Lobular carcinomas in situ display intra-lesion genetic heterogeneity and clonal evolution in the progression to invasive lobular carcinoma

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ABSTRACT (Word count: 248)

Purpose: Lobular carcinoma in situ (LCIS) is a pre-invasive lesion of the breast. We sought to define its genomic landscape, whether intra-lesion genetic heterogeneity is present in LCIS, and the clonal relatedness between LCIS and invasive breast cancers.

Experimental Design: We reanalyzed whole-exome sequencing (WES) data and performed a targeted amplicon sequencing validation of mutations identified in 43 LCIS and 27 synchronous more clinically-advanced lesions from 24 patients (nine ductal carcinomas in situ (DCIS), 13 invasive lobular carcinomas (ILCs) and five invasive ductal carcinomas (IDCs)). Somatic genetic alterations, mutational signatures, clonal composition and phylogenetic trees were defined using validated computational methods.

Results: WES of 43 LCIS lesions revealed a genomic profile similar to that previously reported for ILCs, with CDH1 mutations present in 81% of the lesions. Forty-two percent (18/43) of LCIS were found to be clonally-related to synchronous DCIS and/or ILCs, with clonal evolutionary patterns indicative of clonal selection and/or parallel/branched progression. Intra-lesion genetic heterogeneity was higher among LCIS clonally-related to DCIS/ILC than in those non-clonally related to DCIS/ILC. A shift from aging to APOBEC-related mutational processes was observed in the progression from LCIS to DCIS and/or ILC in a subset of cases.

Conclusions: Our findings support the contention that LCIS has a repertoire of somatic genetic alterations similar to that of ILCs, and likely constitutes a non-obligate precursor of breast cancer. Intra-lesion genetic heterogeneity is observed in LCIS and should be considered in studies aiming to develop biomarkers of progression from LCIS to more advanced lesions.
We investigated the somatic genetic alterations affecting all protein coding genes in lobular carcinoma in situ (LCIS) and synchronously diagnosed ductal carcinomas in situ (DCIS) and invasive lobular (ILC) or ductal carcinomas (IDC). Our analyses revealed that LCIS is a genetically advanced lesion, often displaying intra-lesion genetic heterogeneity, with minor subclones of LCIS becoming the dominant clone in ILCs. An APOBEC-related mutational signature coupled with overexpression of APOBEC3B was found to be present in LCIS subclones progressing to more advanced lesions. Our findings support the notion that LCIS is a non-obligate precursor of ILC, and suggest that the development of robust molecular predictors of the risk of LCIS progression/evolution into more aggressive forms of breast cancer may benefit from the assessment of intra-lesion genetic heterogeneity in LCIS.
INTRODUCTION

Lobular carcinoma in situ (LCIS) is a pre-invasive lesion of the breast, which is often multifocal and bilateral (1). Over the last three decades, LCIS has been clinically perceived as a risk indicator and managed accordingly (1). There is, however, burgeoning phenotypic and genetic evidence to suggest that LCIS is a non-obligate precursor of invasive breast cancer, akin to ductal carcinoma in situ (DCIS) (2).

LCIS and invasive lobular carcinomas (ILCs) are phenotypically and genetically similar. Both lesions are preferentially of the luminal A molecular subtype (i.e. estrogen receptor (ER)-positive, HER2-negative, low-grade and low-proliferation), and harbor recurrent gains of 1q and losses of 16q, encompassing the CDH1 gene locus, as well as recurrent CDH1 somatic mutations (1,3-7). In fact, loss of E-cadherin, the protein product of the CDH1 gene, is a hallmark feature of these lesions (3,6) and has been shown to result in the development of ILCs in conditional mouse models (8). Analyses of the genomic features of ILCs by The Cancer Genome Atlas consortium (TCGA) (6) and individual investigators (9) have revealed the genes most commonly mutated in this subtype of breast cancer, and identified molecular differences between invasive ductal carcinomas (IDCs) of no special type and ILCs, including a higher rate of FOXA1 mutations and a lower rate of GATA3 mutations in those with lobular histology. Additional whole-exome (WES) (7) and targeted (10) sequencing analyses focused on paired LCIS and ILCs demonstrated comparable rates of mutations affecting CDH1, PIK3CA and CBFB, among other genes.

Previous studies have demonstrated that synchronous LCIS and invasive breast cancers may be clonally related and share a common ancestral lesion (4,7,10). In most studies, however, clonal relatedness was inferred using limited genomic information derived from copy number (4) or targeted sequencing analyses (10). By combining copy number and WES data, Begg et al. provided evidence of clonal relatedness between LCIS and associated lesions (7). These studies, however, did not...
investigate the basis of the clonal relatedness between LCIS and ILC, and whether the progression from LCIS to ILC would involve the selection of specific subclones or happen through multiclonal invasion (11,12). Given that not only invasive breast cancers (13) but also pre-invasive lesions (11) may be genetically heterogeneous at diagnosis, and that tumor progression/stromal invasion may stem from clonal selection (11,13), it is plausible that LCIS may display intra-lesion genetic heterogeneity and that the progression from LCIS to more clinically advanced lesions, such as DCIS or invasive breast cancer, may result from the selection of pre-existing subclones.

