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Report

Biomarker profile and genetic abnormalities in lobular carcinoma *in situ*

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Summary

The predisposition of patients with lobular carcinoma *in situ* (LCIS) to develop invasive breast cancer (IBC) is well known. However, relatively little is known about the biologic characteristics, which may be involved in the development and progression of LCIS. This study evaluated 59 cases of LCIS (29 pure, 30 with synchronous IBC) for five biomarkers known to be important in IBC (ER, PgR, c-erbB-2, p53 and Ki-67 proliferation rate) by immunohistochemistry. A comprehensive analysis of loss-of-heterozygosity (LOH) was performed in 12 cases (10 pure, 2 with synchronous IBC) at 15 genetic loci on 9 chromosomes. LCIS demonstrated a low grade/favorable biophenotype that was not significantly different in cases with and without synchronous IBC (ER 98%, PgR 84%, c-erbB-24%, p53 19% and proliferation rate 2%). LOH was present in 80% of pure LCIS and the highest rates of LOH were at loci on 9p (30%), 16q (63%), 17p (33%) and 17q (50%). The clustering of LOH at these four foci suggests that inactivated tumor suppressor genes in these regions may be particularly important. LOH was present in both cases of LCIS with synchronous IBC and the LOH phenotype was shared by LCIS and IBC. Our findings suggest that five known prognostic factors in IBC do not have prognostic utility in LCIS. Multiple genetic mechanisms may be involved in the development of LCIS.

Introduction

Since 1941, when lobular carcinoma *in situ* (LCIS) was first described [1], a considerable amount of controversy has been generated surrounding the natural history of and appropriate therapy for this lesion. Retrospective studies suggested that subsequent invasive breast cancer (IBC) in patients with LCIS was nearly as likely to occur in the contralateral breast as the ipsilateral breast [2–4] and the IBC that developed was as likely to be ductal as lobular [5–7]. In contrast, the risk of developing IBC associated with ductal carcinoma *in situ* (DCIS) is mostly ipsilateral [8]. This has led to the belief in the minds of treating physicians that DCIS is a precursor but LCIS is only a marker of risk for the development of subsequent IBC. Therefore, these two types of *in situ* cancers are managed very differently. DCIS is primarily treated by surgical excision. On the other hand, patients with LCIS have been historically treated either with close follow-up or sometimes offered either unilateral or bilateral mastectomies in order to completely reduce the risk of developing breast cancer, based on the notion that LCIS is only a marker of risk for IBC [6, 7, 9]. However, even historical studies suggest that when LCIS is found with synchronous IBC, up to 90% IBC are of lobular type or show prominent lobular differentiation [10, 11], suggesting a possible precursor-product relationship. In addition, a few studies have demonstrated either sharing of LOH phenotype or mutation in E-cadherin gene in LCIS with adjacent IBC, lending support to the hypothesis that at least some LCIS lesions may be precursors for IBC [12, 13]. Similarly, there is recent evidence suggesting that there may be differences in mammographically detected LCIS [14], and there is a substantial risk of finding cancer if core needle biopsy contains LCIS [15]. These more recent but scant studies have led some clinicians to reconsider their traditional approach to manage patients diagnosed with LCIS. It is therefore important now to study the biological characteristics and factors involved in progression of LCIS in more detail.

As compared to DCIS, relatively little is known about the biological and genetic mechanisms involved in the development and progression of LCIS. In order to better describe this lesion, we have studied five biomarkers (ER, PgR, c-erbB-2, p53 and Ki-67 proliferation rate) in LCIS by IHC. These five markers are known to be important in prognosis in IBC and our objective is to assess their usefulness in prognosis of LCIS. In order to assess this, we have compared the biomarker profile of LCIS without IBC to LCIS with synchronous IBC. The idea is that the pure LCIS at the

time of diagnosis has not acquired the full biologic profile of LCIS with adjacent IBC, and this comparison may identify those factors which may be important in progression of LCIS to IBC. We also analyzed LOH at 15 loci on 9 chromosomes, including several foci not previously studied in LCIS but are known to be important in IBC, to identify new genetic alterations that may be involved in pathogenesis of LCIS.

