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PIK3CA mutations in ductal carcinoma *in situ* and adjacent invasive breast cancer

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Abstract

PIK3CA is one of the most frequently mutated genes in invasive breast cancer (IBC). These mutations are generally associated with hyper-activation of the phosphatidylinositol 3-kinase signaling pathway, which involves increased phosphorylation of AKT (p-AKT). This pathway is negatively regulated by the tumor suppressor PTEN. Data are limited regarding the variant allele frequency (VAF) of PIK3CA, PTEN and p-AKT expression during various stages of breast carcinogenesis. Therefore, the aim of this study was to gain insight into PIK3CA VAF and associated PTEN and p-AKT expression during the progression from ductal carcinoma *in situ* (DCIS) to IBC. We isolated DNA from DCIS tissue, synchronous IBC and metastasis when present. These samples were pre-screened for PIK3CA hotspot mutations using the SNaPshot assay and, if positive, validated and quantified by digital PCR. PTEN and p-AKT expression was evaluated by immunohistochemistry using the Histo-score (H-score). Differences in PIK3CA VAF, PTEN and p-AKT H-scores between DCIS and IBC were analyzed. PIK3CA mutations were detected in 17 out of 73 DCIS samples, 16 out of 73 IBC samples and 3 out of 23 lymph node metastasis. We detected a significantly higher VAF of PIK3CA in the DCIS component compared to the adjacent IBC component ($P = 0.007$). The expression of PTEN was significantly higher in DCIS compared to the IBC component in cases with a wild-type (WT) PIK3CA status ($P = 0.007$), while it remained similar in both components when PIK3CA was mutated. There was no difference in p-AKT expression between DCIS and the IBC component. In conclusion, our data suggest that PIK3CA mutations could be essential specifically in early stages of breast carcinogenesis. In addition, these mutations do not co-occur with PTEN expression during DCIS progression to IBC in the majority of patients. These results may contribute to further unraveling the process of breast carcinogenesis, and this could aid in the development of patient-specific treatment.

Key Words

- ▶ PIK3CA
- ▶ mutation
- ▶ amplification
- ▶ PTEN
- ▶ ductal carcinoma *in situ*
- ▶ invasive breast cancer
- ▶ progression

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Introduction

Invasive breast cancer (IBC) is a heterogeneous disease, consisting of several molecular subtypes, all with a specific tumor biology and clinical outcome (Perou

et al. 2000). Each of these molecular subtypes, which are based on microarray-based gene expression studies, also have an immunohistochemical surrogate subtype based

on estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER2) and Ki-67 (Goldhirsch *et al.* 2013). The somatic mutation spectrum varies widely across these different subtypes (Perou *et al.* 2000). One of the most frequently described mutations in IBC are present in phosphatidylinositol-4,5-bisphosphate-3-kinase, catalytic subunit alpha (PIK3CA) and occur in 16–45% of all patients (Cancer Genome Atlas Network 2012, Ang *et al.* 2014, Pang *et al.* 2014, Abba *et al.* 2015, Mukohara 2015). The majority of these cases are E542K and E545K mutations, located in exon 9, and H1047R mutations, located in exon 20 of the gene (Vogt *et al.* 2007). These mutations are mainly observed in ER+ IBC subtypes, which generally have a good prognosis (Kalinsky *et al.* 2009, Cizkova *et al.* 2012, Pang *et al.* 2014, Zardavas *et al.* 2018). PIK3CA mutations are associated with hyper-activation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway, which involves increased phosphorylation of AKT (p-AKT) and results in increased cell growth and survival (Vivanco & Sawyers 2002, Chalhoub & Baker 2009). This pathway is negatively regulated by phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (Chalhoub & Baker 2009). Therefore, impaired PTEN function or loss of PTEN expression also results in uncontrolled activation of the PI3K signaling pathway. PTEN loss is generally associated with a worse disease-free and overall survival (Bose *et al.* 2002, Lee *et al.* 2004, Iqbal *et al.* 2012, Li *et al.* 2017). On the other hand, PIK3CA mutations are generally associated with longer recurrence-free survival in IBC patients, though associated with reduced therapy response in HER2+ IBC subtypes (Berns *et al.* 2007, Loi *et al.* 2010, Cizkova *et al.* 2012, Sueta *et al.* 2014). A reduced therapy response in HER2+ IBC subtypes was also shown in cases with an aberrant PTEN expression (Nagata *et al.* 2004, Gschwantler-Kaulich *et al.* 2017).