Here, we performed a re-analysis of WES data generated from a unique series of frozen LCIS samples from prospectively accrued, consecutive patients subjected to prophylactic or therapeutic mastectomy, previously published by Begg et al. (7). We performed a high-depth targeted capture sequencing validation of the mutations identified by WES in that study, using the same DNA samples and employed state-of-the-art bioinformatics algorithms with a Bayesian clustering model (PyClone) to infer subclone structure and with construction of clone based phylogeny, seeking to define the clonal composition and mutational processes in LCIS synchronously diagnosed with ILC, DCIS and/or IDC, and to ascertain whether changes in the clonal composition are observed in the progression from LCIS to DCIS or ILC.

MATERIAL AND METHODS

Subjects and samples

This study is based on the 24 cases with available WES out of the 30 cases previously subjected to microarray-based comparative genomic hybridization and/or WES by Begg et al. (7). Eight out of the 24 cases included in this study were also included in the targeted sequencing analysis previously reported by Sakr et al. (10). The cases subjected to WES include 43 LCIS and 27 synchronous more clinically-advanced lesions (Table 1) (Supplementary Methods).

Immunohistochemistry
Immunohistochemistry for ER, progesterone receptor (PR) and HER2 was performed essentially as previously described (11) (Supplementary Methods), and analyzed according to the American Society of Clinical Oncology (ASCO)/ College of American Pathologists (CAP) guidelines (14,15).

**Whole-exome sequencing data analysis**

Previously-generated (7) WES data from tumor-normal DNA samples were retrieved and re-analyzed. Neoplastic samples were sequenced to a median depth of 192x (range 95x-369x) and matched normal samples were sequenced to a median depth of 154x (range 105x-238x; **Supplementary Table S1**).

WES data analysis was performed as described in *Ng et al.* (16) and detailed in the Supplementary Methods. In brief, after aligning the reads to the reference human genome GRCh37, somatic genetic alterations were detected using state-of-the-art bioinformatics algorithms and filters were subsequently applied. In addition to the identification of single nucleotide variants (SNVs) and insertions and deletions (indels), for samples from a given patient, mutations that were identified in at least one sample were subsequently interrogated in all related samples (Supplementary Methods). Given that *CDH1* germline mutations have been shown to be causative of familial gastric and breast cancer syndrome (17), the germline DNA samples from each patient were evaluated for the presence of pathogenic *CDH1* germline mutations (Supplementary Methods). The potential functional effect of each somatic mutation was defined using a combination of mutation function predictors shown to have high negative predictive value (18), as previously described (19), and genes were annotated according to their presence in three cancer gene datasets, *Kandoth et al.* (20), the Cancer Gene Census (21) and *Lawrence et al.* (22). Allele-specific copy number alterations (CNAs) and loss of heterozygosity (LOH) for specific genes were defined using FACETS (23), as previously described (16), and purity and ploidy estimations were calculated using ABSOLUTE (24) (Supplementary Methods).

**Targeted amplicon re-sequencing validation of somatic mutations**
A validation of the mutations found with WES was performed for cases with sufficient DNA material (n=11), using a custom designed AmpliSeq panel on an Ion Torrent Personal Genome Machine. This validation was not included in Begg et al. (7). Out of 4,061 somatic mutations identified by WES, 1,796 were investigated in five LCIS, five DCIS, eight ILCs, and two IDCs from cases 1, 2, 4, 7, 8, 10, 11, 12, 13, 14 and 15. 1,492 (83%) mutations were successfully validated. Mutations that had sufficient coverage in the validation experiment (minimum of 50 reads) but were not validated (allele frequency <1%) were excluded from the list of mutations used in the downstream analyses.

Clonality analysis

To infer the clonal relatedness between synchronous lesions, we defined the “clonality index” (CI) as the probability of two lesions sharing mutations not expected to have co-occurred by chance based on a previously validated method (25) (Supplementary Methods).

Clonal frequencies

To estimate the clonal architecture and composition of the lesions from each patient, mutant allelic fractions from all somatic mutations were adjusted for tumor cell content, ploidy, local copy number and sequencing errors using PyClone, as previously described (26) (Supplementary Methods).

Truncal and branch mutations

For each patient displaying at least one LCIS sample clonally-related to other lesions (LCIS, DCIS or ILC), we categorized the mutations into truncal and branch using PyClone (26) (Supplementary Methods). Truncal mutations were defined as those concurrently present in the modal populations of all LCIS and their clonally-related other lesions from a given patient. Branch mutations were defined as those comprising all non-truncal mutations.

Measure of diversity
To quantitate the intra-lesion genetic heterogeneity of each sample analyzed, we used the Shannon diversity index (27) and Gini-Simpson index (28) (Supplementary Methods).

**Phylogenetic tree construction**

Maximum parsimony trees were built using binary presence/absence matrices built from the somatic genetic alterations, including synonymous and non-synonymous SNVs, indels and CNAs, within the clonally-related lesions from each patient, essentially as described by Murugaesu et al. (16,29) (Supplementary Methods). We have also employed Treeomics as an alternative approach for the reconstruction of phylogenetic trees (30). Treeomics reconstructs phylogenies using a Bayesian inference model and determines the probability that a variant is either present or absent in a given sample.

**Reverse transcription quantitative PCR (RT-qPCR)**

Total RNA was extracted using TRIZOL and reverse transcribed using SuperScript VILO Master Mix (Life Technologies, Thermo Fisher Scientific) according to the manufacturer’s instructions from cases for which sufficient frozen tissue samples were available. RT-qPCR was performed to analyze the expression levels of *APOBEC3B*, *APOBEC3H* and *REV1* genes using TaqMan Assay-on-Demand (Supplementary Methods).

