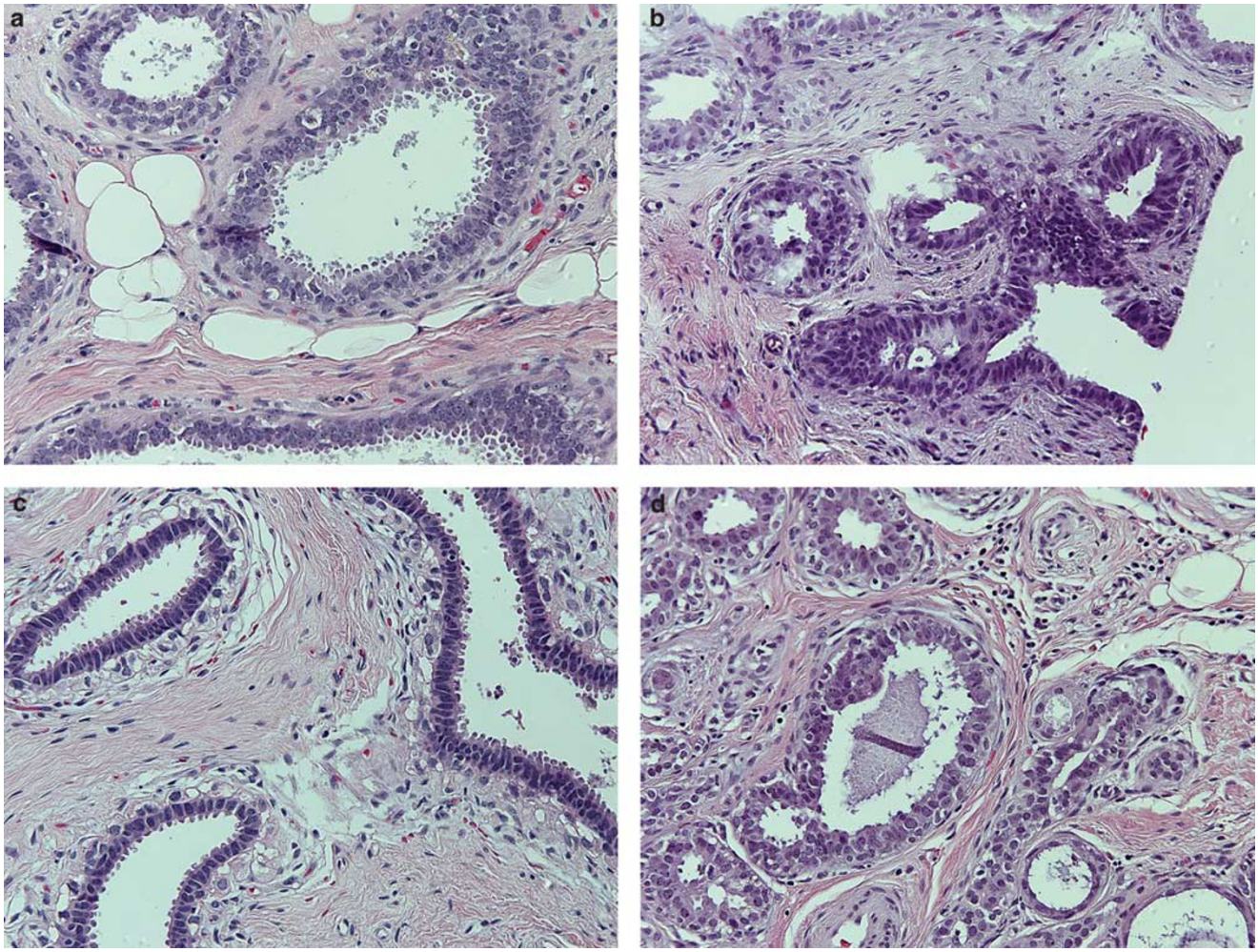

Figure 1 Histopathology of representative columnar cell lesions. (**a**) Patient 4, columnar cell hyperplasia, borderline for atypia, with *PIK3CA* H1047R mutation (see Figure 2). (**b**) Patient 12, columnar cell hyperplasia without atypia, with *PIK3CA* C420R mutation (see Figure 2). (**c**) Patient 7, columnar cell lesion without atypia, with *PIK3CA* H1047R mutation. (**d**) Patient 20, columnar cell lesion with atypia, wild type for all mutations tested.

carcinoma (5/14 = 35%, four wild-type patients 20–22, 11R; 1 *PIK3CA* H1047R-patient 4; Figure 2 and Table 1). Interestingly, five patients had mutations in columnar cell lesion with wild-type ductal carcinoma *in situ* or invasive carcinoma (patients 5–7 *PIK3CA* H1047R; patients 11L–12 *PIK3CA* E542K, *PIK3CA* C420R; Table 1); two patients had different point mutations in columnar cell lesion and carcinoma (patients 8, 11L; Figure 2 and Table 1). Only three cases had wild-type columnar cell lesion and mutated carcinoma (patients 13–15; Table 1).