Approximately 50% of all IBCs are associated with a synchronous precursor lesion, of which ductal carcinoma *in situ* (DCIS) is the most common (Barnes *et al.* 2012, Bleyer & Welch 2012). PIK3CA mutations already arise in these pre-malignant lesions. They are reported in 30% of patients with pure DCIS (without an invasive component) and in 24–43% of all DCIS cases with an adjacent invasive component (Li *et al.* 2010, Miron *et al.* 2010, Troxell 2012, Ang *et al.* 2014). These reported PIK3CA mutation frequencies in DCIS are similar to those reported in IBC (Li *et al.* 2010, Miron *et al.* 2010). Furthermore, the type of PIK3CA mutation remains the same throughout breast cancer progression. Next to these concordances, several studies also reported a discordant PIK3CA mutation status

in synchronous DCIS and IBC (Miron *et al.* 2010, Dupont Jensen *et al.* 2011, Hernandez *et al.* 2012). However, data are limited with respect to the variant percentage of PIK3CA mutations during progression from DCIS to IBC.

Loss of PTEN expression has been described in 11–44% of cases with pure DCIS, in 35% of cases with synchronous DCIS and IBC and in 12–38% of cases with IBC (Bose *et al.* 2002, Song *et al.* 2010, Knudsen *et al.* 2012, Sakr *et al.* 2014, Wang *et al.* 2017). In synchronous DCIS and IBC, PTEN expression was similar in the DCIS and the invasive component (Sakr *et al.* 2014). Studies correlating PIK3CA and PTEN mutations suggest mutual exclusivity (Saal *et al.* 2005, cBioPortal 2015), although data regarding PTEN expression during breast cancer progression are limited.

Current literature regarding p-AKT expression is mainly focused on IBC. Expression of p-AKT has been reported in up to 75% of IBC cases (Esteva *et al.* 2010, Sakr *et al.* 2014, Sueta *et al.* 2014, Cossu-Rocca *et al.* 2015, Azim *et al.* 2016, Guarneri *et al.* 2019). PIK3CA mutations are associated with high p-AKT expression in triple negative and metastatic breast cancer (Dupont Jensen *et al.* 2011, Cossu-Rocca *et al.* 2015, Azim *et al.* 2016). Expression of p-AKT has also been described in 42% of cases with pure DCIS and in 70% of cases with synchronous DCIS and IBC (Sakr *et al.* 2014). In matched DCIS and IBC, the expression was similar (Sakr *et al.* 2014). The effect of PIK3CA mutations on p-AKT expression at the DCIS stage has to be investigated.

Since PIK3CA mutations are early events in breast carcinogenesis, they might be critical in the earliest steps of breast cancer initiation. Furthermore, it is of interest to know how this might be associated with PTEN and p-AKT expression. Added knowledge on the variant allele frequency (VAF) of PIK3CA and its association with PTEN and p-AKT expression during breast cancer progression could facilitate future development of early intervention studies. Therefore, the aim of this study was to have a better understanding of breast carcinogenesis by in-depth analyses of the presence and VAF of PIK3CA mutations and their relation to PTEN and p-AKT expression during the progression from DCIS to IBC.

Materials and methods

Patient selection

Patients diagnosed with synchronous DCIS and adjacent IBC and treated at the Erasmus Medical Center – Cancer Institute in the period between 2010 and 2015 were

included ($n=73$). Patients with neoadjuvant treatment, previous breast irradiation or a history of breast cancer were excluded. The tumor cell percentage was estimated beforehand by a pathologist and only samples with an estimated tumor cell percentage $\geq 50\%$ were included. DCIS grade was assessed primarily based on cytonuclear differentiation and IBC grade was assessed according to the modified Bloom Richardson score (Elston & Ellis 1991). Immunohistochemistry (IHC) was performed on each IBC, using ER (ER SP1; Ventana Medical Systems, Inc.), PR (PR 1E2; Ventana Medical Systems, Inc.) and HER2 (HER2 4B5; Ventana Medical Systems, Inc.) antibodies. ER and PR status were scored positive when $\geq 10\%$ of the tumor cells were positive, according to the Dutch Breast Cancer Guideline (NABON 2017). HER2 status was scored according to the international guidelines (Wolff *et al.* 2014). For this study, coded leftover patient material was used in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands (FEDERA 2011). Therefore, there was no need for an informed consent or study approval by an ethical committee. Figure 1 provides an overview of the workflow.

