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Multiplexed imaging reveals heterogeneity of PI3K/MAPK network signaling in breast lesions of known *PIK3CA* genotype

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Abstract

Purpose—Activating genetic changes in the phosphatidylinositol-3-kinase (PI3K) signaling pathway are found in over half of invasive breast cancers (IBC). Previously, we discovered numerous hotspot *PIK3CA* mutations in proliferative breast lesions. Here, we investigate the spatial nature of PI3K pathway signaling and its relationship with PI3K genotype in breast lesions.

Methods—We identified PI3K phosphosignaling network signatures in columnar cell change (CCL), usual ductal hyperplasia (UDH), ductal carcinoma in situ (DCIS) and IBC in 29 lesions of known *PIK3CA* genotype from 10 human breast specimens using a hyperspectral-based multiplexed tissue imaging platform (MTIP) to simultaneously quantitate PI3K/MAPK pathway targets (pAKT473, pAKT308, pPRAS40, pS6, and pERK) in FFPE tissue, with single cell resolution.

Results—We found that breast lesional epithelia contained spatially heterogeneous patterns of PI3K pathway phosphoprotein signatures, even within microscopic areas of CCL, UDH, DCIS and IBC. Most lesions contained 3–12 unique phosphoprotein signatures within the same microscopic field. The dominant phosphoprotein signature for each lesion was not well-correlated with lesion genotype or lesion histology, yet samples from the same patient tended to group together. Further, 5 UDH/CCL lesions across different patients had a common phosphosignature at the epithelial-stromal interface (possible myoepithelial cells) that was distinct from both the adjacent lesional epithelium, and distinct from adjacent stroma.

Conclusion—We present the first spatial mapping of PI3K phosphoprotein networks in proliferative breast lesions, and demonstrate complex PI3K signaling heterogeneity that defies simple correlation between *PIK3CA* genotype and phosphosignal pattern.

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Keywords

PIK3CA; phosphatidylinositol-3-kinase; usual ductal hyperplasia; columnar cell change; hyperspectral imaging

Introduction

Genomic study of breast cancer has greatly increased our knowledge of its molecular landscape and complexity. Numerous studies have demonstrated that well over half of breast cancers have genetic changes in components of the phosphatidylinositol-3-kinase (PI3K) signaling cascade, predicted to result in pathway activation [1–5]. These genomic changes include amplification, mutation or activation of receptor tyrosine kinases (HER2, EGFR), mutation or amplification of the catalytic subunit (*PIK3CA*), mutation or amplification of *AKT1*, or loss of inhibition, impacting the *PIK3R1* regulatory subunit, or phosphatase and tensin homolog (*PTEN*) function [2–5]. Considerable efforts have been directed to pharmacologic targeting of aberrant PI3K pathway activity [2–4, 6].

Activating ‘hotspot’ point mutations in *PIK3CA* are present in about 25% of invasive breast cancers, and are even further enriched in luminal-A cancers including invasive lobular carcinoma [1–5]. Accumulating data from a number of different studies shows that concurrent invasive and in-situ breast carcinoma (ductal carcinoma in-situ, DCIS) in the same patient frequently harbor identical *PIK3CA* mutations [7–11]. This observation fits the prevailing notion of DCIS as direct precursor of invasive breast cancer, and furthers the concept of activating *PIK3CA* mutations as a potential ‘driver’ of breast carcinogenesis. Further, we and others have recently shown that *PIK3CA* mutations are present in both ‘benign’ and ‘atypical’ proliferative breast lesions [11–13]. However, the *PIK3CA* mutation status of these proliferative breast lesions was frequently different than that of concurrent invasive carcinoma sampled from the same tissue specimen. For example, ten patients with *PIK3CA*-wild type (WT) carcinoma each had *PIK3CA* exon-20 mutated proliferative lesions present in the same breast tissue specimen [11]. Activating *PIK3CA* mutations have been shown to evoke downstream PI3K network signaling that fuels cellular transformation and cancer growth *in vitro*, but their *in vivo* activity within lesional tissue is more difficult to establish [14, 15], in light of intra-network inhibitory feedback loops or inter-network cross-talk [2–4, 16, 17].

Given the high prevalence of mutations and potential impact of *PIK3CA* mutation on downstream signaling in breast epithelial proliferation/neoplasia, we sought to capture the spatial nature of PI3K network signaling and its relationship with *PIK3CA* mutations in proliferative breast lesions. To do this, we used MTIP, a new multiplexed hyperspectral tissue imaging platform [18] to identify and map the location of phosphorylated PI3K network signaling nodes (pAKT1 (308, 473), pPRAS40, pS6, and pERK) and identify distinct PI3K network signatures. We examined these PI3K/MAPK network signatures in relation to *PIK3CA* genotype, histologic lesion type, and other spatial features.

