In situ breast cancer profiling

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Invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) are the two most common types of invasive breast carcinoma. IDC is derived from the ducts and accounts for 80% of all invasive breast cancers, whereas ILC arises from cells in the lobules of the breast and accounts for 10-15% of invasive breast cancer. The incidence of ILC is increasing in post-menopausal women, and epidemiological evidence suggests that this is due to the increased use of hormone replacement therapy [1]. Both types of carcinoma can be associated with a pre-invasive lesion, ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) (Figure 1). Since the introduction of screening mammography the diagnosis of DCIS and LCIS has become more common. Approximately 20% of screen-detected tumours are DCIS, and in the UK are generally treated in a similar manner to invasive breast cancer with wide local excision and radiotherapy. By contrast, patients with LCIS alone do not receive any further treatment and, even if incompletely excised, no further surgery is performed. It is therefore extremely important for patients and doctors to know which cases of in situ disease might give rise to invasive disease, so that appropriate screening and treatment can be offered and those at low risk of invasive disease can be confidently spared unnecessary treatment.

The disparity in treatment between the two types of in situ disease arises from evidence that DCIS is a non-obligate precursor of IDC, with similarities in genetic and molecular markers between DCIS and subsequent invasive disease. In contrast the link between LCIS and progression to invasive disease is not so clear and there is still debate as to whether LCIS is a true precursor or merely a risk factor for subsequent disease.

Patients with DCIS previously misdiagnosed with benign breast disease who received no surgical intervention were retrospectively followed up to investigate the natural progression of untreated DCIS. The odds ratio for developing subsequent carcinoma was 20.1 [2]. In studies where DCIS has been treated with breast-conserving surgery alone with no radiotherapy, long-term follow-up shows that up to 30% of women develop a recurrence (half of which will be DCIS and half invasive cancer) by 10 years.

Women with LCIS have 2-11 times higher risk of developing invasive breast cancer compared to women of comparable age who do not harbour LCIS [3]. Interestingly not all invasive disease post LCIS is ILC, although there is an excess of ILC, women also develop ductal and mixed lobular-ductal invasive cancers. These cancers occur in both the ipsilateral and contralateral breast and for this reason LCIS is often considered a risk factor for breast cancer rather than a true precursor lesion. However there is evidence that LCIS has similar genetic changes to ILC, indicating that it should also be considered as a precursor (Figure 2). The timescale for the development of invasive carcinoma after an initial diagnosis of LCIS in either breast varies greatly between individuals. One study demonstrated that two thirds of patients developed invasive disease within 15 years of the in situ lesion, however another study found that 50% of patients developed ILC as long as 15-30 years later [4].

Considering that the cumulative risk of developing invasive disease in the ipsilateral or contralateral breast following LCIS is 18% and 14% respectively, the current recommended treatment is frequent breast examination and mammography [5]. However ILC is often not detected on a mammogram so other options for patients at high risk of developing invasive disease post LCIS include screening with magnetic resonance imaging (MRI) or chemoprevention with drugs such as tamoxifen or the aromatase inhibitor, anastrozole, which have been shown to be effective at reducing invasive disease post LCIS [6]. In order to identify patients who would benefit most from such interventions it is firstly essential to understand the molecular basis of LCIS in order to identify biomarkers of progression to invasive disease.

Historically, molecular profiling of LCIS has been challenging for a number of reasons. As LCIS is not commonly associated with clinical abnormalities, and rarely presents clinically with a palpable mass nor does it occur with micro-calcifications, a diagnosis is frequently incidental on a core biopsy. Furthermore as it is not common practice for treatment to be surgical if LCIS is not associated with ILC there are limited amounts of tissue available for profiling studies.