**Mutational frequencies of TCGA ILCs and luminal-A cancers**

TCGA luminal-A invasive breast cancers (31) and ILCs (6) and their mutations were retrieved from the "Final Full BRCA Sample Summary" and "Mutations - Publicly accessible MAF archives" at https://tcga-data.nci.nih.gov/docs/publications/brca_2012/ and https://tcga-data.nci.nih.gov/docs/publications/brca_2015/, including all non-silent, non-RNA mutations for 209 luminal-A primary invasive breast cancers and 127 ILCs. Previous studies have demonstrated the
equivalence between the TCGA pipeline and the pipeline employed in this study for mutation detection (19,32).

**Mutational signatures**

To define the mutational signatures involved in the development of LCIS, DCIS and ILCs, we employed deconstructSigs (33) based on the set of mutational signatures “signature.cosmic” (34).

**Statistical analysis**

Analyses were performed using R. For comparisons between categorical variables, the Fisher’s exact test was employed, whereas for continuous variables, the Student’s t-test and Mann–Whitney U test were employed as appropriate. A hypergeometric test was performed to estimate the statistical significance of the enrichment for cancer genes (genes present in at least one of the cancer gene lists by Lawrence et al. (Cancer 5000-S) (22), Kandoth et al. (20) and/or Cancer Gene Census (21); n=745) in the genes with truncal mutations (n=559) and branch mutations (n=2,452). For the hypergeometric test, the total number of genes in the genome used was 18,986, as defined as the number of protein-coding genes by the HUGO Gene Nomenclature Committee. The representation (enrichment) factors and the $P$-values of the hypergeometric tests were provided for the analyses performed. All tests were two-sided and $P$-values<0.05 were considered statistically significant, adjusted for multiple comparisons where specified.

**RESULTS**

**LCIS displays a repertoire of somatic genetic alterations consistent with those of ILCs and luminal A-like breast cancers**

This study consists of a re-analysis of previously described WES data (7), followed by a previously unpublished targeted amplicon sequencing validation of approximately 1,800 selected mutations, from 43 LCIS and synchronous DCIS (n=9), ILCs (n=13) or IDCs (n=5) from 24 patients (Table 1). Three
patients underwent bilateral mastectomy, one was therapeutic for bilateral breast cancer and two patients underwent contralateral prophylactic mastectomy; these three patients were found to have bilateral LCIS (Table 1). All LCIS lesions were of classic type and all DCIS were of intermediate nuclear grade. For those patients with invasive lesions, tumor size and ER, PR and HER2 status in invasive tumor cells are described in Table 1. Notably, all invasive carcinomas were ER-positive/HER2-negative.

Somatic mutation analysis of the 43 LCIS lesions revealed a median of 20 non-synonymous somatic mutations/lesion (range 5-333) and a mutation rate of 0.39 mutations/Mb (Figs. 1A and 2A-D), comparable to the number of non-synonymous somatic mutations and the mutation rates of 209 luminal-A invasive breast cancers (31) and 127 ILCs (6) from TCGA (i.e. 27 somatic mutations/lesion (range 7-203) and 0.52 mutations/Mb in luminal-A and 29 somatic mutations/lesion (range 1-1,080) and 0.56 mutations/Mb in ILCs; Mann–Whitney U test, P>0.1). Consistent with the notion that CDH1 inactivation is a driver of lesions with lobular histologic features (1), we observed pathogenic mutations affecting the CDH1 gene in 35 of 43 (81%) LCIS, of which all but three were somatic; patient 13, who had three distinct foci of LCIS, was found to harbor a CDH1 germline mutation. All but two CDH1 mutations were coupled with loss of heterozygosity (LOH) of the wild-type allele (77% (33/43) of all LCIS analyzed, Fig. 1A). Moreover, all LCIS cases lacked E-cadherin expression by immunohistochemical analysis. LCIS lacking CDH1 mutations did not harbor mutations or deletions affecting genes coding for additional proteins that comprise the cadherin-catenin complex, such as CTNNB1 (β-catenin), CTNNA1 (α-catenin) or CTNND1 (p120-catenin), nor somatic or germline genetic alterations in RHOA (Supplementary Data File 1), a gene that has been implicated in the biology of gastric cancer (35), and whose alterations result in neoplastic cells displaying discohesiveness akin to that caused by CDH1 loss of function.
Additional genes identified by TCGA to be significantly mutated in ILCs (6), such as PIK3CA, TBX3, FOXA1 and MAP3K1, were also found to be recurrently somatically mutated in LCIS (Figs. 1A and 2E); however, TP53 somatic mutations, and PTEN somatic mutations and homozygous deletions, present in 8%, 7%, and 6% of ILCs analyzed by TCGA (6), were not found in any of the LCIS analyzed here. Notably, TP53 mutations were significantly more frequently found in luminal A invasive breast cancers from TCGA than in the LCIS analyzed here (12% (25/209) vs 0% (0/43), Fisher’s exact test, P=0.019, Fig. 2E). Moreover, genes identified by TCGA to be significantly mutated in luminal A invasive breast cancers, including CBFB, GATA3, NCOR1 and MED23 were also found be recurrently mutated in LCIS. Interestingly, however, CBFB was found to be mutated in 19% (8/43) of LCIS, a rate significantly higher than that in 2% (2/127) of ILCs and 2% (5/209) of luminal-A breast cancers from TCGA (Fisher’s exact tests, P<0.01, Fig. 2E). Gene CNA analysis revealed recurrent losses of 16q and gains of 1q (Fig. 1B), a pattern also observed in ILCs (4,6) and luminal-A invasive breast cancers (31). Taken together, our findings demonstrate that LCIS synchronously diagnosed with more advanced lesions in this study is a genetically-advanced, neoplastic lesion often driven by E-cadherin loss of function, with a spectrum of somatic genetic alterations affecting genes commonly altered in ILCs and luminal-A invasive breast cancers.

