Human Cancer Biology

PI3K Pathway Activation in High-Grade Ductal Carcinoma In Situ—Implications for Progression to Invasive Breast Carcinoma

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Abstract

Purpose: To assess the prevalence of phosphoinositide 3-kinase (PI3K) pathway alterations in pure high-grade ductal carcinoma in situ (DCIS) and DCIS associated with invasive breast cancer (IBC), and to determine whether DCIS and adjacent IBCs harbor distinct PI3K pathway aberrations.

Experimental Design: Eighty-nine cases of pure high-grade DCIS and 119 cases of high-grade DCIS associated with IBC were characterized according to estrogen receptor (ER) and HER2 status, subjected to immunohistochemical analysis of PTEN, INPP4B, phosphorylated (p)AKT and pS6 expression, and to microdissection followed by Sequenom genotyping of PIK3CA and AKT1 hotspot mutations.

Results: Alterations affecting the PI3K pathway were found in a subset of pure DCIS and DCIS adjacent to IBC. A subtype-matched comparison of pure DCIS and DCIS adjacent to IBC revealed that PIK3CA hotspot mutations and pAKT expression were significantly more prevalent in ER-positive/HER2-negative DCIS adjacent to IBC (P-values, 0.005 and 0.043, respectively), and that in ER-negative/HER2-positive cases INPP4B loss of expression was more frequently observed in pure DCIS (a P value of 0.013). No differences in the parameters analyzed were observed in a pairwise comparison of the in situ and invasive components of cases of DCIS and adjacent IBC. Analysis of the PIK3CA-mutant allelic frequencies in DCIS and synchronous IBC revealed cases in which PIK3CA mutations were either restricted to the DCIS or to the invasive components.

Conclusion: Molecular aberrations affecting the PI3K pathway may play a role in the progression from high-grade DCIS to IBC in a subset of cases (e.g., a subgroup of ER-positive/HER2-negative lesions). Clin Cancer Res; 20(9); 2326–37. ©2014 AACR.

Introduction

Ductal carcinoma in situ (DCIS) is a neoplastic proliferation of epithelial cells of the breast, which is separated from the breast stroma by the presence of an intact basement membrane and a discontinuous layer of myoepithelial cells (1–3). Widespread mammographic screening has led to an increase in the detection of DCIS, which now accounts for approximately 30% of new screen-detected breast cancers (4). Although DCIS has been shown to constitute a non-obligate precursor of invasive breast cancer (IBC; refs. 5–9), with up to 40% of these lesions progressing to invasive disease if untreated, identifying which cases will either recur as in situ disease or progress to IBC has proven challenging. Clinically useful predictors of progression from in situ to invasive disease have yet to be developed or introduced in clinical practice (2, 10, 11). In addition, the molecular mechanisms that underpin the progression from DCIS to IBC have yet to be defined (2, 3).

Previous studies based on immunohistochemistry (IHC), in situ hybridization, array-based comparative genomic hybridization (aCGH), and microarray-based gene expression profiling have demonstrated that DCIS and IBCs are remarkably similar at the molecular level (12–22). It should be noted, however, that most of these studies have not focused on matched DCIS and IBC from the same patient. In those that have focused on synchronous DCIS and IBC, amplification of MYC (19) and FGFR1 (22) has been reported to be more frequent in the invasive component (3). Furthermore, recent studies have demonstrated that intra-tumor genetic heterogeneity is a common phenomenon from the early stages of breast cancer development (23, 24), suggesting that the progression from DCIS to IBC may follow Darwinian evolutionary rules (3, 23, 24). Hence, one...
Translational Relevance

Ductal carcinoma in situ (DCIS) is considered a non-obligate precursor of invasive breast cancer (IBC). The introduction of mammography screening has resulted in a dramatic increase in the incidence of DCIS. The majority of women with DCIS will not develop IBC, yet we currently lack effective tools to predict which lesions are most likely to progress and, as such, current treatment recommendations are based on the notion that every DCIS has the potential to progress to IBC over time. Germaine to the development of biomarkers to differentiate between DCIS that will or will not progress to IBC is the characterization of the mechanisms that drive progression. Although the phosphoinositide 3-kinase pathway plays a pivotal role in breast cancer, here we demonstrate that alterations in key components of this pathway may play a role in the progression from high-grade DCIS to IBC only in a subset of cases.

could posit that this biologic phenomenon may constitute an evolutionary bottleneck, with the selection of non-modal populations of cancer cells harboring specific genetic aberrations in the progression from DCIS to invasive disease.

It is currently accepted that breast cancer comprises multiple entities with distinct risk factors, clinical presentation, histologic features, response to therapy, and outcomes (25). In fact, the transcriptomic differences between estrogen receptor (ER)-positive and ER-negative breast cancers are such that they are likely to constitute completely different diseases that originate in the same microanatomical structure and affect the same anatomical site (25). Despite the molecular differences between ER-positive and ER-negative disease, some molecular pathways seem to be frequently targeted by genetic aberrations in both ER-positive and ER-negative breast cancers (26). For example, the PI3K (phosphoinositide 3-kinase) pathway, which plays pivotal roles in cell survival, proliferation, and migration (27) is altered not only in the majority of ER-positive breast cancers, but also in a large subset of ER-negative breast cancers (26, 28).