Methods

Histologic specimens and genotyping

Tissue specimens of known genotype were derived from our previous study of breast precursor lesions, with an IRB approved protocol [11]. Briefly, these specimens were fixed in formalin, processed, and paraffin-embedded (FFPE), using standard clinical practice methods. Breast lesions of interest on H&E stained glass slides were identified by a breast pathologist (MLT), and 1–2 mm cores were taken from the corresponding tissue block. Cored tissue was re-embedded, the histologic diagnosis confirmed, and paraffin shavings were used for DNA extraction. DNA was screened for a large panel of hotspot mutations as previously described, including 58 substitutions in 34 codons of the *PIK3CA* gene; point mutations were confirmed by sequencing [11]. From this cohort, 29 lesions from 10 patients were selected for image analysis, based on availability of multiple lesions (up to 4) with different *PIK3CA* mutations from the same patient (Table 1).

Multiplexed phosphoprotein staining and imaging

FFPE lesions specimens were cut as 4- μ m thick unstained serial sections from the same tissue block previously used for genotyping. One level was stained by hematoxylin and eosin (H&E); another was used for multiplexed imaging (within 24–48 microns). Lesion sections from each patient were arranged on a single slide. Immunostaining was performed on FFPE specimens using a Benchmark XT autostainer (Ventana Medical Systems, VSMI, Tucson AZ), as reported earlier [18]. All FFPE specimens were subjected to automated deparaffinization in EZprep buffer (VMSI Cat# 950-102) followed by cell conditioning with the CC1 buffer (VMSI Cat#950-124) for 90 min prior to antibody incubation. FFPE specimens were incubated with a cocktail of hapten-conjugated primary antibodies (pS6:BF, pAKT S473:DIG, pPRAS40:NP, pERK 1/2:NCA, pAKT T308:DNP at 5, 15, 20, 30, 12.5 μ g/mL) at 37°C for 32 minutes. Following the primary antibody incubation step, samples were incubated with secondary anti-hapten antibody quantum dot (QD) conjugates (anti-BF:QD525, anti-DIG:QD565, anti-NP:QD6051, anti-NCA:QD625, anti-DNP:QD655 at 40, 50, 70, 40, and 30 nM concentrations respectively) at 37°C for 32 minutes [18]. All primary antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Anti-hapten specific secondary mouse monoclonal antibodies were conjugated to quantum dots (Life Technologies, Eugene, OR), using standard bioconjugation methods [19]. Following automated staining with antibodies, samples were dehydrated in ethanol series, followed by xylene, and mounted using Cytoseal 60 (Thermo Scientific) in preparation for spectral imaging.

Image acquisition and analysis

We mapped PI3K/MAPK network signaling in breast lesions using MTIP, a multiplexed, spectral imaging platform developed for precise, quantitative spatial mapping of phosphosignaling in FFPE tissue [18]. Regions of interest were selected within pathologist-identified breast lesions and spectrally acquired and unmixed. The signaling activity for each phosphoprotein species was quantified from the pixel intensity of unmixed channels. Pixel values that were 1 percent of the brightest signal in the field of view were excluded to remove non-relevant pixel contributions from unstained areas, and pixels above 95 percent

of the maximum intensity were clamped at the 95 percent intensity level to remove spurious contributions from hot pixels. Identification of signaling network signatures was performed using mean-shift algorithms to extract segmented groups of similar signaling patterns; these algorithms are tolerant of irregularly shaped distributions and functions without *a priori* assumptions of a fixed number of distinct phosphosignaling signatures or discrete cut-off points for establishing phosphosignaling signature uniqueness. Standard, non-parametric hierarchical analysis was employed to rank similarities by Euclidean distances among predominant PI3K/MAPK network signatures (top 1–2 signatures occupying the greatest area of tissue) and displayed in dendrogram format (Matlab).

Results

We analyzed twenty nine breast lesions of known *PIK3CA* mutation status from ten patients (Table 1). This cohort focused on patients with proliferative lesions (usual ductal hyperplasia-UDH, columnar cell lesion-CCL) and invasive or in-situ carcinomas of different *PIK3CA* genotypes. The study group included CCL/UDH, n=12; atypical ductal hyperplasia, n=1; carcinoma in situ, n=4 (3 DCIS, 1 lobular, LCIS); invasive carcinoma, n=11 (9 ductal, 2 lobular); and metastatic lobular carcinoma in one lymph node. Multiplexed phosphoantibody staining and spectral imaging of phosphoactivated protein species (pAKT473, pAKT308, pPRAS40, pS6, and pERK) was performed on the cohort (see Methods). Staining and imaging was successful in 26 of 29 specimens. The phosphosignaling intensity of each antibody was quantified within regions of interest and PI3K/MAPK network signatures were identified as distinct patterns of reactivity across all 5 phosphoprotein signaling species (Figure 1). Pseudocolored maps were generated to show the location of distinct phosphoprotein signatures in lesion tissue (Figure 1).