**LCIS is often clonally-related to DCIS and ILCs**

WES of nine DCIS (a non-invasive precursor lesion perceived clinically to be more advanced than LCIS (36,37)), 13 ILCs, and five IDCs collected synchronously with the LCIS analyzed above demonstrated that overall these lesions displayed similar number of mutations/case, mutation rates, repertoires of CNAs and non-synonymous somatic mutations to those of the LCIS analyzed in this study (Fig. 1, 2A-D), with exception of CDH1 somatic mutations that were exclusively found in LCIS and ILCs.

We reasoned that the somatic mutations and CNAs found in anatomically distinct foci of LCIS, ILC, DCIS and IDC could provide a basis for defining their clonal relatedness. Consistent with the analysis
reported by Begg et al. (7), but based on distinct bioinformatics and biostatistical approaches (Supplementary Methods), here we demonstrate that all multifocal LCIS originating in the same breast quadrant (8/8 samples, four patients; cases 4, 7, 9, 23) were clonally-related, harboring several identical somatic mutations and CNAs (Fig. 3A, Supplementary Figs. S1-S3). Sixty-seven percent (16/24) of multifocal LCIS affecting distinct quadrants of the breast were also clonally-related (Fig. 3A). Further, 10/13 (77%) ILCs and 5/9 (56%) DCIS samples were found to be clonally-related to at least one synchronous LCIS analyzed (Fig. 3A). Interestingly, none of the five IDCs studied were found to be clonally-related to a LCIS (Fig. 3A), however, in all three cases where synchronous DCIS and IDC samples were analyzed, the DCIS and IDC were found to be clonally-related (Fig. 3A, Supplementary Figs. S1-S2). As expected, no clonal relatedness was observed between lesions arising in distinct breasts (bilateral cases; Fig. 3A, Supplementary Figs. S1-S2). In addition, the clonal relatedness reported by Sakr et al. for the 8 pairs of LCIS and ILC were confirmed in this study (Supplementary Table S2).

Taken together, our findings indicate that the majority of multifocal LCIS lesions are clonally-related, and that the presence of these lesions in distinct quadrants of the breast does not predict their clonal relatedness. LCIS and synchronous DCIS and/or ILC are often clonally-related, corroborating the notion (4,7,38,39) that LCIS is a non-obligate precursor of more clinically-advanced lesions, in particular ILCs. Furthermore, no evidence of clonality between LCIS and IDC was observed here, suggesting that direct progression from CDH1-mutant LCIS to IDC is an uncommon biological phenomenon.

**LCIS foci displaying intra-lesion genetic heterogeneity are more likely to progress to ILCs**

Recent studies have demonstrated that intra-tumor genetic heterogeneity may be present in non-invasive lesions including DCIS (11,40) and pre-invasive lesions arising in other organs (e.g. of the esophagus)(41). In such cases, all neoplastic cells harbor the founder genetic events (i.e. truncal mutations) and subclonal populations of cancer cells display additional genetic alterations (i.e. branch
We posited that LCIS would harbor intra-lesion genetic heterogeneity and that LCIS lesions when clonally-related to DCIS or ILC would be associated with a higher level of intra-lesion genetic heterogeneity than LCIS not clonally-related to more advanced lesions.

To test this hypothesis, we resolved the clonal composition of LCIS, DCIS and/or ILC samples by applying a Bayesian clustering model (PyClone (26)) to mutant allele fractions, incorporating tumor cellularity, ploidy and local copy number obtained from ABSOLUTE (24) and/or FACETS (23) (Supplementary Methods). This analysis revealed that all but two (89%; 16/18) LCIS clonally-related to DCIS/ILC but only 40% (10/25) of LCIS not clonally-related to DCIS/ILC displayed intra-lesion genetic heterogeneity at the sequencing depth analyzed (Fisher’s exact test, \( P=0.0016 \), Supplementary Figs. S4A-B). These findings were further corroborated by an analysis of the Shannon and Gini-Simpson diversity indices (27,28,43), which demonstrated that as a group LCIS clonally-related to DCIS and/or ILC (n=18) displayed significantly higher intra-lesion genetic heterogeneity than LCIS not clonally-related to more advanced lesions (n=25) (Mann–Whitney U test, \( P=0.005 \), Fig. 3B-C, Supplementary Figs. S4C-D). Interestingly, in case 4, composed of two LCIS and one ILC, all sharing a common ancestor, the LCIS lesion displaying heterogeneity was found to be the likeliest direct precursor of the ILC (Fig. 4A).

Given the intra-lesion genetic heterogeneity observed in LCIS, in particular in those related to more advanced lesions, we sought to define whether the branch mutations found in these lesions would affect ‘passenger’ genes or genes significantly mutated in cancer (20-22). Contrary to the notion that heterogeneity would primarily affect passenger genetic events, both truncal and branch non-synonymous somatic mutations detected in LCIS clonally-related to the other lesions were found to target genes significantly enriched for known cancer drivers (20-22) (hypergeometric test, representation factor=2.09, \( P<0.01 \), and hypergeometric test, representation factor=1.5, \( P<0.01 \), respectively; Supplementary Figs. S4E-F). Importantly, however, in agreement with previous multi-
region analyses that suggested that most of the driver genetic alterations are early truncal events (13,16,44), the enrichment for cancer genes was higher in the constellation of truncal than in branch mutations. Truncal mutations included genes found to be significantly mutated in ILCs and/or luminal-A invasive breast cancers, including $CDH1$, $PIK3CA$, $MAP3K1$, $CBFB$, $SF3B1$, $RUNX1$ and $FOXA1$ (Supplementary Data File 1), whereas branch mutations included $GATA3$, $PIK3CA$, $ERBB2$ and $KMT2C$.