Activating mutations in PIK3CA, encoding the PI3K catalytic subunit p110α, and loss of function of the negative regulator of PI3K signaling, PTEN, have been reported in up to 35% and 13% of IBCs, respectively, and vary according to the subtype of the disease as defined by molecular subtyping, and ER and HER2 status (26, 28–30). Loss of protein expression of the putative tumor suppressor INPP4B, which inhibits PI3K signaling, has been found in 7% to 11% of ER-positive and 44% to 58% of ER-negative IBCs, in particular in basallike breast cancer (31, 32). In addition, somatic mutations of AKT1 have been reported in 1% to 8% of IBCs; however, their effect on the PI3K pathway is not yet entirely understood (26, 28, 33). PIK3CA mutations have been reported in approximately 50% of DCIS (23, 34–37), and qualitative comparisons between DCIS and IBC have demonstrated that if a PIK3CA mutation is present in the DCIS, it would also be present in the invasive component in the vast majority of cases (35, 37); however, discordances have also been recorded (36). In a pilot study using semiquantitative methods to infer the percentage of cancer cells harboring specific mutations, we have recently documented the presence of PIK3CA mutations in the modal population of samples of DCIS, which were either present in a non-modal subset of the neoplastic cells of the invasive component or entirely absent in the invasive lesion, providing another line of evidence to support the contention that progression from DCIS to IBCs may result in the selection of genetically distinct clones (3, 23).

Given the non-obligate precursor nature of DCIS, questions that are germane to our ability to develop predictors of progression include whether DCIS that does not progress to invasive cancer harbors distinct molecular aberrations as compared with those that do, and how similar synchronous DCIS and IBCs are at the molecular level. Therefore, defining these molecular differences may offer valuable insights into the mechanisms that result in the establishment of invasive disease. Given the pivotal roles played by the PI3K pathway in both ER-positive and ER-negative breast cancers, here we sought to define the prevalence of PI3K pathway alterations in a matched cohort of high-grade DCIS that did or did not progress to IBC, and to define the differences in the frequency of molecular alterations of this pathway in samples of synchronous DCIS and IBC.

Materials and Methods

Patient and tissue samples

Following approval from the Institutional Review Board, the breast surgical database at Memorial Sloan Kettering Cancer Center (MSKCC; New York, NY) was queried for patients who underwent definitive surgical treatment for either pure DCIS or DCIS with associated IBC from 1999 to 2003. To maximize our ability to obtain adequate material for analysis, we restricted our query to those cases with pure DCIS (i.e., cases in which the most advanced lesion found in the surgical specimen was a DCIS and that did not develop IBC in the ipsilateral breast within 5 years of follow-up) or DCIS and synchronous invasive disease in at least two available archival formalin-fixed paraffin-embedded (FFPE) blocks, and to cases in which the invasive tumor component was at least 1.0 cm in size. In addition, to minimize the impact of known confounding factors, such as histologic grade, we restricted the study to high-grade DCIS. Consecutive cases meeting these criteria were selected. Ultimately, 89 cases of pure high-grade DCIS and 119 cases of DCIS with synchronous adjacent IBC (in the same FFPE block) were available for analysis (Supplementary Table S1). All cases were reviewed by two pathologists (DG and VPA) to confirm the reported histologic features and grade. Histologic grade for in situ lesions was assessed on the basis of nuclear pleomorphism and necrosis. High-grade DCIS corresponded to groups 2 and 3 of the Van Nuys classification for DCIS (38). Invasive lesions were graded on the basis of nuclear pleomorphism using the Black nuclear grading method as modified by Fisher and colleagues (39). Given
that ER-positive and ER-negative breast cancers have been shown to have distinct repertoires of molecular aberrations (25, 26, 28), the comparisons between pure DCIS and DCIS adjacent to IBC were only performed within groups stratified according to ER and HER2 status (i.e., ER-positive/HER2-negative, ER-positive/HER2-positive, ER-negative/HER2-positive, and ER-negative/HER2-negative).