DNA and RNA isolation, sample enrichment and product validation

In addition to the estimated tumor cell percentage assessed by a pathologist, we analyzed *EPCAM*, *ESR1* and *ERBB2* gene expression in all lesions. For this, 8 μm thick formalin-fixed paraffin-embedded (FFPE) tissues of DCIS and adjacent IBC and, if available, corresponding lymph node and distant metastasis were dewaxed and rehydrated, followed by a haematoxylin staining. Subsequently, tissue was microdissected manually, using a sterile scalpel under a stereomicroscope (Zeiss).

Microdissected cells were stored into RNase/Dnase-free tubes containing RNeasy Lysis Buffer (Thermo Fisher) at -80°C . RNeasy Lysis Buffer was removed and replaced by xylene after which RNA and DNA isolation was performed with the AllPrep RNA/DNA FFPE isolation kit (Qiagen) according to the manufacturer's instructions. RNA concentrations were measured by Nanodrop (Thermo Fisher Scientific) and DNA concentrations with picogreen by Qubit (Thermo Fisher Scientific) and stored at -30°C .

The isolated RNA was used to generate cDNA and analyze the *ESR1*, *ERBB2* and *EPCAM* expression by reverse transcriptase RT-PCR as previously described (Doebar *et al.* 2017, Sieuwerts *et al.* 2017). In brief, a gene-specific pre-amplification was performed for *ESR1* (Hs00174860_m1),

Figure 1

Flowchart of the analyzed samples. dPCR, digital PCR; FISH, fluorescent *in situ* hybridization; IHC, immunohistochemistry. Patients were matched on ER, PR, HER2 and tumor grade.

ERBB2 (Hs01001580_m1) and *EPCAM* (Hs00158980_m1) and three reference genes (*GUSB*, Hs99999908_m1, *HMBS*, Hs00609297_m1 and *HPRT1*) using the TaqMan PreAmp Master mix (Thermo Fisher Scientific) for 15 cycles, followed by TaqMan probe-based real-time PCRs according to the manufacturer's instructions in a MX3000P Real-Time PCR System (Agilent). Gene expression levels were quantified relative to the average expression of *GUSB*, *HMBS* and *HPRT1* using the $2^{-\Delta\text{Ct}}$ (average quantitative cycle (Cq) reference genes – Cq target gene) method.

Prior to use, DNA samples were diluted or (Sieuwerts *et al.* 2017) vacuum dried to a working concentration of 1 ng/ μL in case the original concentration was ≤ 0.5 ng/ μL . Next, the PIK3CA targets were enriched in a targeted PCR using TaqMan PreAmp mastermix (Thermo Fisher Scientific) and PIK3CA-specific exon 9 and exon 20 region primers at a final concentration of 10 nM in a Thermal cycler (Bio-Rad Laboratories, Inc.) (Supplementary Table 1, see section on [supplementary data](#) given at the end of this article). These pre-amplified products were then diluted 10 $\times$ in LoTE (3 mM Tris-HCl/0.2 mM EDTA, pH 8.0) before further amplification by SYBR-based quantitative PCR (qPCR), using the same primers at a final concentration of 100 μM and SensiFast low ROX mastermix (Bioline). The resulting qPCR products were used for fragment size validation and quantification of the fragment concentrations by microchip electrophoresis with the DNA-500 kit (MultiNA, Shimadzu) and SNaPshot analysis.

Detection of PIK3CA mutations by SNaPshot assay

SNaPshot is a rapid and simple assay to detect multiple PIK3CA mutations present in down to 5–10% of the tumor cells in a single analysis (Boland *et al.* 2003, Fariña Sarasqueta *et al.* 2011). The SNaPshot Multiplex System for SNP Genotyping (Thermo Fisher Scientific) was therefore used to identify PIK3CA mutations in our cohort. The qPCR products were first diluted in LoTE to a working concentration of 2.5 ng/μL and were then amplified, using the PIK3CA primers at a final concentration of 1 μM for exon 9 and 0.7 μM for exon 20 (Supplementary Table 1). Then, samples were purified using 3 U/Well FastAP and 0.45 U/Well Exonuclease 1 (Thermo Fisher Scientific) after which the SNaPshot ddNTP multiplex probe mix (Supplementary Table 2) was used to extend and terminate the reaction as previously described (Hurst *et al.* 2009). Finally, an ABI PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific) was used to detect the mutations by electrophoresis. Breast cancer cell lines with known PIK3CA mutation status were used as positive and negative controls. Mutation analysis was performed using GeneMarker V2.7.0 Software (SoftGenetics, LLC).