Given the clonal relatedness of LCIS with DCIS and ILC, we posited that progression from LCIS to DCIS/ILC could result in the selection of specific subclones harboring private genetic alterations (11,12,40). In 29% (4/14) of cases where LCIS was clonally-related to DCIS or ILC, we observed that a selected population from the LCIS became dominant in the respective DCIS or ILC (Fig. 4, Supplementary Fig. S1), whereas in the remaining 10 cases our findings suggested parallel progression between LCIS, DCIS and/or ILC. In two cases (cases 4 and 10), a minor subclone from a LCIS was the likeliest substrate for the development of the DCIS or the ILC (Fig. 4). In cases 1, 11 and 16, the biological chronology of the LCIS and DCIS could not be resolved on the basis of the sequencing data available (Supplementary Fig. S1). Analysis of the genes affected by branch somatic mutations restricted to, or enriched in, the DCIS/ILC samples clonally-related to LCIS revealed that in the progression from LCIS to DCIS or ILC, known cancer driver genes were affected by somatic mutations (e.g. $MAP3K1$ (2 cases), $RUNX1$, $NCOR1$, $ARID1A$ and $TBX3$ (2 cases)) or LOH of the wild-type allele (Figs. 1 and Supplementary Fig. S4F, Supplementary Data File 1).

Taken together, our results demonstrate that LCIS clonally-related to DCIS/ILC more frequently displays intra-lesion genetic heterogeneity than LCIS not clonally-related to more advanced lesions, that both truncal and branch mutations are enriched for known cancer drivers, and that known cancer genes are likely targeted by somatic genetic events in the progression from LCIS to more clinically advanced lesions.
Shifts in mutational processes are linked to progression from LCIS to DCIS and ILCs

There is evidence to suggest that the mutational processes that shape the mutational spectra of tumors may change during evolution (16,45). Hence, we sought to define whether changes in mutational spectra were observed in the transition from LCIS to DCIS/ILC. Given that truncal mutations are likely reflective of biological phenomena that took place prior to or during the development of LCIS, and that branch mutations in DCIS/ILC likely stem from mutational processes involved in tumor maintenance and progression, we compared the mutational spectrum of truncal and branch mutations in cases where LCIS was clonally-related to DCIS/ILC. Both truncal and branch mutations were found to be enriched for C>T transitions in the NpCpG context, consistent with a signature ascribed to aging (46), and C>G transversions and C>T transitions in the TpCpW context, suggestive of the mutational processes caused by APOBEC DNA cytosine deaminase activity (47); the latter being predominately found in the branch mutations of case 4 (Fig. 5A) and emerging in the DCIS of case 1 and ILC of case 18 (Fig. 5B-C). Akin to the variations in mutational processes observed in the progression of other cancer types (29,45), in-depth analysis of cases 1, 4 and 18 revealed that a mutational process consistent with the APOBEC signature was active in the progression from LCIS to DCIS or ILC (Fig. 5). Moreover, the mRNA levels of APOBEC3B, a DNA cytosine deaminase that has been causally implicated in the development of APOBEC signature mutations in cancer (47,48), were significantly higher in samples displaying an APOBEC mutational process than in those displaying an aging signature (Fig. 5D). These observations combined to indicate that, at least in a subset of cases, the APOBEC mutational process is likely to be contributing to the development of more advanced lesions.

DISCUSSION

Here we provide direct evidence of the neoplastic and non-obligate precursor nature of at least a subset of LCIS. By performing a clonal decomposition and clonal relatedness analysis of LCIS and synchronously diagnosed DCIS, ILCs and/or DCIS, we have observed that LCIS can display intra-
lesion genetic heterogeneity and be clonally-related to DCIS and ILCs, whereas progression from LCIS to IDC is likely a rare event. Notably, LCIS clonally related to ILCs and/or DCIS were found to display higher levels of intra-lesion genetic heterogeneity than LCIS that were not clonally related to a more advanced lesion, and evidence of clonal selection in the progression from LCIS to ILCs and/or DCIS was documented in a subset of patients. In these patients, the APOBEC mutational process, which has been implicated in genetic instability and intra-tumor genetic heterogeneity, appears to be present later in the evolution of LCIS and may be involved in its progression to more advanced lesions. Interestingly, the samples enriched for APOBEC mutation process displayed higher expression levels of \textit{APOBEC3B}, whose activity has been shown to be mutagenic (47). Therefore, one hypothesize that in a subset of LCIS, upregulation of \textit{APOBEC3B} results in increased mutagenesis and intra-tumor genetic heterogeneity, ultimately promoting subclonal expansions and progression to ILC.

