Loss of heterozygosity in lobular carcinoma in situ of the breast

S R Lakhani, N Collins, J P Sloane, M R Stratton

Abstract

Aims—(1) To investigate whether loss of heterozygosity identified at various loci in invasive breast carcinoma or is present in lobular carcinoma in situ (LCIS). (2) To investigate whether LCIS is a monoclonal (neoplastic) or a polyclonal (hyperplastic) proliferation.

Methods—Forty three cases of LCIS (30 with associated invasive carcinoma or in situ ductal carcinoma (DCIS) and 13 cases of pure LCIS) were investigated for loss of heterozygosity on chromosomes 16q, 17q, 17p, and 13q using a microdissection technique, polymorphic DNA markers, and the polymerase chain reaction (PCR).

Results—Loss of heterozygosity was detected in both subgroups of LCIS at all the loci examined. There was no significant difference in the frequency of the loss between the group associated with invasive carcinoma and the pure LCIS group. The frequency of loss of heterozygosity ranged from 8% on 17p to 50% on 17q.

Conclusions—Because of the nature of the technique employed, our findings show that LCIS is a monoclonal (neoplastic) proliferation rather than a hyperplastic proliferation. The incidence of loss of heterozygosity on 17p (D17S796) is lower than we have observed previously in DCIS, suggesting that LCIS and DCIS are different genetically as well as clinically and morphologically. The similar incidence of loss of heterozygosity on 16q and 17q, however, suggests that DCIS and LCIS may share a common pathway of evolution.


Keywords: Breast cancer, lobular carcinoma in situ, loss of heterozygosity.

Lobular carcinoma in situ (LCIS) of the breast is an uncommon lesion with a distinctive histological appearance characterised by masses of loosely adherent cells with small round, monotonous, hyperchromatic nuclei that distend acini of the lobular unit. In contrast to ductal carcinoma in situ (DCIS), the disease is often multicentric and bilateral. Over 80% of the cases are diagnosed between 40 and 50 years of age, usually as an incidental finding in a biopsy taken for other palpable or mammography detected benign or malignant lesions. LCIS is not palpable and rarely visible on mammography. Over the 25 years following diagnosis, approximately one fifth of patients with LCIS will develop invasive cancer. Invasive cancers are equally likely to occur in the contralateral breast and in the breast known to contain LCIS. This is in contrast to partially resected DCIS in which the invasive cancer usually develops in the same quadrant of the same breast as the DCIS. Approximately 50% of invasive cancers developing upon a background of LCIS are lobular in type, the remainder being a mixture of ductal-NST (no special type), tubular, and others.

The biological nature of LCIS and its relationship to invasive carcinoma are ill defined. LCIS has been regarded solely as a risk indicator for invasive cancer. By this hypothesis, the presence of LCIS indicates that the whole breast epithelium is at increased risk of neoplastic transformation, and the invasive cancer itself does not necessarily arise from LCIS cells. An alternative postulate (which is generally accepted for DCIS) is that LCIS cells are intermediates in the progression to invasive cancer (the “transitional” hypothesis). By this view, the apparent bilaterality of the risk of invasive cancer in patients with LCIS is attributable to the multifocality of the disease. In this report we have studied loss of heterozygosity in LCIS in order to determine if it is a clonal proliferation and to clarify its relationship to DCIS and invasive cancer.

Methods

Cases of LCIS were obtained from the histopathology archives of the Royal Marsden NHS Trust. Criteria for diagnosis were those adopted for the UK National Breast Screening Programme. Forty three cases of LCIS were examined. In 27, LCIS was associated with an invasive cancer, of which 20 were classified as purely lobular, four as purely ductal, and three as showing features of both. In five of these cases DCIS was also present and in three cases there was DCIS but no invasive tumour. In the remaining 13 cases, LCIS was associated with benign changes only. Three cases were bilateral.
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<table>
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<tr>
<th></th>
<th>16q D16S413</th>
<th>17p D17S796</th>
<th>17q D17S250</th>
<th>13q D13S267</th>
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<tbody>
<tr>
<td>LCIS + Inv or DCIS (n=30)</td>
<td>8/24 (33%)</td>
<td>2/26 (8%)</td>
<td>2/2 (100%)</td>
<td>1/1 (100%)</td>
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<tr>
<td>Pure LCIS (n=13)</td>
<td>2/10 (20%)</td>
<td>1/11 (9%)</td>
<td>2/6 (33%)</td>
<td>2/8 (25%)</td>
</tr>
<tr>
<td>LCIS (both groups) (n=43)</td>
<td>10/34 (29%)</td>
<td>3/37 (8%)</td>
<td>4/8 (50%)</td>
<td>3/9 (33%)</td>
</tr>
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LCIS = lobular carcinoma in situ; DCIS = ductal carcinoma in situ; Inv = invasive carcinoma.