Quantifying PIK3CA mutation by dPCR assay

Digital PCR (dPCR) is a highly sensitive technique that can detect and, in addition to the SNaPshot assay, quantify mutations present in down to 0.1% of tumor cells (Beaver *et al.* 2013, Sueta *et al.* 2014). We therefore used this technique to validate the SNaPshot results and quantify the relative number of PIK3CA-mutated copies in both the DCIS and IBC component of those patients with a PIK3CA mutation identified by SNaPshot analysis. Additionally, we also performed a dPCR assay on samples with a wild-type (WT) PIK3CA mutation ($n=33$ samples) outcome by SNaPshot to ensure we did not overlook mutations. Mutation-specific TaqMan SNP Genotyping Assays (Thermo Fisher Scientific) were used for dPCR analysis. These assays consist of two TaqMan probes, one for the WT sequence and the other one specific for E542K, E545K, H1047R or H1047L PIK3CA mutation (Supplementary Table 3). Starting with 1 ng/μL DNA, the PIK3CA targets where first enriched using Taqman PreAmp mastermix (Thermo Fisher Scientific) and the mutation-specific TaqMan SNP Genotyping Assays in a Thermal cycler (Bio-Rad Laboratories, Inc.), followed by a qPCR with the same assays and SensiFast Universal Mastermix (Bioline) to determine the optimum loading concentration in de dPCR chip (Supplementary Table 4). The mutation-specific

TaqMan SNP Genotyping assay was then added to these pre-amplified DNA samples along with the Digital PCR V2 Master Mix (Thermo Fisher) (Supplementary Table 5). Next, the samples were loaded to a Digital PCR 20K chip and sealed using the QuantStudio 3D Digital PCR Chip Loader, according to the manufacturer's instructions. After loading, a dPCR reaction was performed in a Thermo Fisher ProFlex PCR system. For optimal signal detection, two chip reads were performed using the Thermo Fisher QuantStudio 3D Digital PCR Chip Reader. The samples were individually analyzed online using the QuantStudio 3D AnalysisSuite. PIK3CA mutations in DCIS, IBC and lymph node metastasis were quantified as VAF, that is defined as the number of PIK3CA-mutated copies divided by the number of PIK3CA WT copies plus the number of PIK3CA mutated copies. Samples were scored as PIK3CA mutant when the VAF was at least 5%.

Assessing PIK3CA amplification by fluorescent *in situ* hybridization (FISH)

Because the relative number of PIK3CA mutations might be influenced by amplification or deletion of the *PIK3CA* gene, we performed FISH on all patients with a PIK3CA mutation ($n=18$), using a PIK3CA-specific FISH (ZytoLight SPEC PIK3CA/CEN 3 Dual Color Probe; Catalog number: Z-2140-200, Zytovision). For this assay, consecutive slides were used from the same tissue blocks as previously used for microdissection. First, the 4 μm-thick FFPE tissues were dewaxed, dehydrated and cooked for 13 min in citrate buffer. Subsequently, tissue was treated with pepsin for 20 min and dehydrated, followed by probe hybridizing for 10 min at 75°C and overnight at 37°C in a Hybridizer (Dako Agilent). The slides were washed in a stringent wash buffer at 73°C, rinsed in 2× SSC, dehydrated air-dried, sealed with a cover glass and stored at 4°C until further use.

For visualization and analysis, the stained slides were scanned by a wide field fluorescent microscope (Zeiss, Jena). At least three areas of the hybridized region, with the highest tumor cell content were imaged at a 63× magnification using a Z-stack of three layers and a 3-by-3 tile. The images were analyzed using a Fiji (ImageJ 1.49s) cell counter plugin. The PIK3CA and CEN3 signals were counted in at least 100 cells containing both signals. PIK3CA amplification was defined as a PIK3CA/CEN3 ratio ≥ 2.0 and/or when the average number of PIK3CA alleles per tumor cell nucleus was ≥ 4.0 (Shi *et al.* 2012, Kim *et al.* 2016).