LCIS has been historically considered a less advanced lesion as compared to DCIS, and is usually managed conservatively, not mandating surgical excision (1). Accordingly, in the latest version of the TNM staging system, LCIS is no longer staged as an \textit{in situ} carcinoma (pTis) as DCIS is (49). It should be noted that although we detected clonal relatedness between LCIS and DCIS, and the LCIS as the potential substrate for the development of the DCIS (i.e. case 10), the directionality of the evolution was not clear in three cases (i.e. cases 1, 11 and 16). Hence, we cannot rule out the possibility that in a subset of cases, LCIS may have arisen from a preexistent DCIS or a common precursor (e.g. flat epithelial atypia). In fact, due to the molecular similarities between low-grade LCIS and DCIS (2), inactivation of \textit{CDH1} in a DCIS subclone would be the likeliest explanation for such a phenotypic shift. Bi-directional progression between lesions of lobular (atypical lobular hyperplasia and LCIS) and ductal phenotype (atypical ductal hyperplasia and DCIS) is entirely consistent with the proposed concept of a low nuclear grade breast neoplasia family (2), which encompasses a group of low-grade, ER-positive neoplasms of the breast that not uncommonly affect the same segment of the breast, if not the same terminal ductal-lobular unit, and share a remarkably similar genomic landscape, having concurrent 1q
gains and 16q losses, and PIK3CA mutations, as their genetic signature (2). Alternatively, both LCIS and DCIS might arise from a common precursor (i.e. case 1), such as flat epithelial atypia (2). Taken together, these findings support the notion that the progression of LCIS and DCIS might be bidirectional, or that these lesions may evolve in parallel from a common ancestor.

Our findings demonstrate that LCIS displays a genomic landscape comparable to that of invasive breast cancers of luminal A subtype (31) and/or of lobular histology (6), lesions unequivocally more advanced and that mandate therapeutic intervention. Akin to ILCs (6), LCIS harbors recurrent bi-allelic inactivation of CDH1 (77%), and recurrent mutations affecting genes commonly mutated in breast cancer, including PIK3CA, FOXA1 and TBX3, among other genes. It should be noted, however, that genetic alterations affecting TP53 and PTEN, previously found as recurrent events in ILCs (6), were not identified in the LCIS samples analyzed in this study. These differences might be related to the fact that our cohort included only classic LCIS, but given that progression may occur via clonal selection, and that not only truncal, but also branch mutations are enriched for known cancer genes, it is plausible that acquisition of genetic alterations, including those resulting in inactivation of these two bona fide tumor suppressor genes, may play a role in the progression to ILC. Indeed, we (7,11) and others (13) have demonstrated previously that loss of PTEN may be associated in the progression from DCIS to IDC.

The finding that LCIS is unlikely clonally-related to IDCs is in contrast with previous publications, including that from Begg et al. (7), who reported two LCIS lesions clonally-related to IDCs based on a limited number of shared mutations (one and three mutations in Patients 9 and 14, respectively), which is substantially lower than the number of shared mutations observed in clonally-related LCIS-ILC or LCIS-DCIS lesions in this study (median 12, range 2-171). The mutations described by Begg et al. (7) found to be shared between LCIS and IDC samples may have constituted sequencing artifacts, germline mutations or common single nucleotide polymorphisms (SNPs) (Supplementary Table S2, Supplementary Data File 1), as they were filtered out in our more conservative somatic mutation
analysis. Although we did not detect direct clonal relatedness between LCIS and IDC in this study, we cannot rule out the possibility that a subset of synchronous IDC and LCIS may share a common early precursor or that ductal lesions and LCIS may arise from a common earlier precursor lesion and undergo parallel evolution. In addition, it is also plausible that a subset of LCIS may stem from DCIS harboring 16q losses, but the CDH1 inactivation takes place later in the evolution of the lesion.

Although our findings define LCIS as a non-obligate precursor of ILC, they do not imply that changes in the clinical management of patients presenting with LCIS are necessary, as the rate of subsequent breast cancer development in a large cohort of patients with a diagnosis of LCIS as reported by the SEER database demonstrates a risk of approximately 1% per year (50). Nonetheless, our study might provide a framework for the identification of markers to define LCIS cases that have a greater likelihood to progress. Although some of the pathologic characteristics of LCIS, such as volume of disease, are associated with a greater likelihood of progression to DCIS/ILC, there has yet to be a validated biomarker to predict the behavior of classic LCIS. Based on our results, one could posit that assessing the levels of intra-lesion genetic heterogeneity and/or APOBEC3B activity in LCIS may help select patients that should be counseled more proactively towards surgical excision and/or hormonal chemoprevention, akin to the current management of low- to intermediate-grade DCIS. Increasingly, treatment of early ER-positive breast cancer relies on pathologic features, tumor burden and genomic profiles; our findings suggest that with continued investigation a combination of clinical features, histologic classification, assessment of volume of disease and intra-lesion genetic heterogeneity may allow a more personalized risk assessment for patients with LCIS.

Our study has important limitations. The prospective accrual of frozen samples of LCIS adequate for detailed molecular studies is remarkably challenging; hence the sample size of the present study is small. In addition, our study may not be representative of incidental cases of LCIS, given that the patients included in this study were accrued in a prospective protocol for the multiregional sampling of
prophylactic and/or therapeutic mastectomies from patients with a previous diagnosis of LCIS.

Moreover, we only performed WES analysis, hence we cannot rule out that non-coding alterations and/or epigenetic changes may play a role in the development and progression of LCIS. More comprehensive analyses may also be required to define the alternative drivers of CDH1 wild-type LCIS.

Finally, we used tumor bulk sequencing and state-of-the-art computational approaches to infer the clones present in each sample/case and their phylogeny. Single-cell sequencing analyses of LCIS and synchronous lesions are warranted to confirm our findings and provide direct evidence of the clonal composition of LCIS and of clonal selection in the evolution to more advanced lesions.