**Immunohistochemistry**

IHC for ER, progesterone receptor (PR), HER2, PTEN, INPP4B, phosphorilated (p)AKT(Ser473), and pS6 (Ser240/244) was performed on representative 5-μm-thick FFPE sections containing either pure DCIS, or DCIS and adjacent IBCs, as described previously (31, 32, 40, 41). In brief, monoclonal antibodies against ER (clone 1D5; Dako), PR (clone PgR636; Dako), HER2 (HercepTest; Dako), and PTEN (clone 6H12.1; Dako) were diluted 1:100, against pAKT (Ser473; clone D9E; Cell Signaling Technology) 1:40, against INPP4B (clone EPR3108; Abcam), and against pS6 (Ser240/244; clone D68F8; Cell Signaling Technology) 1:1,000. ER, PR, PTEN, HER2, INPP4B, and pS6 staining was performed using the Dako Autostainer Plus. pAKT staining was performed manually using an overnight primary antibody incubation at 4°C, and immunodetection with an avidin–biotin–peroxidase complex (Vectastain; Vector). All sections were counterstained with hematoxylin and reviewed by four observers (VPA, FCM, C14C, and CTD-3211L18, Red-dUTP labeled) and CEP17 (plasmid clone p17H8, Green-dUTP labeled; Abbott Molecular). Following the ASCO/CAP guidelines (42), cases were considered amplified if the HER2/CEP17 ratio was greater than 2.2, equivocal between 2.2 and 1.8, and not amplified if the ratio was less than 1.8.

**Microdissection and DNA extraction**

Eight representative 10-μm-thick sections were cut from each case and manually microdissected with a sterile scalpel under a stereomicroscope to ensure a tumor cell content of >70%. DNA of DCIS and IBC components was extracted separately using the QuickGene DNA Tissue Kit (FujiFilm) as previously described (41). DNA quantification was performed with Quant-IT Picogreen (Invitrogen; Life Technologies).

**Mutation detection**

DNA samples extracted from microdissected pure DCIS and from each component of the cases with adjacent DCIS and IBC were subjected to Sequenom MassARRAY (Sequenom) analysis to detect PIK3CA hotspot (H1047R, E542K, E545K or N345K) and AKT1 (E17K) mutations, as previously described (41, 45). The multiplexed assays were designed using the Assay Design 3.1 Sequenom software. In brief, pre-PCR amplification (15 ng gDNA) using the same primers as for Sequenom was performed before the iPLEX Gold genotyping assay, and 7 nL of the purified primer extension reaction was loaded on a matrix pad of a SpectroCHIP (Sequenom) for analysis and measured by laser desorption/ionization of time-of-flight mass spectrometry. The prevalence of mutant alleles was estimated by calculating the ratio of the area of the raw spectra of the mutant allele to its wild-type, as previously described (23).

**Statistical analysis**

The association between PI3K pathway aberrations, type (DCIS vs. IBC), and ER and HER2 status was assessed using Fisher’s exact and χ² tests for categorical data, and the two-tailed Student t test for comparison of mean values. Of note, 95% confidence intervals were adopted, and P values <0.05 were considered significant. Statistical analyses were performed using SAS software, version 9.1 (SAS Institute, Cary, NC).

**Results**

The clinical and pathologic characteristics of the 89 patients with pure high-grade DCIS were similar to those of the 119 patients with DCIS adjacent to invasive cancer (Supplementary Table S1). In brief, of 89 cases of pure DCIS, 31 (35%) were ER-positive/HER2-negative, 15 (17%)

**HER2 fluorescence in situ hybridization**

HER2 amplification assessed by HercepTest was confirmed by fluorescence in situ hybridization (FISH) analysis using probes for HER2 (ERBB2, BAC clones RP11-94L15 and CTD-3211L18, Red-dUTP labeled) and CEP17 (plasmid clone p17H8, Green-dUTP labeled; Abbott Molecular). Following the ASCO/CAP guidelines (42), cases were considered amplified if the HER2/CEP17 ratio was greater than 2.2, equivocal between 2.2 and 1.8, and not amplified if the ratio was less than 1.8.
ER-positive/HER2-positive, 36 (40%) ER-negative/HER2-positive, and 7 (8%) ER-negative/HER2-negative, whereas of 119 cases of DCIS associated with invasive cancer, 33 (28%) were ER-positive/HER2-negative, 42 (35%) ER-positive/HER2-positive, 31 (26%) ER-negative/HER2-positive, and 13 (11%) ER-negative/HER2-negative. All DCIS and IBC cases included in this study had nuclear grade 3; the IBC and 13 (11%) ER-negative/HER2-negative. All DCIS and IBC cases were of histologic grade 3 (IBC cases included in this study had nuclear grade 3; the IBC and 13 (11%) ER-negative/HER2-negative.