Quantifying PTEN and p-AKT expression by IHC

In order to assess the potential negative regulation of the PI3K pathway during progression, we performed PTEN and p-AKT IHC on consecutive slides. We included all patients with a PIK3CA mutation ($n=18$) and matched patients with a WT PIK3CA status ($n=18$). Matching was based on ER, PR, HER2 expression and tumor grade. OptiView DAB IHC Detection Kit (v1.00.0117, Ventana Medical Systems, Inc.) was used to detect PTEN expression. For this assay, we used the same tissue blocks as previously used for microdissection. Initially, 4 μ m-thick FFPE tissues of DCIS and adjacent IBC were incubated on a hot plate over the weekend for optimal sealing of the slides. The staining was then carried out by a BenchMark ULTRA instrument (Ventana Medical Systems, Inc.), using a customized protocol for the PTEN monoclonal antibody (D4.3 XP, 1:200, Cell Signaling Technology) and p-AKT monoclonal antibody (Ser473, D9E XP, 1:100, Cell Signaling Technology). The slides were incubated in citrate buffer at 97°C for antigen retrieval and then with the primary antibody, each for 32 min. Skin and breast epithelial cells were used as positive controls for PTEN. IBC and a breast cancer cell line (MM468) were used as positive controls for p-AKT. DCIS and IBC components were scored separately, blinded for PIK3CA status. PTEN and p-AKT expression was reviewed by two observers and assessed semi-quantitatively using the Histo-score (H-score) (Sakr *et al.* 2010). The total H-score varied from 0 to 300 and was calculated as the staining intensity (0–3) multiplied by the percentage of positive cells (0–100).

Statistical analysis

SPSS Statistics 21 (IBM) was used for statistical analysis. After testing for normal distribution, we used non-parametric tests to analyze the differences in PIK3CA VAF, PTEN and p-AKT expression. The Wilcoxon signed-ranks test was used to analyze the differences between paired samples and the Mann–Whitney test was used to analyze the differences in PTEN expression between patients with mutated and WT PIK3CA. To correlate linear variables, the Spearman rank correlation test was used. A Chi-square test was used to analyze the associations between groups. P values <0.05 were considered statistically significant.

Results

Clinicopathological characteristics

A total number of 73 patients with paired DCIS and adjacent IBC were included (Table 1). Median age at diagnosis was 56 years, ranging from 28 to 102 years. Approximately half of the patients (53.4%) underwent breast-conserving surgery and the majority (94.5%) of patients underwent lymph node staging. Local metastasis was found in half of the examined lymph nodes and six patients developed a distant metastasis. In total, 23 lymph nodes metastasis and 3 distant metastatic lesions were included for PIK3CA mutation analysis.

Presence of PIK3CA mutations in DCIS and adjacent IBC by SNaPshot analysis

Overall, a PIK3CA somatic hotspot mutation was detected in 24.7% (18 out of 73) of patients (Fig. 2). The detected mutations were mainly located in H1047R ($n=9$), followed by E545K ($n=5$) and E542K ($n=4$). A PIK3CA mutation

Table 1 Patient and tumor characteristics.

Patient and tumor characteristics ($n=73$)	Frequency (%)
Age at diagnosis (in years)	
Median (range)	56.7 (28–102)
Diameter IBC (cm)	
Median (range)	2.10 (0.60–11.0)
IBC histological grade	
Low	2 (2.7)
Intermediate	28 (38.4)
High	43 (58.9)
Diameter DCIS (cm)	
Median (range)	3.00 (0.10–15.0)
DCIS histological grade	
Low	1 (1.4)
Intermediate	23 (31.5)
High	49 (67.1)
IBC IHC subtype	
ER+ HER2–	34 (46.6)
ER+ HER2+	15 (20.5)
ER– HER2+	9 (12.3)
ER–, PR–, HER2–	15 (20.5)
Lymph node status	
No metastasis or isolated tumor cells	41 (58.9)
Micro- or macrometastasis	28 (35.6)
No LN procedure	4 (5.5)
Distant metastasis	
Yes	6 (5.5)
No	67 (94.5)

Figure 2

The number of PIK3CA mutations during breast cancer progression. Overall, 18 patients with a mutated PIK3CA status (black bars) and 55 patients with a WT PIK3CA status (white bar) were identified.

was identified in both the DCIS and the IBC component of 14 patients. A discordant PIK3CA mutation status was found in four patients, whereby three patients had a PIK3CA mutation in the DCIS component only and one patient had a PIK3CA mutation in the IBC component only. A PIK3CA mutation was detected in 3 out of the 23 patients with a lymph node metastasis. In these three patients, a PIK3CA mutation was also present in both the DCIS and IBC stage. In our cohort, none of the patients with a PIK3CA mutation in either the DCIS component and/or the invasive component developed a distant metastasis. Furthermore, no PIK3CA mutations were identified in the three available distant metastases of three patients without a PIK3CA mutation in the DCIS and IBC component. When a PIK3CA mutation was detected in the DCIS and/or IBC component of a patient, this concerned the same mutation in all cases. We never observed two or more different mutations in the material of one patient.