Despite these limitations, this proof-of-principle study demonstrates that LCIS is a neoplastic non-obligate precursor of DCIS and ILC, with a repertoire of somatic genetic alterations similar to that of ILCs and luminal-A invasive breast cancers, but lacking TP53 and PTEN mutations. LCIS at diagnosis often displays intra-lesion genetic heterogeneity, and, in a subset of cases, the progression from LCIS to DCIS and ILC may involve the selection of clones, which may harbor distinct active mutational processes such as APOBEC. Our findings suggest that early documentation of intra-lesion genetic heterogeneity may be central to developing robust molecular predictors of the risk of LCIS progression/evolution into more aggressive forms of breast cancer.

DATA AVAILABILITY

WES data have been deposited in the database of Genotypes and Phenotypes (dbGaP) under the accession phs001006.v1.p1.

AUTHORS’ CONTRIBUTIONS

DBS, BW, JSR-F and TAK conceived the study and supervised the work. RAS and TAK provided samples. DDG and JVSC reviewed the cases. Sample processing was performed by RAS, ADP, JVSC and RT. Massively parallel sequencing was carried out by AV. Bioinformatics analysis was performed
by JYL, MS, PS, RSL and CKYN. CKYN and JSR-F coordinated the bioinformatics analyses. JYL, MS, FCG, SP, CKYN, RAS and RSH performed data analysis and data interpretation. SP performed statistical analyses. JYL, FCG, BW and JSR-F wrote the first draft of the manuscript, which was initially reviewed by MS, SP, CKYN, RSH and TAK. All authors edited and approved the final draft.

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REFERENCES


TABLE 1: Clinico-pathologic characteristics of the 24 patients included in the study.

<table>
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<th>Breast laterality</th>
<th>Frozen tissue blocks analyzed (n)</th>
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<th>Tumor size (invasive, mm)</th>
<th>Lymph node status</th>
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<th>PR</th>
<th>HER2</th>
<th>Sakr et al.</th>
<th>Begg et al.</th>
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DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma of no special type; ILC, invasive lobular carcinoma; LCIS, lobular carcinoma in situ; ER, estrogen receptor; PR, progesterone receptor; HER2, epidermal growth factor receptor 2; +, positive; -, negative; N/A, not available; *all LCIS are of classic type; **all DCIS are of grade 2; Y, the case was analyzed in the previous study; N, the case was not analyzed in the previous study.
FIGURE LEGENDS

Fig. 1: Landscape of somatic genetic alterations of lobular carcinoma in situ (LCIS) and associated lesions.

A) Heatmap illustrating the recurrent (n≥2) somatic mutations and selected gene amplifications in LCIS (n=43), invasive lobular carcinomas (ILCs, n=13), ductal carcinoma in situ (DCIS, n=9) and invasive ductal carcinomas (IDC, n=5), subjected to whole-exome sequencing. Cases are shown in columns, grouped according to histologic category color-coded according to the legend, and genes in rows. Somatic mutations affecting cancer genes listed in Kandoth et al. (20), the Cancer Gene Census (21) and/or Lawrence et al. (22) are ordered from top to bottom in decreasing order of frequency, followed by selected gene amplifications. Genes highlighted in bold and/or red represent significantly mutated genes in ILCs and/or luminal-A invasive breast cancers from The Cancer Genome Atlas breast cancer studies (6,31). B) Heatmap illustrating the copy number alterations found in LCIS (n=43), ILC (n=13), DCIS (n=9) and IDC (n=5). For A and B, mutation type, copy number states, and/or type of lesion are indicated according to the color keys on the right of the figure. Indel, insertion and deletion; SNV, single nucleotide variant.

Fig. 2: Comparison of mutation rate and frequency of mutations at gene level affecting lobular carcinoma in situ (LCIS) and invasive lobular carcinomas (ILCs) and Luminal A breast cancers from The Cancer Genome Atlas (TCGA) breast cancer study.

A and B) Boxplots showing the mutation burden and C and D) mutation rate (Mutation/Mb) in LCIS samples (n=43) and ILC (n=13), DCIS (n=9) and IDC (n=5) from this study and ILC (n=127) and Lumina A tumors (n=209) from TCGA (31). NS: not significant *p-value>0.05, **p-value>0.1 (Mann–Whitney U test). E) Heatmap depicting the most recurrently mutated genes affecting cancer genes identified in LCIS samples from this study and ILCs (6) and luminal A invasive breast cancers (31) from TCGA. Cases are shown in columns, genes in rows. Fisher’s exact test comparisons of mutational frequencies of the mutated genes were performed between LCIS from this study (n=43) and 127 ILCs
and 209 Luminal A breast cancers (31) from TCGA. The significantly different mutation frequencies between LCIS and TCGA ILCs and/or luminal A breast cancers (TCGA) are highlighted with an asterisk, where **p-value < 0.01 and *p-value < 0.05 (Fisher’s exact test).

**Fig. 3:** Clonal relatedness and intra-lesion genetic heterogeneity in lobular carcinoma *in situ* (LCIS), invasive lobular carcinoma (ILC), ductal carcinoma *in situ* (DCIS).

A) Schematic representation of the anatomical locations (breast quadrants) of all sequenced samples in each patient and their clonal relatedness. Clonally-related lesions are connected by orange or green lines, while those in black represent lesions without a clonal relationship with any other lesion from the respective patient. In cases of unilateral LCIS, only the left or right breast was represented, and in cases of bilateral LCIS, both breasts were schematically depicted. Boxplot illustrating the distribution of B) the Shannon diversity index and C) the Gini-Simpson diversity index in LCIS not clonally-related to DCIS/ILC (n=25) and in LCIS clonally-related to DCIS and/or ILC (n=18). The colored dots indicate the cases. *P*-values of unpaired t-test with Welch’s correction are indicated at the top of each figure. NS: not significant, p-value >0.05.