In pure DCIS, significant differences in both the prevalence of PTEN and INPP4B loss of expression, PIK3CA and AKT1 hotspot mutations, and pAKT and pS6 expression. Consistent with previous observations (23, 34–37), alterations of the PI3K pathway were frequently found in both pure DCIS and DCIS adjacent to IBC (26, 28, 31). We posited that different subtypes of DCIS would differ in the prevalence of PTEN and INPP4B loss of expression, PIK3CA and AKT1 hotspot mutations, and pAKT and pS6 expression. Consistent with previous observations (23, 34–37), alterations of the PI3K pathway were frequently found in both pure DCIS and DCIS adjacent to IBC (Table 1; Fig. 1; Supplementary Table S2; Supplementary Fig. S1). In pure DCIS, significant differences in both the prevalence of PIK3CA hotspot mutations and loss of INPP4B expression were observed according to the subtypes, with 0%, 0%, 5%, and 28% of ER-positive/HER2-negative, ER-positive/HER2-positive, ER-negative/HER2-negative, and ER-negative/HER2-negative lesions harboring PIK3CA mutations, respectively (4 × 2 Fisher exact test, \( P = 0.0220 \); Table 1), and 5%, 0%, 30%, and 34% of ER-positive/HER2-negative, ER-positive/HER2-positive, ER-negative/HER2-positive, and ER-negative/HER2-negative lesions, showing loss of INPP4B expression, respectively (4 × 2 Fisher exact test, \( P = 0.0271 \); Table 1). Differences in the prevalence of pAKT expression were also observed in pure DCIS, with 42%, 67%, 75%, and 57% of ER-positive/HER2-negative, ER-positive/HER2-positive, ER-negative/HER2-positive, and ER-negative/HER2-negative lesions displaying pAKT expression, respectively (4 × 2 Fisher exact test, \( P = 0.0427 \); Table 1). Of note, the only PIK3CA mutation observed in pure DCIS was the oncogenic H1047R kinase domain mutation (Supplementary Table S3). No significant differences in the prevalence of PTEN loss of expression, AKT1 mutations or pS6 expression were observed.

In contrast, in high-grade DCIS adjacent to IBC, significant differences between subtypes, as defined by ER and HER2 status, were found in relation to PTEN and INPP4B loss of expression (4 × 2 Fisher exact test, \( P < 0.001 \) and \( P = 0.0336 \), respectively; Table 1; Supplementary Table S2). No other significant differences in the prevalence of pAKT and pS6 expression, and PIK3CA or AKT1 mutations were observed. In fact, AKT1 mutations, albeit previously reported in a subset of DCIS (4%) and adjacent IBC (35), were shown to be remarkably rare in our study (0.5% of all lesions analyzed), suggesting that mutations affecting this gene may not constitute an important driver of high-grade DCIS.

Consistent with previous studies, which have demonstrated that PIK3CA mutations and PTEN loss are generally mutually exclusive in IBCs (26, 28), here we show that alterations affecting these genes were largely mutually exclusive in all subtypes of both pure DCIS and DCIS adjacent to IBC. In fact, PTEN loss of expression and PIK3CA mutations were concurrently found only in one pure DCIS of ER-negative/HER2-negative phenotype, and in two DCIS adjacent to IBC, one ER-positive/HER2-negative, and another ER-negative/HER2-negative (Figs. 1 and 2). We also observed that INPP4B loss of expression was preferentially found in cases lacking PTEN loss of expression and/or PIK3CA hotspot mutations. In fact, concurrent INPP4B and PTEN loss of expression was found only in two pure DCIS and two DCIS adjacent to IBC, whereas concurrent INPP4B loss of expression and PIK3CA hotspot mutations were found only in one case of pure DCIS (Fig. 1).

Comparative analysis of the cases of pure DCIS and DCIS adjacent to IBC matched according to nuclear grade and subtype revealed remarkable similarities in the prevalence of PTEN and INPP4B loss of expression, presence of PIK3CA hotspot mutations, AKT1 mutations, and pAKT and pS6 expression (Table 1). In fact, significant differences were only observed in the group of ER-positive/HER2-negative lesions, in which significantly higher frequencies of PIK3CA hotspot mutations and pAKT expression were found in DCIS adjacent to IBC than in pure DCIS (Fisher’s exact test \( P \) values 0.005 and 0.043, respectively; Table 1), and in ER-negative/HER2-positive lesions, in which a significantly higher prevalence of INPP4B loss of expression was found in pure DCIS than in DCIS adjacent to IBC (30% vs. 4%, respectively; Fisher’s exact test \( P = 0.013 \); Table 1). No significant differences were found between pure DCIS and DCIS adjacent to IBC in ER-positive/HER2-positive and ER-negative/HER2-negative lesions.

These observations demonstrate that in a way akin to IBC, different subtypes of DCIS, as defined by ER and HER2 status, have different patterns of PI3K pathway alterations, and that this pathway is altered in similar ways in pure DCIS and DCIS adjacent to IBC. Although PIK3CA mutations and pAKT expression were more frequently found in DCIS adjacent to IBC than in pure ER-positive/HER2-negative DCIS, in ER-negative/HER2-positive lesions, a significantly higher frequency of loss of INPP4B expression was found in pure DCIS than in DCIS adjacent to IBC. Taken together, our findings suggest that alterations in the PI3K pathway may play a role in the progression from in situ to invasive disease in a subset of ER-positive/HER2-negative DCIS.