There were no significant associations between the presence of a PIK3CA mutation in either the DCIS or IBC component and clinicopathologic characteristics. However, the majority of samples with a PIK3CA mutation, 11 out of 18 patients, were ER+ and HER2-. This result was not affected by using a cut-off for ER/PR positivity of 1%.

VAF of PIK3CA in DCIS and adjacent IBC by dPCR analysis

All PIK3CA hotspot mutations, as previously detected by SNaPshot analysis, were also detected by dPCR. However, dPCR detected one additional patient with a 6.15% PIK3CA VAF in an IBC lesion, which was not detected by the SNaPshot assay. In this patient, the SNaPshot assay only detected a mutation in the DCIS component. Other samples with a WT PIK3CA outcome by SNaPshot remained WT after sample check with dPCR. In summary,

after validation by dPCR, the total number of discordant PIK3CA mutation status between DCIS and IBC was reduced from four to three patients: two patients had a PIK3CA mutation in the DCIS component only and one patient had a mutation in the invasive component only.

After quantification, we found a higher PIK3CA VAF in the DCIS stage (45.8%) than in the IBC stage (31.7%), P value=0.007 (Fig. 3), which could not be caused by differences in tumor cell percentage as the mean tumor cell percentage in DCIS was similar to that in IBC (P value=0.24) and results were not affected by correction for tumor cell percentage (P value=0.018). Additionally, the expression of the epithelial marker EPCAM, ESR1 expression and ERBB2 expression were similar in DCIS and IBC (P =0.910, P =0.177 and P =0.381 respectively).

All three patients with a PIK3CA mutation in the lymph node metastases also had a PIK3CA mutation in both the DCIS and the IBC component. The PIK3CA VAF in lymph node metastases were similar to that found in the IBC stage (33.8 vs 30.9%). In one patient with a PIK3CA mutation in the DCIS stage only, no PIK3CA mutation was detected in the lymph node metastasis (Supplementary Fig. 1).

Assessing PIK3CA amplification by fluorescent *in situ* hybridization (FISH)

Using FISH we detected PIK3CA amplification in 3 out of 18 patients (Fig. 4; patients 3, 4 and 8). The PIK3CA VAF by dPCR in these patients was 70.5, 51.1 and 77.0% respectively. The number of mutant PIK3CA DNA copies in patients 3 and 8 were up to three times higher than the number of WT PIK3CA DNA copies. In patient 4, the number of WT and the mutated PIK3CA DNA copies were similar (data not shown). With regard to breast cancer progression, PIK3CA amplification was observed only in the DCIS component. No PIK3CA amplification was

Figure 3

The percentage PIK3CA VAF in paired DCIS (white bars) and IBC (black bars). In total, 2 patients (patient number 4 and 18) had a PIK3CA mutation in the DCIS component only and one patient (patient number 1) had a mutation in the invasive component only. The X-axis displays the number of analyzed patients and the Y-axis displays the PIK3CA VAF in %. DCIS, ductal carcinoma *in situ*; IBC, invasive breast cancer.

observed in IBC lesions or lymph node metastasis. The differences in VAF remained significant after removal of PIK3CA-amplified cases.

PTEN expression during breast cancer progression

PTEN was expressed in the cytoplasm in the majority (29 out of 36) of examined patients. Nuclear PTEN expression was observed in two cases. Both patients had a WT PIK3CA status. Overall, PTEN expression had a significantly lower H-score in IBC compared to DCIS (mean $\pm$ s.d. of 111.11 ± 112.43 vs 138.57 ± 120.78 respectively; P value = 0.004). **Figure 5** illustrates a case with a higher PTEN expression in the DCIS component with a WT PIK3CA status as compared to the adjacent invasive component. When stratified by PIK3CA mutation status, PTEN expression was lower in the IBC component compared to the DCIS component in patients with a WT PIK3CA status (mean H-score $\pm$ s.d. of 90.28 ± 105.20 vs 129.72 ± 118.61 , respectively; P value = 0.007). In patients with a PIK3CA mutation on the other hand, there was no significant difference between PTEN expression in the DCIS component and the IBC component (P -value = 0.14).

p-AKT expression during breast cancer progression

p-AKT expression was evaluated on 36 patients (two DCIS samples were excluded due to tissue exhaustion). In total, there was cytoplasmic p-AKT expression in 15 out of 36 patients. Overall, p-AKT expression was low with a median H-score of 0 (range: 0–205 in DCIS and 0–45 in IBC). Seven out of the 15 patients with p-AKT expression had a PIK3CA mutation; the remaining eight cases were WT. Six out of 15 patients had p-AKT expression in DCIS and adjacent IBC. There was no difference in the level of p-AKT expression between these two components. The remaining nine cases showed a discordant p-AKT expression; four patients had expression in the DCIS component only and five patients had expression in de IBC component only. **Figure 6** provides a heat map of these analyses.