Epithelial Heterogeneity

Multiplexed imaging and identification of PI3K/MAPK phosphoprotein network signatures within breast tissue showed that stromal areas and epithelium differed; epithelia showed higher levels of phosphoactivation in all 5 phosphoprotein species compared to surrounding stromal areas (e.g. cluster 1 plots and white-coded clusters, Figure 1A, 1B). Interestingly, multiplexed imaging also revealed considerable diversity of PI3K pathway signatures within breast epithelium (each signature characterized by unique patterns of activation of each of 5 phosphoprotein species). As many as 12 distinct network signatures were identified within the small areas of lesional epithelium, while most samples had 3–4 or more distinct signatures spatially mapped (example of CCL and UDH, Figure 1A, 1B). These images also show varied spatial patterns of signaling and heterogeneous grouping of subpopulations of cells with distinct signaling (e.g. clusters 3, 5, 12, Figure 1A and clusters 3 and 4, Figure 1B).

PI3K/MAPK network signatures at the epithelial-stromal interface

Inspection of phosphoprotein signaling maps revealed a distinct PI3K/MAPK network signature at the epithelial-stromal interface in 5 non-invasive lesions from different patients (4 columnar cell lesions, some accompanied by usual or atypical hyperplasia; and one usual ductal hyperplasia.) Review of serial sections stained with H&E showed prominence of the

myoepithelial cell layer in the 4 columnar cell lesions (arrows in H and E, green cluster in pseudocolor map, and cluster 3 in plot, Figure 1B). The network signature at this epithelial-stromal cellular interface was characterized by moderate levels of pERK and pAKT T308. The adjacent lesional epithelium had relatively higher levels of pAKT T308 compared to the interface signature, yet had moderate levels of pERK (Figure 1B, Figure 2).

Clustering of PI3K/MAPK network signatures analyzed by PIK3CA genotype and lesion type

We investigated similarities between PI3K/MAPK network signatures and their relationship to genotype and histologic type across all imaged breast lesions by computing Euclidean distances. Dendrograms showing similarities and distances, and heatmaps showing relative intensity of each phosphoprotein were constructed using the dominant network signature for each lesion (signature occupying the largest area of each lesion –1 or rarely 2 network signatures each; Figure 2). The analysis revealed that lesions of the same *PIK3CA* genotype from different patients did not cluster together, indicating that *PIK3CA* genotype did not correlate in a simple manner with specific PI3K pathway signatures. For example, lesions with the *PIK3CA* exon 20 H1047R mutation, coded in yellow in the rightmost column of Figure 2, were spaced throughout the dendrogram tree. Furthermore, lesions of the same histology did not cluster together. For example, the bottom portion of the dendrogram contains a branch that contains IDC, DCIS, and columnar cell lesions (some mixed with UDH and ADH) from patients 21R, 44 and 4, respectively. We did, however, find similarity in PI3K/MAPK signatures at the epithelial-stroma interface in 5 CCL lesions across different patients with different genotypes: PIK3CA E545K and H1047R (epithelial-stromal interface signatures are denoted by an asterisk, as described above and shown in Figure 1B and Figure 2). In some instances PI3K/MAPK network signatures from several lesions from the same patient showed some similarity, even if the lesions were of different genotype and histologic type. For example, patient 21R shows network signatures in normal and lesional epithelium grouped in the upper portion of the dendrogram (color olive green in patient # column, Figure 2). In summary, breast lesions contain a diverse set of unique PI3K/MAPK phosphosignatures irrespective of *PIK3CA* genotype and histologic type.

Discussion

We have characterized PI3K/MAPK network activation in a cohort of premalignant and malignant breast lesions of known *PIK3CA* genotype. The data show that even small breast lesions contain a wide diversity of unique PI3K network signatures. Moreover these networks are distributed heterogeneously through the epithelium. Lesion genotype is not directly related to a predominant PI3K/MAPK network signature. However, we identified a unique phosphoprotein signature at the epithelial-stromal interface that appears to be common across some proliferative CCL breast lesions with morphologically prominent myoepithelial cells.