Materials and methods

Tumor specimens

Twenty-nine [29] consecutive unselected cases of pure LCIS were identified in our pathology files from 1986 to 1995. These were matched with 30 cases of LCIS with synchronous IBC. Patient's with pure LCIS ranged in age from 33 to 76 (median 49 years), while those with IBC ranged in age from 40 to 76 (median 53 years). All the cases were reviewed by one pathologist (ALL) and the diagnosis was confirmed prior to inclusion of the cases in this study. The diagnostic criteria of Page et al. were used to define LCIS [16]. These are: (1) the involved acini are populated exclusively by the characteristic cells of lobular neoplasia and (2) these cells, fill, distend, and distort at least one half of the acini within the lobular unit. These criteria define what is now referred to "classical LCIS". None of the variants of LCIS, such as pleomorphic LCIS, or those with synchronous DCIS or those cases with histological features overlapping between LCIS and DCIS were included in this study. Similarly, cases of atypical lobular hyperplasia (ALH) were excluded from the study. The study was conducted with approval of the local Institutional Review Board.

Immunohistochemistry

All fifty-nine cases of LCIS were evaluated for ER, PgR, c-erbB-2, p53 and Ki-67 by immunohistochemistry. Routinely processed formalin-fixed tissue was used. Antibodies 6F11 (Novocastra, UK) for ER, 1A6 (Novocastra, UK) for PgR, Tab 250 (Zymed, CA) for c-erbB-2, DO-1 (Dakocytomation, CA) for p53, and MIB1 (Dakocytomation, CA) for Ki-67 proliferation rate were used with a strept-avidin–biotin–peroxidase detection system (Dakocytomation, CA) and DAB/H_2O_2 chromogen. Heat-induced antigen retrieval (citrate buffer at pH 6.0 boiled in a pressure cooker for 5 min) was employed when assessing ER, PgR, p53, and MIB1.

Slides immunostained for ER, PgR, c-erbB-2, and p53 were scored using the "Allred Score", which has been used in several previous studies from our laboratory assessing these four biomarkers in IBC [17–21]. Briefly, this scoring system is based on estimating the proportion and average intensity of positive tumor cells (proportion score: 0 $\Rightarrow$ none, 1 $\Rightarrow$ $<1/100$, 2 $\Rightarrow$ 1/100-1/10, 3 $\Rightarrow$ 1/10-1/3, 4 $\Rightarrow$ 1/3-2/3, 5 $\Rightarrow$ $>2/3$; intensity score: 0 $\Rightarrow$ none, 1 $\Rightarrow$ weak, 2 $\Rightarrow$ intermediate, 3 $\Rightarrow$ strong). The proportion and intensity scores are added to obtain a total score (ranging from 0, 2–8). Cases with total scores ≥ 3 were considered positive for ER and PgR, as we have validated this cutoff in IBC [19, 22]. Total scores >0 were used to define p53 and c-erbB2 positive as previously done in studies of IBC from our laboratory [17, 23]. Slides immunostained for Ki-67 proliferation rate were evaluated by point-counting at least 200 tumor cells at intersects on a 10×10 1 mm^2 ocular grid, and reported as percent positive cells. No cut-offs were used to define low versus high proliferation rates and percent staining was assessed and analyzed as a continuous variable. Representative examples showing positive phenotypes for each of the five markers assessed with this methodology are illustrated in Figure 1.

Analysis of LOH

Histological slides from routine archival (formalin-fixed and paraffin-embedded), clinical cases were screened microscopically for adequate amounts of normal