**Fig. 4:** Clonal composition of clonally-related lobular carcinoma *in situ* (LCIS) and ductal carcinoma *in situ* (DCIS) or invasive lobular carcinoma (ILC) and potential clonal selection during progression.

A and B) Decomposition of genetically distinct clones and clonal evolution in lesions from A) case 4 and B) case 10 using the results from PyClone (26). On the top left, a schematic representation of the quadrants from which each sequenced lesion was sampled is shown, and on the top right, the clonal frequency heatmap of mutations within the lesions of each case, grouped by their inferred clonal/subclonal structure (clusters). Non-synonymous somatic mutations are shown. The clusters inferred by PyClone are shown below the clonal frequency heatmap, and the Shannon index measuring intra-lesion genetic heterogeneity for each lesion is specified within parentheses after the sample names in
the heatmap. On the bottom left, the parallel coordinates plot generated by PyClone and, in the middle right, a cluster-based phylogenetic tree based on the clusters identified by PyClone are shown. The color of the trunk and branches matches the color of their respective clusters shown in the parallel coordinates plot. On the bottom right, a histologic lesion-based phylogenetic tree constructed using Treeomics (30) is depicted. The mutations affecting cancer genes (colored in orange) and the hotspot mutations (colored in red) that define a given clone are illustrated alongside the branches. The length of the branches is proportional to the number of mutations that distinguish a given clone from its ancestor. The numbers alongside the branches represent the total number of somatic mutations.

**Fig. 5: Mutational signatures of trunk and branch mutations in lobular carcinoma in situ (LCIS) clonally-related ductal carcinoma in situ (DCIS) or invasive lobular carcinoma (ILC).**

Evolution of the mutational processes with a schematic representation of the subclone structure in **A)** case 4, **B)** case 1 and **C)** case 18. Each black line represents the acquisition of somatic genetic alterations that define a given clone and each arrow depicts the divergence of a cell population from one lesion to another along with the acquisition of a set of somatic genetic alterations. The mutational signature representative of newly acquired mutations by a given subclone is depicted adjacent to each circle. The pie chart depicts the proportion of mutational signatures detected and signature 1 (aging, blue), signature 2 (APOBEC, violet) and signature 13 (APOBEC, green) are shown, with the remaining mutational signatures merged as ‘Others’ (dark gray). The number alongside the branches is the total number of somatic mutations. **D)** Reverse transcription quantitative PCR (RT-qPCR) of APOBEC3B (left), APOBEC3H (middle) and REV1 (right) genes in samples displaying the APOBEC-related and aging-related signatures, where tissue samples were available for RNA extraction. The error bars represent the standard deviation of mean of RT-qPCR data (n=3).
Figure 2

(A) Box plots showing the distribution of all mutations (n) and non-synonymous mutations (n) for different breast cancer subtypes. LCIS (n=43), DCIS (n=9), ILC (n=13), IDC (n=5), TCGA ILC (n=127), TCGA Luminal A (n=209). 

(B) Box plots showing the distribution of all mutations (n) and non-synonymous mutations (n) for different breast cancer subtypes. LCIS (n=43), DCIS (n=9), ILC (n=13), IDC (n=5), TCGA ILC (n=127), TCGA Luminal A (n=209). 

(C) Box plots showing the mutation rate of all mutations (mutations/Mb) for different breast cancer subtypes. LCIS (n=43), DCIS (n=9), ILC (n=13), IDC (n=5), TCGA ILC (n=127), TCGA Luminal A (n=209). 

(D) Box plots showing the mutation rate of non-synonymous mutations (mutations/Mb) for different breast cancer subtypes. LCIS (n=43), DCIS (n=9), ILC (n=13), IDC (n=5), TCGA ILC (n=127), TCGA Luminal A (n=209). 

* non significant (ns) p-value > 0.05
** non significant (ns) p-value > 0.1

(E) Heatmap showing the mutation type distribution for different breast cancer subtypes. CDH1 (n=43), TCGA ILC (n=127), TCGA Luminal A (n=209). 

Mutation Type: Hotspot Mutation, Truncating SNV, Frame-shift indel, Missense SNV, In-frame indel, Splice site mutation

* p-value < 0.05
** p-value < 0.01
Figure 3

A

Cases 1 to 24 show various patterns of lesions, with labels indicating different types of lesions such as LCIS, ILC, IDC, and DCIS. Cases are numbered from 1 to 24.

B

Panel B contains a box plot with the following data groups:
- LCIS not clonally related to ILC/DCIS (n=25)
- LCIS clonally related to ILC/DCIS (n=18)
- LCIS clonally related to ILC (n=12)
- LCIS clonally related to DCIS (n=6)

The plot shows a comparison of Shannon Index, with a significance level of P = 0.005.

C

Panel C contains a box plot with the following data groups:
- LCIS not clonally related to ILC/DCIS (n=25)
- LCIS clonally related to ILC/DCIS (n=18)
- LCIS clonally related to ILC (n=12)
- LCIS clonally related to DCIS (n=6)

The plot shows a comparison of Gini-Simpson Index, with a significance level of P = 0.005.

ns: non significant (p-value > 0.05)