Discussion

PIK3CA mutations have been frequently described in IBC. However, data are limited regarding its presence and quantity during progression from DCIS to IBC.

Figure 4

Representative FISH images (63 $\times$ magnification) of cases with a PIK3CA amplification (number of PIK3CA/nucleus ≥ 4) in DCIS of patient 3 (A), patient 4 (B) and patient 8 (C). Blue represents the cell nuclei, red dots represent CEN3 and the green dots represent PIK3CA.

Figure 5

Illustrative case with a higher IHC expression of PTEN in the DCIS component (H-score of 300) as compared to the adjacent IBC component (H-score of 115). (A) An overview image of PTEN expression in DCIS at a 5× magnification, with a red square indicating the enlarged region (B) at 20× magnification. (C) An overview image of PTEN expression in IBC at a 5× magnification, with a red square indicating the area enlarged region (D) at 20× magnification.

Therefore, the aim of this study was to have a better understanding of PIK3CA mutations during breast cancer progression. We identified a PIK3CA mutation in 24.7% of our cohort (18 out of 73 patients) by SNaPshot analysis. The majority of these patients (14 out of 18) had a PIK3CA mutation in both the DCIS and the IBC component. A discordant PIK3CA mutation status was found in four patients by SNaPshot analyses. This number was reduced to three patients after validation and quantification by dPCR. Our results are in line with previous reports on PIK3CA mutations in DCIS and adjacent IBC, regardless of the different detection methods used in these studies (Li *et al.* 2010, Miron *et al.* 2010, Kalinsky *et al.* 2011, Ang *et al.* 2014, Sakr *et al.* 2014). Our data support the presence of PIK3CA mutation heterogeneity in synchronous DCIS and IBC as previously demonstrated (Miron *et al.* 2010, Ang *et al.* 2014). Besides, this underlines the early development of PIK3CA mutations during breast cancer carcinogenesis, specifically in ER+ tumors. However, late development of PIK3CA mutations have also been observed (Dupont Jensen *et al.* 2011, Gonzalez-Angulo *et al.* 2011, Sueta *et al.* 2014).

In those cases, a PIK3CA mutation was found in the metastases and not in the primary tumor, suggesting a role in maintenance of breast cancer metastatic lesions. In our cohort, the low frequency of PIK3CA mutations in lymph node metastasis and the absence of a PIK3CA mutations in distant metastasis suggests otherwise, although the number of metastases was too small for defining conclusions.

After pre-screening by SNaPshot analyses, we used dPCR to validate and quantify our results. All mutations identified by the SNaPshot assay were confirmed by dPCR. However, we identified one additional mutation in the IBC stage, which was not detected by the SNaPshot assay. This detection failure by the SNaPshot assay is most likely due to its detection limit of 5–10% (Fariña Sarasqueta *et al.* 2011). The PIK3CA VAF in this sample was rather low (6.2%). We further quantified the PIK3CA mutation frequency in synchronous DCIS and IBC. Our data showed a significantly higher PIK3CA VAF in the DCIS component compared to the adjacent IBC component, which could not be contributed to differences in the

Figure 6

Heat map of DCIS and IBC results with an overview of performed assays. %VAF, % variant allele frequency of PIK3CA; dPCR, digital polymerase chain reaction; FISH, fluorescent *in situ* hybridization; IHC, immunohistochemistry; qPCR, quantitative PCR. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0019>.

EPCAM, *ESR1* or *ERBB2* expression as quantified by RT-qPCR. Previous studies on this topic either focused on the IBC stage only or on high-grade DCIS and synchronous DCIS and IBC (Sakr *et al.* 2014, Sueta *et al.* 2014). Sakr and colleagues suggested that PIK3CA mutations in high-grade synchronous DCIS and adjacent IBC are present in a non-modal population of the DCIS component versus a modal population of the IBC cells. These results are rather contradictory to ours, since our data shows the opposite. This outcome could be explained by the use of different detection methods or inclusion criteria. Our results suggest that PIK3CA mutations could be essential in early stages of breast carcinogenesis but might not be necessary for progression from DCIS to IBC. Furthermore, a higher PIK3CA VAF in DCIS compared to IBC suggests subclonal heterogeneity, whereby different clones of malignant cells might have progressed into IBC. However, since PIK3CA mutations are present in both the DCIS stage and the IBC stage in the majority of patients, it could be needed for maintenance of the tumor in the majority of breast cancers.