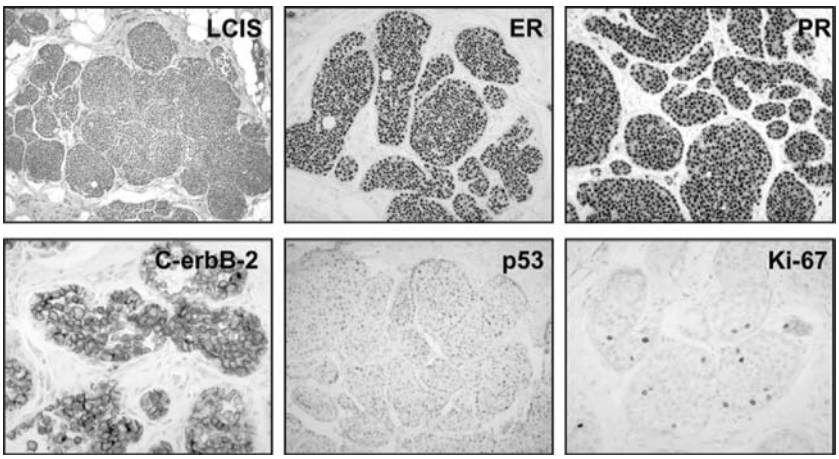

Figure 1. Biomarker Profile of LCIS. These are representative examples of histological features (Hematoxylin and eosin stain) and the five biomarkers, i.e. ER, PgR, erbB-2, p53 and Ki-67 proliferation rate of LCIS (magnification 400×).

(control) tissue, LCIS, and IBC. A total of 16 cases (14 with pure LCIS and 2 with LCIS and IBC) had enough tissue to perform macrodissection of both normal and neoplastic tissue and were therefore candidates for LOH studies. In approximately 75% of cases, normal tissue consisted of benign breast tissue (terminal-duct lobular units [TDLUs] or large ducts) within the same specimens. Skin (epidermis) or lymph nodes from the same specimens were used as normal controls in the remaining cases. On the basis of data suggesting that closely adjacent breast cancer and morphologically normal TDLUs may occasionally share LOH for certain loci [24], we may be slightly underestimating rates of LOH for some markers in cases where adjacent TDLUs were the only source of normal tissue available.

Alternating 3-μm and 10-μm histologic sections were cut from selected formalin-fixed, paraffin-embedded tissue blocks and float-mounted onto glass slides. Areas of interest on the hematoxylin–eosin-stained 3-μm slides were outlined as a template to guide the independent manual microdissection of corresponding regions on the unstained 10-μm slides. Cellular enrichment was about 90% in the majority of samples.

DNA was liberated from the samples by a modification of the method of Wright and Manos [25]. The methods used in this study have been previously described [26]. Samples were independently evaluated for LOH at each of 15 highly polymorphic microsatellite loci known to be important in IBC [26]. Genetically informative samples showed two distinguishable alleles. On average, about 90% of samples were informative for each locus**.** Overall, two cases of pure LCIS were considered uninformative and were not included in any further analyses. Only 5–10% of cases for each locus showed evidence of microsatellite instability, and information from such cases were omitted from further analysis of those loci. The intensity ratio of the two allelic bands of DNA from normal tissue relative to DNA from lesions in the same case was obtained from digitized data collected with a phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA) and analyzed with Image-Quant software (Molecular Dynamics, Inc.). A conservative ratio of greater than or equal to 1.5:1 (ratio of normal tissue to tumor) was used to define LOH in this study. Our 75% or more level of target cell enrichment is adequate to meet or exceed this ratio in cases with pervasive LOH. In addition, all cases showing LOH in the original assay were repeated twice, and only those showing losses in all three assays were considered as positive for LOH.

Statistical analysis

LCIS with synchronous IBC was compared to pure LCIS for each biomarker, using Fisher's exact test.

Results

Biomarker profile of LCIS

The results of immunohistochemical staining for all five biomarkers (ER, PgR, c-erbB-2, p53 and Ki-67 proliferation index) are summarized in Table 1. Nearly all cases of LCIS were not only positive for ER (98%), but they expressed very high level of ER (TS ≥ 6 in 75% of the cases). A large number of cases expressed PgR (84%), however, unlike ER, PgR expression was evenly distributed over a wide range of expression. C-erbB-2 and p53 expression were seen in only a handful of LCIS cases (4 and 19%, respectively) and in these cases, the level of expression was generally low (TS ≤ 4). LCIS demonstrated a low proliferation rate (average = 2%; range 0–11%). The results for all 5 biomarkers were evaluated separately in LCIS with and without synchronous IBC and no statistically significant differences were found between these two groups of LCIS.