Of the few pairs of ductal carcinoma *in situ* and invasive carcinoma tested, two of the three were discordant for mutation status (patients 11L and 14, both of these patients had different mutations in each of the several different lesions). Of the three wild-type metastatic carcinomas, two were associated with wild-type invasive carcinoma, while one was associated with ductal carcinoma *in situ* harboring the *PIK3CA* exon 9 E545K mutation (patient 8), without invasive carcinoma available for analysis.

Discussion

The *PIK3CA* mutation prevalence of 25–30% in invasive as well as *in situ* breast carcinoma is fairly well established in the literature, and is perhaps slightly higher in estrogen receptor-positive subgroups.[12–15,30–43] However, the mutation prevalence in breast columnar cell lesions, as well as the evolution of activating point mutations across different breast lesions in a single patient, has not been well studied to date. In a cohort of 24 columnar cell lesions, encompassing columnar cell change, columnar cell hyperplasia, and flat epithelial atypia, we found *PIK3CA* mutations in 13 samples. In addition, we found little concordance between mutation status of columnar cell lesions and accompanying carcinoma.

The 54% *PIK3CA* mutation frequency in columnar cell lesions in our study is higher than the mutation frequency of invasive carcinoma, both in our study (3/8, 37%), and higher than reported in the

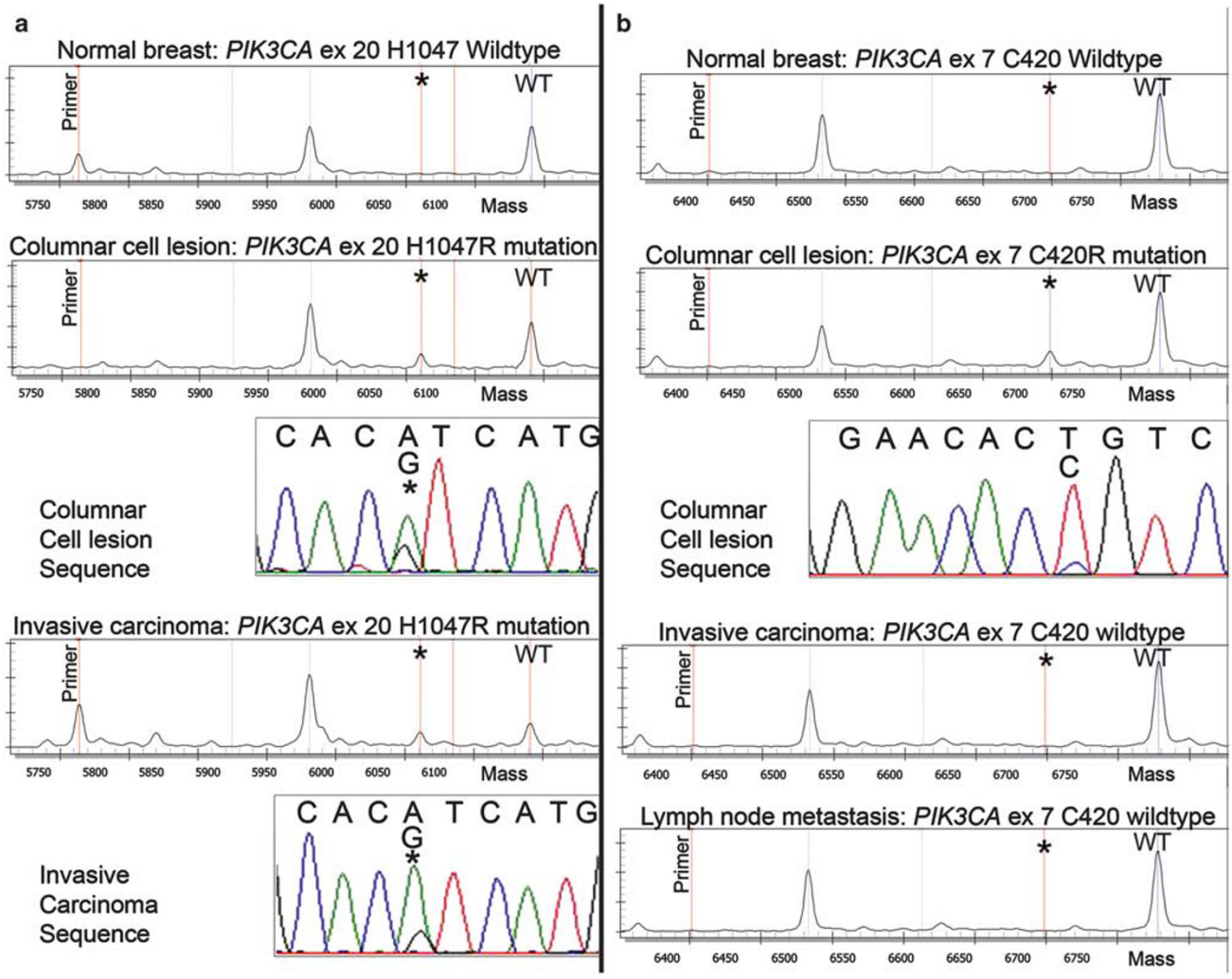

Figure 2 Mutation status across multiple samples from the same patient. (**a**) Patient 4. Top: Normal breast tissue shows a single wild-type (WT) peak for *PIK3CA* H1047 by PCR-mass-spectroscopy assay (PCR-MS). Middle: Columnar cell lesion (see Figure 1a) shows a WT and smaller H1047R mutant (*) peak by PCR-MS analysis, and is confirmed by direct sequencing below (CAT→CGT). The relatively small mutant peak is due to sample heterogeneity, including lesional columnar cell epithelium, myoepithelial cells, and stroma. Bottom: Invasive ductal carcinoma demonstrates the same H1047R mutation (*) peak by PCR-MS