**PI3K pathway alterations differ in subtypes of pure DCIS and DCIS adjacent to IBC**

Previous studies have reported PI3K pathway alterations in IBC and DCIS (23, 26, 28, 34–37), and have demonstrated that the mechanisms resulting in PI3K pathway activation vary according to the subtype of IBC (26, 28, 31). We posited that different subtypes of DCIS would differ in the prevalence of PI3K pathway alterations affecting these genes were largely mutually exclusive in all subtypes of both pure DCIS and DCIS adjacent to IBC. In fact, PTEN loss of expression and PIK3CA mutations were concurrently found only in one pure DCIS of ER-negative/HER2-negative phenotype, and in two DCIS adjacent to IBC, one ER-positive/HER2-negative, and another ER-negative/HER2-negative (Figs. 1 and 2). We also observed that INPP4B loss of expression was preferentially found in cases lacking PTEN loss of expression and/or PIK3CA hotspot mutations. In fact, concurrent INPP4B and PTEN loss of expression was found only in two pure DCIS and two DCIS adjacent to IBC, whereas concurrent INPP4B loss of expression and PIK3CA hotspot mutations were found only in one case of pure DCIS (Fig. 1).

Comparative analysis of the cases of pure DCIS and DCIS adjacent to IBC matched according to nuclear grade and subtype revealed remarkable similarities in the prevalence of PTEN and INPP4B loss of expression, presence of PIK3CA hotspot mutations, AKT1 mutations, and pAKT and pS6 expression (Table 1). In fact, significant differences were only observed in the group of ER-positive/HER2-negative lesions, in which significantly higher frequencies of PIK3CA hotspot mutations and pAKT expression were found in DCIS adjacent to IBC than in pure DCIS (Fisher’s exact test \( P \) values 0.005 and 0.043, respectively; Table 1), and in ER-negative/HER2-positive lesions, in which a significantly higher prevalence of INPP4B loss of expression was found in pure DCIS than in DCIS adjacent to IBC (30% vs. 4%, respectively; Fisher’s exact test \( P = 0.013 \); Table 1). No significant differences were found between pure DCIS and DCIS adjacent to IBC in ER-positive/HER2-positive and ER-negative/HER2-negative lesions.

These observations demonstrate that in a way akin to IBC, different subtypes of DCIS, as defined by ER and HER2 status, have different patterns of PI3K pathway alterations, and that this pathway is altered in similar ways in pure DCIS and DCIS adjacent to IBC. Although PIK3CA mutations and pAKT expression were more frequently found in DCIS adjacent to IBC than in pure ER-positive/HER2-negative DCIS, in ER-negative/HER2-positive lesions, a significantly higher frequency of loss of INPP4B expression was found in pure DCIS than in DCIS adjacent to IBC. Taken together, our findings suggest that alterations in the PI3K pathway may play a role in the progression from in situ to invasive disease in a subset of ER-positive/HER2-negative DCIS.

**PI3K pathway alterations are largely maintained in the progression from DCIS to IBC**

Despite the qualitative similarities between synchronous DCIS and IBC, recent pairwise comparisons of these lesions have demonstrated that DCIS and IBC may differ by the presence of specific genetic aberrations (3, 23, 24).
sought to determine whether PTEN and INPP4B loss of expression, PIK3CA and AKT1 hotspot mutations, and pAKT and pS6 expression would differ between the in situ and invasive components of cases of synchronous DCIS and IBC. Although differences in PTEN loss of expression, presence of PIK3CA mutations, and pAKT and pS6
expression were observed between matched DCIS and IBC, these changes were not unidirectional (Fig. 1). For example, of the 8 cases with differences in PTEN loss of expression, 4 showed PTEN loss in the IBC component but not in the DCIS, whereas in the remaining 4 cases, PTEN was expressed in the invasive component but absent in the DCIS (Figs. 1 and 3A). Changes in INPP4B loss of expression, on the other hand, were unidirectional; in the progression from DCIS to IBC, 6 cases displayed loss of INPP4B expression, 5 of which were ER-negative lesions (Fig. 1). Consistent with previous observations (23, 34–37), no significant qualitative differences were observed between the DCIS and invasive components of the cases analyzed (Table 2; Supplementary Table S2). When stratified according to ER and HER2 status, again, the DCIS and invasive components of each case were remarkably similar in regard to PTEN and INPP4B loss of expression, presence of \(\text{PIK3CA}\) and \(\text{AKT1}\) mutations, and pAKT and pS6 expression (Table 2).

Changes in \(\text{PIK3CA}\) mutation status in the progression from DCIS to IBC

Although \(\text{PIK3CA}\) mutations have been reported at similar frequencies in DCIS and IBC (23, 34–37), recent studies have described changes in the \(\text{PIK3CA}\) status in the progression from in situ to invasive disease (23, 36). Here we have observed that in 3 cases the H1047R \(\text{PIK3CA}\) mutation was present in the invasive component but not detectable in the synchronous DCIS areas, and in 5 additional cases, the \(\text{PIK3CA}\) mutation was present in a non-modal population of the DCIS cells (\(\text{PIK3CA}\)-mutant allele frequencies ranging from 25% to 33.3%), but likely present in the modal population of the IBC (\(\text{PIK3CA}\)-mutant allele frequencies ranging from 46.2% to 52.8%; Table 3; Figs. 1 and 3C). In 2 additional cases, the H1047R \(\text{PIK3CA}\) mutation was restricted to the DCIS component but absent in the IBC (Figs. 1, 3B and C, Table 3).