LOH analysis in LCIS

Table 2 summarizes the rates of LOH observed at 15 genetic loci known to have high rates of loss (i.e. >25%) in fully developed IBC [26]. The highest rates of loss in LCIS were observed on 9p/D9S157 (30%), 16q21/D16S265 (63%), 17p13/D17S960 (33%), and 17q11/NF1 (50%). Relatively high rates of LOH were also found at 8p/D8S264 (20%) and 11q23/D11S1328 (25%). Among 10 cases of LCIS without synchronous IBC, 80% showed LOH with equal numbers showing LOH at 1, 2, 3 and 4 foci. Two cases of LCIS were evaluated from breasts with synchronous IBC. Both the LCIS and IBC components of each case showed LOH at one locus, and shared their LOH phenotype. A representative electrophoretic gel showing shared phenotype

Table 1. Biomarker phenotypes for LCIS with and without synchronous IBC

	ER % pos (*n*)	PgR % pos (*n*)	c-erbB-2 % pos (*n*)	p53 % pos (*n*)	Ki-67* avg % (*n*)
All cases	98% (57)	84% (56)	4% (56)	19% (52)	2% (52)
LCIS	96% (28)	89% (28)	7% (28)	18% (28)	2% (27)
LCIS with IBC	100% (29)	79% (28)	0% (28)	21% (24)	2% (25)
p-value	NS	NS	NS	NS	NS

NS = Not significant.

* Average % represents average of Ki-67 percent positive cells of all cases in each category.

Table 2. Summary of LOH at 15 genetic loci on 10 chromosomes in LCIS

Marker/location	% LOH (*n*)
TPO/2pter	0 (7)
D2S362/2q35	0 (6)
D4S192/4q25	0 (11)
D6S417/6qter	0 (5)
D8S264/8p	20 (10)
D9S157/9p	30 (10)
D11S988/11p15	0 (7)
D11S1328/11q23	25 (8)
D13S137/13q13	10 (10)
D14S62/14q24	0 (10)
D16S265/16q21	63 (8)
D17S960/17p13	33 (9)
NF1/17q11	50 (8)
D17S597/17q21	0 (9)
D17S787/17q25	13 (8)

of LCIS and adjacent synchronous IBC is illustrated in Figure 2.

Discussion

Relatively little is known about the natural history of LCIS. Foote and Stewart first described LCIS in 1941 as a premalignant lesion [1] and since then, several long term- retrospective studies have shown that the relative risk of developing IBC following a biopsy showing LCIS ranges from 5.0 to 17.0 [9, 10, 27, 28]. However, the data about the laterality of breast cancer risk has been confusing. Several earlier studies suggested that the risk of subsequent breast cancer is not significantly different for either breast [3, 4, 6, 7, 9, 10, 27, 29, 30]. Based on data from the earlier studies, most physicians today believe that LCIS is only a ''marker'' of increased risk for subsequent invasive carcinoma rather than a direct precursor lesion [5–7], unlike DCIS, which is considered a direct precursor of IBC.

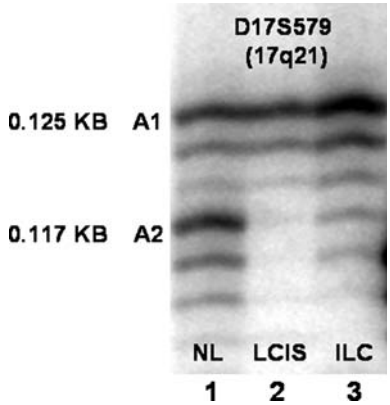

Figure 2. Loss of Heterozygosity Assay. Shown is an LOH assay with marker D17S579 (17q21). Formalin-fixed, paraffin-embedded archival specimens from a single patient were microdissected. DNA from normal tissue (1, NL), *in situ* (2, ILCV), and invasive (3, ILC) cancers were polymerase chain reaction (PCR) amplified with D17S579 primes radiolabeled with γ-ATP^{32}. PCR products were subjected to denaturing polyacrylamide gel electrophoresis and autoradiography. Normal tissue DNA is heterozygous for alleles A1 (0.125 kb) and A2 (0.117 kb). The DNA from the LCIS and ILC are homozygous for allele A1, indicating somatic loss of allele A2. Loss of allele A2 in the both the LCIS and ILC suggests clonal evolution. The allele ''laddering'' is characteristic of dinucleotide repeat markers.