In one, there was pure LCIS on both sides, while in two cases, one breast contained pure LCIS while the other showed LCIS with an invasive lobular carcinoma. Uninvolved lymph nodes or normal breast tissue clear of neoplastic cells were used to provide constitutional DNA from each patient. All cases were reviewed by two pathologists (SRL and JPS).

Figure 1 Case 279. (A) Classical pure lobular carcinoma in situ (LCIS) from the right breast. (B) Left breast biopsy with an infiltrating lobular carcinoma (ILC) and pagetoid spread of LCIS within a duct. (C) Autoradiograph following PCR using the microsatellite D17S250. The right hand lane represents the control DNA from normal uninvolved lymph node and the two alleles are arrowed. A number of "stutter" or "slippage" bands are present beneath the alleles and are due to replication errors by the polymerase of the microsatellite. The first lane represents the LCIS in (B) and shows loss of the bottom allele. No loss is seen in the corresponding ILC. The third lane represents the pure LCIS from the right breast (A) and shows loss of the top allele. The results are confirmed using a phosphorimager (see fig 2). LN=lymph node; R=right; L=left.
DISSECTION OF NEOPLASTIC TISSUE
Paraffin sections (3 × 15 μm) were cut from each case onto double sided clear adhesive tape and placed upon glass slides. They were stained lightly with toluidine blue to highlight the relevant areas. Under a dissection microscope, a fine scalpel blade was used to cut around the area of LCIS and the same area from the three slides was peeled off and placed into a 1-5 ml eppendorf tube; 5 μm sections taken immediately before and after the 15 μm sections were stained with haematoxylin and eosin. These sections were used to identify the relevant lesions and to estimate the level of contamination by normal stromal and inflammatory cells.

DNA EXTRACTION
Dissected fragments were incubated for 16 hours at 37°C in 10 mM tris HCl pH 7-5, 1 mM EDTA, 1%(w/v) sodium dodecyl sulphate, and 500 μg/ml proteinase K. The mixture was heated to 100°C for 10 minutes to inactivate the proteinase K and aliquots were used directly in the polymerase chain reaction (PCR).

ANALYSIS OF POLYMORPHIC DINUCLEOTIDE REPEATS
Aliquots of the digested material were amplified using the PCR in 50 μl of 50 mM KCl, 10 mM Tris-HCl pH 9-0, 0-1%(v/v) TritonX-100, 2-5 mM MgCl₂, 0-5 M Taq polymerase, 0-1%(w/v) bovine serum albumin, 2 mM each dNTP and each primer at 5 μg/μl. In a typical experiment 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute were performed. One primer of the pair for each locus was 5’ endlabelled using γ[32P] ATP and T4 polynucleotide kinase. The amplified products were electrophoresed through 6% denaturing polyacrylamide gels and exposed to x ray film for 16 hours to 5 days. Cases showing evidence of allele loss were repeated at least once to reaffirm the loss. Allele loss was confirmed by comparing the relative intensities of the alleles using scanning densitometry or by exposure on a phosphorimag...
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Discussion

By dissecting small foci of disease and subsequently amplifying polymorphic dinucleotide repeats (microsatellites) using PCR, we have shown loss of heterozygosity in LCIS. Some of the cases were associated with either invasive cancer or DCIS. The presence of an invasive component is important because it raises the possibility that the in situ disease represents intralobular and intraductal spread of a clone that has already acquired invasive capability, rather than a precursor of such a clone. We therefore also studied loss of heterozygosity in a group of pure LCIS identified in otherwise benign breast biopsies. We demonstrated loss of heterozygosity at all the loci studied in pure LCIS and in that associated with invasive cancer, the overall frequency of loss ranging from 8% at D17S796 to 50% at D17S250. Although the numbers were small, there was no obvious correlation between loss of heterozygosity and the type of associated disease.

Previous reports indicate that LCIS is commonly multifocal. The nature of these foci is not entirely clear, although their cytological features suggest that they are neoplastic, this being reflected in the nomenclature. Nevertheless, the presence of multiple foci could also result from hyperplasia. It is possible to determine allelic loss by the method we have described only when the sequence amplified by the PCR is lost from the great majority of cells in the sample. Where significant numbers of cells have retained the relevant allele, the PCR will amplify it sufficiently to produce a second band and hence obscure the deletion. The most likely explanation for the presence of allelic loss in the cell population is that the majority of cells are descendants of a single cell in which the genetic lesson developed. The alternative explanation that all cells acquired the identical genetic lesson independently is highly improbable. Consequently our findings indicate that each focus of LCIS is monoclonal and therefore a neoplastic rather than a hyperplastic (polyclonal) proliferation. In seven cases, we studied multiple foci of LCIS from the same breast. In one case, two separate foci of pure LCIS from the same breast showed a different pattern of loss of heterozygosity, suggesting the presence of independent clones. Similarly in one bilateral case, there was loss of different alleles in each side. Taken together with the clinical information, our results suggest that, even in the same breast, multifocal LCIS represents multiple independent neoplastic proliferations.