Taken together, our results demonstrate the existence of intra-tumor genetic heterogeneity in DCIS and suggest that in the progression from DCIS to IBC, subclones of neoplastic cells harboring specific repertoires of genetic aberrations may be selected. Furthermore, our data support the contention that although \(\text{PIK3CA}\) mutations may play a role from the early stages of breast tumorigenesis, their role as driver of the progression from in situ to invasive disease is less clear, given that examples of both \(\text{PIK3CA}\) wild-type DCIS adjacent to \(\text{PIK3CA}\)-mutant IBC and of \(\text{PIK3CA}\) wild-type IBC adjacent to \(\text{PIK3CA}\)-mutant DCIS were observed.

**Discussion**

Here we demonstrate that both pure high-grade DCIS and high-grade DCIS adjacent to IBC often harbor molecular alterations that result in activation of the PI3K pathway, and that in a way akin to IBCs, different subtypes of DCIS, as defined by ER and HER2 status, display different patterns of alterations affecting genes in the PI3K canonical pathway. We have also confirmed previous observations demonstrating that synchronous DCIS and IBCs display remarkably similar patterns of alterations of
this pathway; however, we also provide direct evidence of the existence of intra-tumor genetic heterogeneity in DCIS and that the \textit{PIK3CA} mutation status may change in the progression from \textit{in situ} to invasive disease.

PI3K pathway alterations have previously been documented in breast cancer (23, 26, 28, 31, 32, 34–37). Here we not only confirmed that a subset of DCIS do harbor \textit{PIK3CA} mutations, but also provided an integrative analysis combining an assessment of the most common mechanisms of activation of this pathway, and an immunohistochemical assessment of the PI3K pathway activity using pAKT and pS6 as surrogates of activation of this pathway. In both pure DCIS and DCIS adjacent to IBC, we have observed that the presence of PTEN and INPP4B loss of expression and/or mutations in \textit{PIK3CA} or \textit{AKT1} varied significantly according to subtype (Table 1). Importantly, the observation that \textit{PIK3CA} hotspot mutations and pAKT expression were significantly more frequent in ER-positive/HER2-negative DCIS adjacent to IBC than in pure DCIS is consistent with the notion that PI3K pathway activation may impart increased risk of or association with invasive progression in this disease subtype. Additional studies to test this hypothesis are warranted.

Using a subtype-matched approach, we have observed a significantly higher prevalence of \textit{PIK3CA} mutations and pAKT activity in high-grade ER-positive/HER2-negative DCIS adjacent to IBC than in pure DCIS. In high-grade ER-negative/HER2-positive lesions, however, INPP4B loss of expression, an event that can potentially activate the PI3K pathway, was more frequent in pure DCIS than in high-grade DCIS adjacent to IBC. These observations demonstrate that loss of PTEN and INPP4B expression, and mutations affecting \textit{PIK3CA} and \textit{AKT1} are present in a subset of both pure high-grade DCIS and high-grade DCIS adjacent to IBC, providing additional evidence to support the role of this pathway in the early stages of breast cancer development.

In pure high-grade DCIS, \textit{PIK3CA} mutations were relatively infrequent and were not found in ER-positive lesions; on the other hand, 5% of ER-negative/HER2-positive and 28% of the ER-negative/HER2-negative high-grade DCIS harbored the H1047R \textit{PIK3CA} mutation. In high-grade

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**Figure 2.** \textit{PIK3CA} mutations and PTEN loss of expression may not be mutually exclusive in breast cancer. Representative micrograph of a pure DCIS concurrently harboring an H1047R \textit{PIK3CA} mutation and PTEN loss of expression as defined by IHC (case D094). Note that INPP4B and pAKT were expressed at moderate-to-high levels, whereas pS6 expression was absent. H&E, hematoxylin and eosin. Magnification of micrographs, ×200.
DCIS adjacent to IBC, however, *PIK3CA* mutations were present in all subtypes, ranging from 8% in ER-negative/HER2-negative lesions to 24% in ER-positive/HER2-positive disease. Out of all ER-positive DCIS analyzed in this study, only 11% harbored *PIK3CA* hotspot mutations. Albeit at first glance at variance with the notion that *PIK3CA* mutations are more frequently found in ER-positive IBCs, these seemingly unexpected findings can be reconciled by...
the fact that we have focused on high-grade ER-positive lesions, which have been reported to less frequently harbor PIK3CA mutations than low-grade ER-positive IBCs (24%–49%; refs. 26, 36, 46), low-grade pure DCIS (34%; ref. 36), and early precursors of low-grade forms of DCIS (54%; ref. 47). The high prevalence of PIK3CA mutations in non-obligate precursors of low-grade DCIS (e.g., columnar cell lesions) and low-grade DCIS, in conjunction with the low frequencies of PIK3CA mutations in high-grade ER-positive DCIS analyzed in this study are consistent with the notion that the molecular pathways involved in the development and progression of low- and high-grade DCIS are likely distinct (1), and that in high-grade DCIS, PIK3CA mutations may only play a role in a minority of cases.