analysis. In this assay, the primer peak is non-zero, yet the mutation is confirmed by direct sequencing below. (The large peak in the middle of the PCR-MS tracing represent WT peaks from an unrelated, multiplexed assay). (**b**) Patient 12. Top: Normal breast tissue shows a single WT peak for *PIK3CA* C420 by PCR-MS assay. Second from top: Columnar cell lesion (see Figure 1b) shows a WT and smaller C420R mutant (*) peak by PCR-MS analysis, and is confirmed by direct sequencing below (CTG→CCG). Second from bottom: Invasive ductal carcinoma is WT for *PIK3CA* C420R. Bottom: Lymph node metastasis is also WT for *PIK3CA* C420R. (The large peak in the middle of the PCR-MS results represent WT peaks from an unrelated, multiplexed assay).

literature, including recent studies using very sensitive techniques, similar to those employed in this study.[14,31,32,44–46] However, our group previously identified *PIK3CA* or *AKT1* mutations in 65% of benign or atypical breast papillary lesions,[16] suggesting that a variety of proliferative lesions of the breast may harbor a high frequency of *PIK3CA* pathway mutations. In contrast, Li *et al*[13] studied a spectrum of proliferative and atypical breast lesions and found relatively fewer mutations. They screened usual ductal hyperplasia, Ductal Intraepithelial Neoplasia 1A (flat epithelial atypia), and Ductal Intraepithelial Neoplasia 1B (atypical ductal hyperplasia), as well as ductal carcinoma *in situ* and invasive carcinoma using direct sequencing, with laser capture microdissection. They found mutations in only 1/20 (5%) cases of Ductal Intraepithelial Neoplasia 1A and 2/32 (6%) cases of Ductal Intraepithelial Neoplasia 1B, and none in 16 cases of usual ductal hyperplasia.[13] Although the frequency of mutations identified by Li *et al*[13] in ductal carcinoma *in situ* and invasive carcinoma was comparable to other reports in the literature (24.5 and 26.8%, respectively), direct sequencing is known to have lower sensitivity for small samples, as compared with Sequenom mass-array, SNaPshot PCR, and other methods.[15,45,47,48]