Genomic heterogeneity within malignant tumors has gained considerable attention recently, and is postulated to account for some degree of therapy resistance [4, 10]. Classic studies demonstrated genomic differences between metastases in renal cell carcinoma [20], and a

growing body of data is revealing genetic and protein expression heterogeneity within breast primary carcinomas, and between primary and metastatic carcinoma, including *PIK3CA* genotype [4, 10, 21–25]. We previously demonstrated spatial heterogeneity of PI3K phosphoprotein signatures in invasive breast cancers. In the present study, we show that microscopic foci of proliferative and precursor breast lesions also contain a diversity of unique PI3K signaling signatures, with heterogeneous spatial localization across the tissue. Mathematical algorithms delineated up to 12 unique PI3K pathway phosphoprotein signatures within microscopic fields of breast epithelial proliferations; most lesions contained 3–4 signatures. While UDH is morphologically heterogeneous lesion as compared to in-situ breast carcinoma, [26, 27], our multiplexed spatial mapping showed phosphosignaling heterogeneity within small UDH, DCIS and other breast lesions. Our results show not only the range of activated phosphoprotein species in each lesion, but also the spatial relationships of these multiple downstream phosphorylated (activated) species within the same signaling cascade. Phosphofunctional signaling pathways in proliferative breast lesions have not been previously mapped, to our knowledge.

Our study group included breast lesions of different *PIK3CA* genotype in order to investigate the correlation between *PIK3CA* mutations and PI3K phosphoprotein signaling. Our analysis of phosphoprotein signatures in 26 breast lesions demonstrated that lesions of the same *PIK3CA* genotype did not cluster together (Figure 2). For this study, genotype was established by sequencing DNA extracted from serial sections of lesional tissue, which does not preserve spatial information. Application of multiplex PI3K pathway imaging, together with *PIK3CA* mutation specific antibodies would allow for more precise mapping of genotype-phenotype associations (or lack thereof), as genotype may also theoretically be heterogeneous in proliferative lesions [28, 29]. Unfortunately, such *PIK3CA* antibodies are not currently available, and we were unable to correlate with *PIK3R1*, *PTEN* genotype, or other pathway elements that could also influence signaling. Further, we did not observe clustering by histologic type of lesion (proliferative vs. malignant for instance). Instead, different lesions from the same patient tended to cluster nearby. This observation could derive from differences in hormonal status or other systemic or field effects between patients. As shown in Table 1, patient age, and thus pre-menopausal/post-menopausal status, was highly variable across the study group. All of the lesions from an individual patient were combined on a single slide for staining and imaging (lesions from patients 23 & 26 were combined on a slide, likewise 29 & 41). Based on reproducibility data from our laboratory [18], it is unlikely that variability in staining would account for the observed clustering within patients. We cannot exclude the possibility, however, that pre-analytic factors played a role. This retrospective study included historical specimens which preceded the recording or controlling of ischemic time in our laboratory; specimen fixation times are listed in Table 1, and are generally within ASCO/CAP guidelines [30]. Despite this caveat, the specimens maintained a robust spectrum of phosphoprotein signatures.

The observed patterns of phosphoprotein activation were spatially complex, and seemingly ‘randomly’ distributed within breast epithelial lesions. Nevertheless, there was one recurring PI3K signature, which was seen in 5 proliferative, non-invasive breast lesions at the epithelial-stromal interface (Figure 1b, Figure 2). This particular signature was characterized by moderate levels of pERK and pAKT T308, with pAKT T308 lower than the adjacent

epithelium. Interestingly, unsupervised clustering placed the epithelial-stromal interface signature from different patients fairly closely together (Figure 2). We also noted that myoepithelial cells were prominently seen between the epithelium and basement membrane/stroma in lesions with an interface signature, which could potentially represent a myoepithelial signature. The role of epithelial-stromal interactions and epithelial-myoeplithelial-stromal interactions in cancer progression is increasingly recognized, and this interface phosphoprotein signature offers an avenue for further investigation. Analysis of additional lesions and multiplex staining incorporating myoepithelial cell markers (such as p63, calponin, smooth muscle myosin heavy chain) would be a next step to investigate this hypothesis [31, 32].

In conclusion, we demonstrate a heterogeneous and complex PI3K phosphoprotein signature in premalignant and malignant breast lesions, without clear *PIK3CA* genotypic correlation. These findings support the notion that protein expression and pathway activation should be tested in addition to genotype, in designing precision medicine treatments for breast cancer [4]. We have illustrated the importance of spatial mapping and the potential of quantitative multiplexed image techniques to enrich the study of breast neoplasia in subsequent larger studies.