Our analysis of PI3K pathway activation using pAKT and pS6 as surrogate markers suggested that activation of this pathway is more frequent than alterations of PTEN, INPP4B, PIK3CA, and AKT1 in pure high-grade DCIS and high-grade DCIS adjacent to IBC. In all subtypes, pAKT expression was more prevalent than pS6 expression in both pure high DCIS and DCIS adjacent to IBC (Table 1). Concurrent expression of pAKT and pS6 was observed in 13% and 38% of pure DCIS and DCIS adjacent to IBC, respectively. The vast majority of cases (69% of pure DCIS and 75% of DCIS adjacent to IBC) harboring PTEN or INPP4B loss of expression, or PIK3CA or AKT1 mutations, displayed pAKT expression, whereas pS6 expression was found in 6% and 21% of pure DCIS and DCIS adjacent to IBC harboring these molecular aberrations. The lack of pAKT and pS6 expression in cases with alterations in these genes may stem from the fact that we have (i) assessed pAKT and pS6 expression in surgical specimens and that previous analyses have shown that their immunohistochemical assessment is affected by preanalytic variables, and its expression levels are significantly lower in surgical specimens than in core biopsies (48, 49); and (ii) used antibodies that recognize only a few phosphorylation sites of AKT (i.e., Ser473) and S6 (i.e., Ser240/244). Although our study may have underestimated the prevalence of PI3K pathway activation in DCIS, our results do demonstrate that mechanisms other than PTEN or INPP4B loss of expression, or PIK3CA or AKT1 mutations may result in activation of this pathway in DCIS, and warrant further studies investigating the causes of PI3K pathway activation in these lesions.

Recent studies based on aCGH, FISH, and Sequenom analysis, or on multiprobe FISH analysis of synchronous DCIS and IBC, have demonstrated that from a qualitative standpoint, DCIS and IBC samples from a given patient have strikingly similar genomic profiles (23, 24). These studies, however, have revealed not only intra-lesion genetic heterogeneity but also differences in the prevalence of amplifications affecting specific loci and in the prevalence of mutations between the DCIS and invasive samples. In the study by Hernandez and colleagues (23), in 3 of 13 cases of synchronous DCIS and IBC harboring PIK3CA mutations, these mutations were either restricted to the DCIS component (n = 2) or the frequency of the PIK3CA-mutant allele...
was decreased in the IBC when compared with the DCIS component (23). Our results confirm and expand on previous observations, given that in 2 cases, PIK3CA mutations were present in the DCIS but absent in the IBC component, whereas in 3 cases, mutations affecting this gene were found in the invasive component but not in the DCIS. In addition, differences in the frequencies of the mutant allele varied from the DCIS to the invasive component in 5 cases. Taken together, these observations are consistent with a model in which DCIS is composed of a mosaic of tumor cells that, in addition to the founder genetic aberrations, harbor private mutations, and that clonal selection is likely to take place in the progression from in situ to invasive disease (3). These results provide another line of evidence to suggest that PIK3CA mutations may play a role in the progression from in situ to invasive disease in a small subset of cases.

Our study has several limitations. First, the retrospective identification of tissue specimens and the selection criteria that included samples with at least two FFPE blocks available may limit the generalizability of the results; however, we have assembled a large cohort of carefully analyzed cases and our findings should be considered as exploratory and hypothesis generating. Second, the sample size of some of the subtypes of DCIS investigated in this study (i.e., ER-negative/HER2-negative DCIS) is small; hence, we cannot rule out type II or β-errors in the comparative analyses performed in this subgroup. Third, given the evidence to suggest that low- and high-grade DCIS and IBCs are likely to evolve through distinct pathways (reviewed in ref. 1), we have focused only on the subset of high-grade lesions; hence, our conclusions should be considered relevant only to high-grade disease. Fourth, the surrogate markers used to determine activation of the PI3K pathway, pAKT and pS6 IHC, have been shown to be affected by preanalytic parameters, including delayed fixation and the type of specimen (48, 49). Although the frequency of PI3K pathway activation may have been underestimated in this study due to the use of surgical specimens, given that all tissues were collected during the same time frame at a single institution, the reduction in pAKT and pS6 expression driven by preanalytic parameters should be equally prevalent among all groups. Finally, we have only investigated a limited number of PIK3CA hotspot mutations; hence, we may not have captured all cases harboring activating mutations in this gene. It should be noted, however, that in IBC the PIK3CA mutations included in the Sequenom MassARRAY (i.e., H1047R, E542K, E545K or N345K) assay used here account for 87% of all mutant cases reported by The Cancer Genome Atlas (26).