LCIS is far less common than DCIS and makes it difficult to study this lesion in a comprehensive manner in a large series of cases. Therefore, the biophenotype of LCIS has been previously studied in relatively small data sets and in a largely piecemeal fashion. Only two previous small studies ($n = 19$ and 23) have evaluated all five biomarkers in a single data set [31, 32]. We studied LCIS in a relatively large series of cases to significantly improve our understanding of biophenotype of this lesion. LCIS demonstrated a low-grade/well-differentiated phenotype for the biomarkers evaluated in this study. Our findings are compared to those of previous studies in Table 3 [14, 31, 33–45]. The biophenotype of LCIS is similar but in general better differentiated than low-grade DCIS, and markedly different from intermediate and high grade DCIS [46]. Ninety-eight percent of our cases were ER positive. These findings are similar to three previous studies, which also utilized IHC [32, 39, 44]. The lower values obtained by Rudas et al., Giri et al. and Pertschuk et al. [31, 34, 36] are likely related to the different cut-off values used to define a positive result (10, 25 and 10%, respectively). The relatively low value obtained by Pallis et al. [40] can be explained by their use of enzyme immunoassay (EIA), which requires tissue homogenates rather than tissue sections as well as a very small sample size. The findings for PgR are similar with the exception of the unexplained finding of 47% positivity by Querzoli et al. [32].

C-erbB2 is the most widely studied marker in LCIS. Previous studies assessing c-erbB-2 overexpression in LCIS have shown either no overexpression or low rates of overexpression similar to our finding of overexpression in 4% cases [14, 31–33, 35, 37, 38, 41, 42, 44]. Our finding of 19% positivity for p53 is similar to the findings of Querzoli et al. [32]. Rudas et al. and Siziopikou et al. [31, 45], who used the same antibody but a cutoff level of 10% in their studies found no positive cases (combined $n = 33$). Use of cutoff value of 10% in our study does not affect the 4% cases being classified as c-erbB-2 overexpressors. Domagala et al. [43] used the same antibody that we used in this study with a similar cutoff level and found no positive cases, probably related to the small number of cases in their study ($n = 7$). Only three other studies have assessed proliferation rate in LCIS [31, 32, 44]. Their findings are consistent with ours, showing a low proliferation rate in LCIS.

Our LOH findings as well as the findings from a handful of previous studies of genetic alterations in LCIS [12, 13, 47–49] indicate that LCIS is a clonal (neoplastic) proliferation. Lakhani et al. [47] evaluated 43 cases of LCIS for LOH at only 4 loci. They found

Table 3. Estrogen receptor, progesterone receptor, c-erbB-2, p53 and proliferation rate in LCIS – a review of the literature

Reference	ER % pos (*n*)	PgR % pos (*n*)	c-erbB-2 % pos (*n*)	p53 % pos (*n*)	Proliferation rate
This study	98 (57)	84 (56)	4 (56)	19 (52)	2% (52)[1]
(44)	100 (NA)	100 (NA)	0 (15)		[2]
(31)	80 (23)	90 (23)	4 (23)	0 (23)	1.3% (23)[1]
(32)	100 (19)	47 (19)	11 (19)	21 (19)	0% (19)[3]
(40)	67 (6)	60 (5)			
(34)	60 (10)				
(36)	86 (7)				
(39)	100 (5)				
(67)			0 (16)		
(35)			0 (5)		
(37)			0 (48)		
(42)			0 (10)		
(41)			0 (9)		
(38)			2 (57)		
(43)				0(7)	
(45)				0 (10)[4]	
(14)	50 (10)	[6]	[6]	0 (10)	10 (10)[6]

NA = Number of cases not available.
[1] Ki-67 (IHC) using MIB-1 clone.
[2] S-phase reported as "normoproliferative" in an undefined number of cases.
[3] % of cases with proliferation rate greater than 13% as assessed by Ki-67 (MIB-1 clone).
[4] % cases with >10% of cells staining for p53.
[5] ALH and LCIS.
[6] Exact number of positive cases was not documented for ER, PgR or c-erbB-2. A cut-off value of 20% was used to define high proliferation rate. The proliferation rate ranged from 2 to 15%.