We have previously studied loss of heterozygosity in DCIS11 and atypical ductal hyperplasia.12 The proportion of DCIS and atypical ductal hyperplasia showing loss of heterozygosity at D16S413 and D17S796 (approximately 40% and 30% at each locus) did not differ significantly from that of invasive cancers in these genomic regions.13 14 This observation suggests that the majority of genetic abnormalities at these loci in breast cancer take place before invasion. In LCIS, however, the frequency of loss of heterozygosity on chromosome 17p appears to be lower than in DCIS or invasive carcinoma, with only 3/37 cases (2/26 LCIS + invasive carcinoma and 1/11 pure LCIS) showing this phenomenon at D17S796.

Immunohistochemical studies have also shown differences between LCIS and DCIS. Increased p53 protein expression is detected in 30–50% of in situ and invasive ductal carcinomas but rarely in invasive lobular car-

![Figure 3: Examples of loss of heterozygosity at various markers. Case 138: These foci of lobular carcinoma in situ (LCIS) were associated with invasive lobular carcinoma (ILC). Both foci of LCIS show loss of the top allele. Case 272: Two separate foci of pure LCIS from the same breast. The first lane shows loss of the bottom allele (with some residual signal) but no loss of heterozygosity is seen from the second focus. Review of the sections showed minimal contamination of the second sample, hence the lack of loss of heterozygosity is taken to represent a real result. Case 275: A focus of pure LCIS showing loss of the top allele. Case 277: A case of bilateral LCIS. The two foci of LCIS, one associated with ILC and the pure LCIS from the right breast both show loss of the top allele. Again the ILC does not show evidence of LOH. Case 278: A focus of pure LCIS showing loss of heterozygosity involving the bottom allele. This marker has particularly numerous slippage bands, but the reduction in intensity of the lower allele is clear. The gels shown in these examples also illustrate the difficulties with interpretation and hence all suspected losses are checked by repitition and by analysis on a phosphorimager or by scanning densitometry. LN=lymph node; NT=normal tissue; LCIS=lobular carcinoma in situ; ILC=invasive lobular carcinoma; R=right; L=left.]

16q-D16S413 17q-D17S250 13q-D13S267 13q-D13S267 16q-D16S413

Case 138 Case 272 Case 275 Case 277 Case 278

of heterozygosity involving different alleles (figs 1 and 2), suggesting that the two foci of LCIS were different clones. Despite the use of microdissection techniques, loss of heterozygosity was not found in invasive lobular carcinoma and the phosphorimager trace for this carcinoma (fig 2) is identical to the normal control, indicating significant contamination from the stroma. Further examples of loss of heterozygosity are illustrated in fig 3.
cinomas or LCIS. Since increased p53 protein is often associated with p53 mutations, these findings, taken together, suggest that p53 mutation in LCIS is rare. Other genetic or biochemical features that differ between LCIS and DCIS are the greater frequency of c-erb-B2 overexpression and expression of the epithelial cell specific adhesion molecule, E-cadherin, in DCIS. These data, together with our data on loss of heterozygosity on chromosome 17p, show that the morphological, epidemiological, and clinical differences between LCIS and DCIS are reflected at the biochemical and genetic level. The sharing of allelic imbalance at other loci, however, suggests that LCIS and DCIS may have a common pathway of evolution.

Although this study was aimed at the analysis of in situ breast neoplasia, it is in fact the first (to our knowledge) that provides substantial information concerning molecular genetic changes in lobular carcinoma. The reason for the difficulty in studying this group of disorders is highlighted in the phosphorimagery trace (fig 2). The invasive lobular carcinomas are extremely difficult to isolate in pure form and despite microdissection the degree of contamination remains high; hence the trace for invasive lobular carcinoma is effectively identical to the normal control sample. In contrast, LCIS, despite being a small lesion, can be dissected relatively cleanly allowing the demonstration of loss of heterozygosity.

Two major hypotheses can explain the bilateral and multifocal pattern of disease: exposure to an exogenous carcinogen, and genetic susceptibility. At present there is insufficient evidence to discriminate between them. However, there is at least one report of a substantially increased risk of breast cancer in relatives of patients with LCIS, suggesting that a proportion may arise as a result of inherited predisposition. LCIS is not, however, a common feature in breast tissue from patients with familial breast cancer due to the BRCA1 gene on chromosome 17q and BRCA2 gene on chromosome 13q, and almost all invasive cancers arising in these individuals are ductal in type (Gusterson B, Lakhani SR, and Stratton MR, unpublished data). Therefore if multifocal/bilateral LCIS is attributable to a genetic susceptibility, it could well be as a result of mutations in another gene.

SRL is a research fellow supported by the South Thames Regional Health Authority. We would also like to acknowledge the support from the Cancer Research Campaign and the Medical Research Council. We are grateful to Ann Ward and Professor B Gusterson for help in retrieving cases from the in situ carcinoma data base which was funded by the Stebbings Trust and the Royal Marsden Hospital Breast Diagnostic Unit. We thank Apple Computer UK Ltd for the generous donation of computer equipment to SRL.