In conclusion, here we demonstrate that PTEN and INPP4B loss of expression, PIK3CA hotspot mutations, and AKT1 mutations are found in a subset of pure high-grade DCIS and high-grade DCIS adjacent to IBC, and that the prevalence of alterations affecting these genes vary more according to the ER/HER2 subtype of DCIS than to its association with synchronous IBC. PTEN loss of expression was infrequent in subtypes other than high-grade ER-negative/HER2-negative DCIS, INPP4B loss of expression was preferentially found in ER-negative/HER2-positive and ER-

### Table 3. Cases with discordant PIK3CA-mutant frequencies in the high-grade DCIS and synchronous IBC components

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Component</th>
<th>ER</th>
<th>PR</th>
<th>HER2</th>
<th>PIK3CA mutation</th>
<th>Allele</th>
<th>WT frequency</th>
<th>Mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 I037</td>
<td>DCIS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>E545K</td>
<td>A</td>
<td>0.731</td>
<td>0.269</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>E545K</td>
<td>A</td>
<td>0.538</td>
<td>0.462</td>
</tr>
<tr>
<td>2 I071</td>
<td>DCIS</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>E545K</td>
<td>A</td>
<td>0.744</td>
<td>0.256</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>E545K</td>
<td>A</td>
<td>0.508</td>
<td>0.492</td>
</tr>
<tr>
<td>3 I038</td>
<td>DCIS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>H1047R</td>
<td>G</td>
<td>0.750</td>
<td>0.250</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>H1047R</td>
<td>G</td>
<td>0.525</td>
<td>0.475</td>
</tr>
<tr>
<td>4 I050</td>
<td>DCIS</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>H1047R</td>
<td>G</td>
<td>0.667</td>
<td>0.333</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>H1047R</td>
<td>G</td>
<td>0.472</td>
<td>0.528</td>
</tr>
<tr>
<td>5 I106</td>
<td>DCIS</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>H1047R</td>
<td>G</td>
<td>0.715</td>
<td>0.285</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>H1047R</td>
<td>G</td>
<td>0.530</td>
<td>0.470</td>
</tr>
<tr>
<td>6 I002</td>
<td>DCIS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>None</td>
<td></td>
<td>1.000</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>None</td>
<td></td>
<td>1.000</td>
<td>0</td>
</tr>
<tr>
<td>7 I044</td>
<td>DCIS</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>H1047R</td>
<td>G</td>
<td>0.549</td>
<td>0.451</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>H1047R</td>
<td>G</td>
<td>0.344</td>
<td>0.580</td>
</tr>
<tr>
<td>8 I072</td>
<td>DCIS</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>None</td>
<td></td>
<td>1.000</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>None</td>
<td></td>
<td>1.000</td>
<td>0</td>
</tr>
<tr>
<td>9 I068</td>
<td>DCIS</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>H1047R</td>
<td>G</td>
<td>0.692</td>
<td>0.308</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>None</td>
<td></td>
<td>1.000</td>
<td>0</td>
</tr>
<tr>
<td>10 I129</td>
<td>DCIS</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>H1047R</td>
<td>G</td>
<td>0.698</td>
<td>0.302</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>None</td>
<td></td>
<td>1.000</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviation: WT, wild-type.
negative/HER2-negative DCIS. PIK3CA mutations were relatively uncommon in all subtypes of high-grade in situ disease (0%–28%), and AKT1 mutations were only found in 0.5% of all lesions analyzed, yet activation of the PI3K pathway, as defined by pAKT and/or pS6 expression, was shown to be a more pervasive biologic phenomenon, possibly driven by genetic (e.g., HER2 gene amplification) or epigenetic alterations other than those surveyed in our study. Our findings also demonstrate the qualitative similarities in ER, PR, and HER2 status, and PI3K pathway alterations between DCIS and synchronous invasive IBCs, suggesting that the overall phenotype of a breast cancer is likely to be determined early in tumorigenesis. Intra-tumor genetic heterogeneity and selection of genetically distinct clones in the progression from in situ to invasive disease, however, were documented in a subset cases, and qualitative and quantitative differences in the presence and percentage of PIK3CA-mutant alleles between matched DCIS and IBC. Our findings provide additional evidence to demonstrate the importance of the PI3K pathway in breast cancer and that PI3K pathway aberrations may be associated with a higher risk of progression in a subset of lesions; however, its role in mediating the progression from in situ to invasive disease seems to be more limited.

Disclosure of Potential Conflicts of Interest

N. Rosen has received a commercial research grant from Bayer and is a consultant/advisory board member for Astra-Zeneca, Bayer, and Novartis. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: R. A. Sakr, T. A. King

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