rates of LOH ranging from 8% on 17p to 50% on 17q, and no differences were found in the rate of LOH in LCIS cases with and without synchronous IBC. Nayar et al. [12] evaluated 38 cases containing some combination of ALH, LCIS and infiltrating lobular carcinoma (ILC) for LOH at one locus on 11q13, and found that LCIS associated with synchronous ILC showed LOH in 54% of cases while LCIS without synchronous ILC showed much lower rates of LOH, similar to ALH [12]. Based on their preliminary findings, they postulated that genetic alterations at 11q13 might play a part in the progression to IBC. Lu et al. employed comparative genomic hybridization (CGH) for a global look at genetic alterations in LCIS and found losses at 16p, 16q, 17p and 22q and gains at 6q. We performed a fairly comprehensive survey of LOH in LCIS looking at 15 loci on 10 chromosomes, previously shown to be important in IBC. We found the highest rates of LOH on 9p (30%), 11q23 (25%), 16q (63%), 17p (33%), and 17q (50%). LOH at 9p has not been reported before. It is not clear that our probe for 11q23 is related to 11q13 used by Nayar et al., but it seems that these are most likely detecting the same genetic alteration. Our rates of LOH at 16q and 17p are much higher than 30 and 8% found by Lakhani et al. [47]. Like our study, Lakhani et al. have also reported a 50% LOH at 17q [47], but they were looking at BRCA1 locus.

LOH may result from a loss of tumor suppressor genes, from allelic imbalance due to (onco)gene amplification, or simply from non-specific genomic instability. However, when high rates of loss (i.e., >20%) are repeatedly observed at specific loci, they usually indicate the approximate locations of inactivated tumor suppressor genes [26]. Candidate genes that might be responsible for some of the highest rates of LOH include E-cadherin on 16q, [50] p53 on 17p [51] and NF1 [52] and BRCA1 [53] on 17q. Of these, p53, [54, 55] NF1, [56, 57] and BRCA1 [53] have shown a substantial relationship between mutation and loss in several types of cancer, including IBC, but the significance of these genes in precursor lesions remains speculative. Loss of expression of the intercellular adhesion molecule E-cadherin appears to play an important role in the development of *in situ* and invasive lobular neoplasia [13, 49, 58, 59]. Our finding of 30% LOH at 9p and 50% at NF1 have never been described in LCIS and seem interesting. Candidate genes at 9p include RECK tumor suppressor gene, which is regulated by histone deacetylase complex and is an inhibitor of MMP2. Another interesting finding in our study is that the rates of incidence of LOH are similar to those for DCIS from our previous study [26] and from the literature [60–62].

There is recent debate that some LCIS, which are associated with synchronous IBC, represent direct precursors of IBC, however, the data is conflicting. For example, Nayar et al. examined 12 cases of LCIS with synchronous IBC at one genetic locus (11q13) and found that 5 cases showed a shared LOH phenotype. On the

other hand, Lakhani et al. did not demonstrate any LOH in synchronous IBC, but attributed their findings to contamination of tissue used for LOH analysis with normal stroma [47]. We wanted to compare the LOH patterns between LCIS and IBC but were limited to only two cases with sufficient material to perform the assay for all 15 loci. Based on such limited data, no definitive hypothesis can be generated but the finding of sharing of LOH between LCIS and synchronous IBC would be more in keeping with the notion that some LCIS may be direct precursors of IBC. Studies with larger samples and perhaps using techniques with more comprehensive look at the genome are needed to address this issue. Until very recently, limitation of frozen samples of LCIS has precluded such studies, but with recent developments in technology to use paraffin tissue for gene expression profiling may help perform such studies.

In summary, the purpose of our study was to describe the biological profile of LCIS at the protein and genetic level and we have shown that LCIS has low grade/well differentiated biomarker phenotype in the largest series of cases so far. Our data indicates that multiple genetic mechanisms are likely to be involved in the development of LCIS and have identified two new loci of LOH at 9p and NF1 in LCIS, which may be important in the genetic evolution of LCIS. However, these preliminary studies also show that none of the biologic or genetic alterations are specific enough to discriminate subset of LCIS that is likely a precursor from those that are just a marker of general genetic instability in the breast epithelium and are therefore a marker of risk to develop subsequent invasive cancer. Continued study in this area will hopefully allow us to better predict the premalignant potential of LCIS, with the ultimate goal of preventing the development of IBC.

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