Progression from pre-neoplastic lesions to *in situ* to invasive carcinoma has been associated with accumulation of genetic changes, as is well established in the progression of colonic adenomas to colon carcinoma.[49,50] In breast pathology, comparative genomic hybridization and a number of other techniques have substantiated the known precursor status of ductal carcinoma *in situ*.[23,51,52] These techniques are now being employed to investigate columnar cell change and other proliferative breast lesions as putative precursors to breast carcinoma.[22–28] Previously, Moinfar *et al*[22] analyzed loss of heterozygosity in Ductal Intraepithelial Neoplasia-flat lesions, and found significant alterations, including changes in common with concurrent carcinoma; however, 9 of the 22 studied cases were 'pleomorphic' Ductal Intraepithelial Neoplasia-flat, equivalent to clinging carcinoma (high nuclear grade flat ductal carcinoma *in situ*). In a subsequent study using array comparative genomic hybridization Moinfar's group showed by that Ductal Intraepithelial Neoplasia low grade harbored changes common to lobular neoplasia, low nuclear grade ductal carcinoma *in situ*, and invasive carcinoma (loss of 16q, gain of 1q).[28] Simpson *et al*[24] found chromosomal alterations, as analyzed by comparative genomic hybridization, across the spectrum of columnar cell lesions (including some with architectural complexity that would alternatively be classified as atypical ductal hyperplasia by some experts). They noted a greater number of changes in lesions with cytologic atypia, hyperplasia, and/or architectural complexity. Further, they studied a large number of lesions from three patients and found recurring genetic changes in lesions of different severity, ranging from columnar cell change to low-grade ductal carcinoma *in situ* in two of three patients. The authors suggested that columnar cell lesions might be an important non-obligate precursor of breast carcinoma, even the 'missing link.'[18,24] In further support of that hypothesis, Dabbs *et al*[25] evaluated loss of heterozygosity of 10 microsatellite markers, and also demonstrated an accumulation of alterations across the spectrum from columnar cell lesions, to atypical ductal hyperplasia, to ductal carcinoma *in situ* and invasive carcinoma. Recently, Ellsworth *et al*[27] studied allelic imbalance (loss of heterozygosity) in columnar cell lesions and atypical ductal hyperplasia that were not associated with carcinoma. They found levels of genomic alterations intermediate between normal breast and invasive/*in situ* carcinoma, importantly without recurrent alterations and 16q and 17q. They concluded that these molecular profiles do not support a role for columnar cell lesions and atypical ductal hyperplasia as obligate precursors.[27]

Similar to those previous studies, we found a high percentage of genetic alterations in the form of activating *PIK3CA* mutations in breast columnar cell lesions. However, we found a very low concordance with mutational status of accompanying carcinoma. In fact, only 5/14 cases were concordant, and four of these cases were wild type. More commonly, we found that mutated columnar cell lesion was accompanied by wild-type carcinoma, or carcinoma with a different activating point mutation (9/14). This combination of genotypes does not follow the canonical genetic stepwise progression model from precursor with few key early mutations, to carcinoma after accumulation of additional deleterious mutations. Certainly, these data remain compatible with the hypothesis that columnar cell lesions might be non-obligate or heterogeneous precursors, such that only a small fraction of columnar cell lesions might progress to carcinoma. This and several alternative hypotheses deserve further study, including columnar cell lesions as a marker of susceptibility, columnar cell lesions as incidental unrelated proliferations without precursor potential, etc.

PIK3CA or *AKT1* mutations are thought to have key roles in the biology of breast cancer, in that PIK3CA/AKT pathway activation may account for resistance to trastuzumab or small molecule tyrosine kinase inhibitors in some Her2-positive tumors.[32,53–56] Further, in estrogen receptor-positive breast cancers, a *PIK3CA* mutation-gene signature has been elucidated by expression analysis, paradoxically imparting a relatively favorable prognosis.[39,40,43,44,57] Nevertheless, whether *PIK3CA/AKT1* mutations have a role in breast carcinogenesis, perhaps as an early primary or 'driver' mutation remains unclear.[50] The high frequency of *PIK3CA/AKT1* mutations in columnar cell lesions and benign breast papillomas suggests that *PIK3CA/AKT1* mutations may be related to the development of breast proliferative lesions. If columnar cell lesions represent non-obligate precursors of *in situ* and invasive breast carcinoma, our data suggest the possibility that *PIK3CA/AKT1* mutations may not necessarily confer increased risk of progression, as the *PIK3CA/AKT1* mutation status of columnar cell lesions and concurrent carcinoma were frequently discordant. Thus, it appears that mutant clones may be bypassed, selected against, or mutations may be lost, during progression to carcinoma, allowing speculation that *PIK3CA/AKT1* mutations might be less beneficial in later stage lesions. Similarly, an elegant repressible mouse model system has shown that mammary tumors induced by overexpression of *PIK3CA* H1047R frequently recur independent of the *PIK3CA* mutant transgene, with many recurrent tumors harboring additional genomic alterations, including Met or myc perturbations, among others.[58]

In summary, we have demonstrated a high prevalence of *PIK3CA* mutations in breast columnar cell lesions, as well as a lack of genotypic concordance of columnar cell lesions with paired carcinoma. Although these findings need validation in a larger study, they raise a number of

interesting questions as to the biologic/precursor potential of breast columnar cell lesions, and the role of *PIK3CA/AKT1* mutations in breast carcinogenesis.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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