We detected PIK3CA amplification in the DCIS component of 3 out of 18 patients by FISH. The PIK3CA VAF of two out of these three patients suggests a PIK3CA amplification of the variant PIK3CA allele, since the mutant versus WT allele ratio was up to 3. Additional investigations in other cohorts have shown

important clinical consequences, since acquired PIK3CA amplifications have been associated with resistance to selective phosphoinositide 3-kinase inhibitors in IBC (Huw *et al.* 2013).

With respect to PTEN and p-AKT scoring, there is no standardized scoring system, nor a standardized cut-off point for positive vs negative. We semi-quantified PTEN and p-AKT expression immunohistochemically using the H-score. In general, PTEN expression was significantly lower in the IBC component compared to the DCIS component. Previous studies reported either a similar PTEN expression or a higher frequency of PTEN loss in the IBC stage compared to the DCIS stage using a dichotomous scoring system (Aktepe *et al.* 2009, Sakr *et al.* 2014). The latter study is in line with our finding, suggesting decreased PTEN expression at the IBC stage. When we stratified by PIK3CA mutation status, a lower PTEN expression in IBC compared to DCIS was only observed in WT PIK3CA. In patients with a mutated PIK3CA on the other hand, no significant difference in PTEN expression was observed between these components. In our cohort, we observed co-occurrence of PTEN loss and a PIK3CA mutation in a minority of cases (four IBC samples). Based on these results, we hypothesize that a PIK3CA mutation could contribute to preservation of PTEN expression during progression from DCIS to IBC, since PIK3CA-driven progression does not seem to co-occur with PTEN loss

in the majority of patients. However, we did not observe significant differences in PTEN expression between the PIK3CA-mutated and WT cases, which might be due to the small cohort. In our cohort, p-AKT was expressed in 15 out of 36 patients. There was no association between PIK3CA mutation status and p-AKT expression: seven patients with p-AKT expression had a PIK3CA mutation, eight patients were WT. There was a strong overlap between p-AKT expression in the DCIS component and the adjacent IBC component. In those patients with p-AKT expression in both components, there was no difference in the level of expression between DCIS and IBC, which is in line with a previous study (Sakr *et al.* 2014). To the best of our knowledge, this is the first study focusing on the presence and quantity of PIK3CA mutations during the progression from DCIS to IBC, including the potential association with PTEN and p-AKT expression. We are also one of the first to report PIK3CA amplification in breast cancer, specifically in DCIS. Nevertheless, our study also has several limitations. First, the number of mutated cases in our series was rather small for further subgroup analyses. Next, our study is restricted to hotspot changes in the *PIK3CA* gene only. It is also likely that changes in the *PIK3CA* gene can further interact with other genes, in and outside the PI3K pathway (Martelotto *et al.* 2017, Ramirez-Ardila *et al.* 2017, Wang *et al.* 2017, Casasent *et al.* 2018). Besides, we only included patients with a substantial DCIS component to be able to isolate enough DNA to perform our analyses, which could have resulted in a selection bias.

In conclusion, we detected a significantly higher PIK3CA VAF in the DCIS component compared to the adjacent IBC component, which suggests that PIK3CA mutations could specifically be essential in early stages of breast carcinogenesis rather than progression from DCIS to IBC. Moreover, we have shown maintained PTEN expression in DCIS and loss of PTEN expression at the IBC stage, which is associated with a WT PIK3CA. This association suggests that PIK3CA mutations and loss of PTEN expression do not co-occur during DCIS progression to IBC in the majority of patients. In our cohort, there was no association between PIK3CA mutation status and p-AKT expression. Our data might contribute to further unraveling breast carcinogenesis and heterogeneity, which could facilitate patient-specific treatment.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-19-0019>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M A G, A M S and C D E conceived and designed experiments; D O E and C D E selected the patients and collected clinical data; M A G, E V E and V W E collected and processed the samples; M A G, A M S, C M B, K R R and H S L conducted the experiments; M A G, A M S, D I N, J W M and C D E consulted and deliberated on the results; C D E secured funding for the project; M A G, A M S and C D E wrote the manuscript. All co-authors reviewed the manuscript.

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