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Figure 1. Breast lesions show heterogeneous spatial distribution of many distinct PI3K/MAPK pathway signatures

A) Representative UDH shows a diversity of PI3K signaling signatures (Patient 29, lesion #421). Representative H&E images are shown. Color-codes represent individual PI3K pathway signatures generated by applying a mean shift clustering of the signal intensities for each phosphomarker. Plots showing the intensity distribution for each of the 5 PI3K/MAPK phosphoproteins of each PI3K/MAPK network signature (7 signatures illustrated of 12). Percentages in each plot denote the percentage of tissue area that is associated with each unique network signature (cluster). Scale bar = 50 μ m; ROI is 208 μ m $\times$ 165 μ m, at 0.325 μ m/pixel resolution. **B)** Representative UDH showing distinct PI3K pathway signaling cluster associated with the epithelial/stromal interface (Patient 23, lesion #321). Arrows in B point to nuclei of prominent myoepithelial cells with clear cytoplasm at the epithelial-stromal interface.

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Figure 2. Heatmap and hierarchical ordering of the major PI3K/MAPK network signatures in breast lesions

Unique network signatures are denoted by a network id# and plotted in dendrogram with associated heatmap showing contributions of each phosphoprotein species. Dark to bright color tones in the heatmap depict lower to higher levels of activity for each phosphoprotein species. Asterisks denote PI3K/MAPK network signatures identified at the epithelial-stroma interface. E545K and E542K mutations are in *PIK3CA* exon 9, and H1047R mutation in exon 20. Patient11-lesion 214 *PIK3CA* genotype is H1047R along with *FGFR2* N550K; patient 46L-lesion 164 *PIK3CA* genotype is E542K, Q546E (exon 9). “None” indicates no *PIK3CA* mutations amongst those *PIK3CA* mutations covered by screening panel. ADH-Atypical ductal hyperplasia CCL- Columnar cell lesion; DCIS-ductal carcinoma in situ; FEA-flat epithelial atypia IDC-invasive ductal carcinoma; LCIS-lobular carcinoma in situ; UDH-usual ductal hyperplasia; normal-normal breast tissue.

Table 1

Patients, breast lesions, and *PIK3CA* mutation status

Patient #	Age	Formalin fixation (hours)	Lesion #	Diagnosis	<i>PIK3CA</i> Genotype
4	35	36	376	Columnar cell lesion	E545K (exon 9)
			377	Columnar cell lesion	E545K (exon 9)
			378	ADH & Columnar cell lesion	E545K (exon 9)
			379	Invasive ductal carcinoma scant	E545K (exon 9)
11	64	48	213	Invasive lobular carcinoma	H1047R (exon 20)
			214	Lymph node with metastatic lobular carcinoma	H1047R (exon 20) & <i>FGFR2</i> N550K
			215	Columnar cell lesion	E545K (exon 9)
			216	UDH & Columnar cell lesion	H1047R (exon 20)
21R	48	28	307	Invasive ductal carcinoma	None
			308	Columnar cell lesion	None
			309	Invasive ductal carcinoma	None
			310##	Columnar cell lesion	H1047R (exon 20)
23	47	>72	321	Columnar cell lesion & UDH	H1047R (exon 20)
			324	Invasive ductal carcinoma	None
26	42	48	383#	UDH & Columnar cell lesion	H1047R (exon 20)
			384	Invasive ductal carcinoma	None
29	31	32	421	Columnar cell lesion & UDH	H1047R (exon 20)
			422	DCIS	None
41	42	27	176	Invasive ductal carcinoma	E545K (exon 9)
			177	LCIS & Columnar cell lesion/FEA	None
42	37	48	349	Columnar cell lesion	None
			351	DCIS	E545K (exon 9)
			352##	Invasive ductal carcinoma	E545K (exon 9)
44	53	28.5	225	Columnar cell lesion & UDH	None
			226	DCIS	E545K (exon 9)
			227	Invasive ductal carcinoma	E545K (exon 9)

Patient #	Age	Formalin fixation (hours)	Lesion #	Diagnosis	PIK3CA Genotype
46L	58	48	161	UDH scant	None
			162	Invasive ductal carcinoma	None
			164	Invasive lobular carcinoma	E542K, Q546E (exon 9)

383 unsuccessful imaging, low signal

310 and 352 unsuccessful imaging, analysis yielded one signature with low signal, recorded as artifact

All lesions from a single patient arranged on same slide. Lesions from patients 23 & 26 on same slides; lesions from patients 29 & 41 on same slide

ADH-atypical ductal hyperplasia

DCIS-ductal carcinoma in situ

FEA-flat epithelial atypia

LCIS-lobular carcinoma in situ

UDH-usual ductal hyperplasia

None-no mutations detected with screening panel