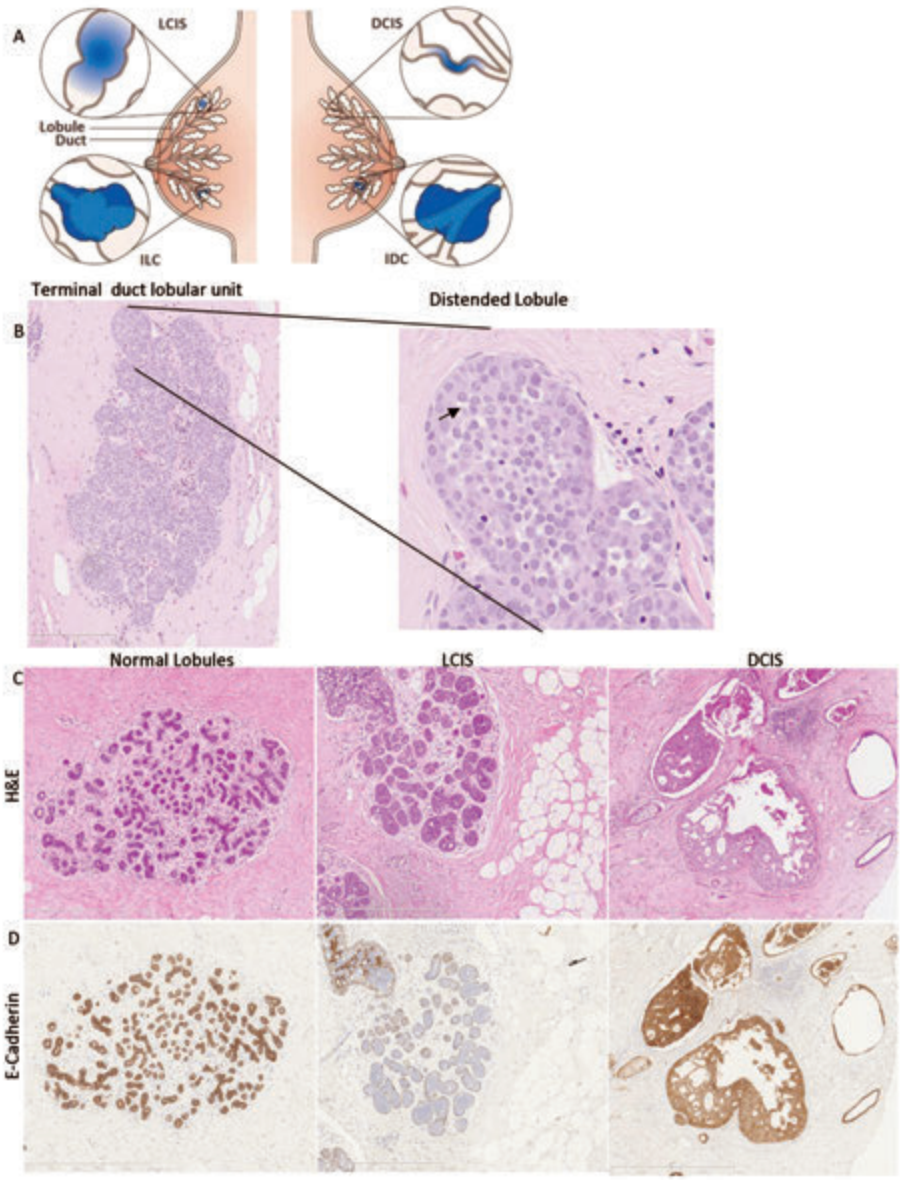

Figure 1. A) Diagrammatic representation of structures contained in the breast indicating the origin of both ductal and lobular variants of *in situ* and invasive carcinoma. Source: Cancer Help UK B) Criteria for the diagnosis of LCIS states that a minimum of half the lobules within a lobular unit must show distension while maintaining the overall structure of the lobule. The arrows indicate discohesion between the cells, a key feature of LCIS. C) Haematoxylin and Eosin stained breast tissue shows distended ducts and lobules. D) Diagnostically, LCIS can be differentiated from DCIS and other non-invasive lesions of the breast by the use of the well characterised marker, E-cadherin. E-cadherin is a transmembrane protein with an important role in intracellular adhesion coded by *CDH1*, a gene located on 16q22.1 Staining of E-cadherin shows loss of expression in LCIS compared to normal lobules and DCIS.

This issue has been addressed by the GLACIER (Genetics of lobular carcinoma in situ in Europe) study, which has recruited over 2000 patients with either LCIS or ILC with an aim of understanding genetic predisposition and progression of LCIS to ILC. Peripheral blood and formalin fixed and paraffin embedded (FFPE) tissue were collected from all patients. This has provided a vast tissue resource from which DNA/RNA can be extracted for profiling. Another significant obstacle is the quality of material obtained from FFPE samples. The key issue is the method of fixation and length of time before fixation of the tissue. Variations in both will cause differing levels of degradation to both the extractable RNA and DNA [7]. DNA and RNA of higher quality can be obtained from fresh frozen tissue, however as tissue banking of fresh frozen tissue from in situ disease has not been routinely performed by established breast tissue banks, this is a scarce resource.

In recent years, there have been vast technical developments, which allow DNA or RNA molecular interrogations to be performed in samples where tissue is scarce or quality of material is relatively poor. For example, molecular inversion probes (MIPs) overcome specific issues arising from genotyping and the assessment of copy number variations in degraded DNA. MIPs are small sequences of DNA, the ends comprise two small sequences which are complementary to two adjacent sequences on the genome, which results in successful probe binding requiring only a 40 bp target binding site. Such assays have been carried out with as little as 40 ng of DNA [8].

In addition when conducting profiling studies the different histopathological subtypes of the lesion under investigation need to be considered. To date LCIS can be classified into three groups, classical (cLCIS), pleomorphic (pLCIS) and florid (fLCIS), all of which have been shown to have differing molecular profiles which have differentially clustered on unsupervised hierarchical clustering of genome copy number profiles. An interrogation of fLCIS by array comparative hybridisation has revealed this lesion to be genetically more aberrant compared to the classical variant. Together with the fact that fLCIS is more frequently found with invasive disease has highlighted a need for differential clinical treatment of variants of the same lesion. A region of amplification, 11q13.3 containing the CCDN1, cyclin D1 gene was also identified and validated immunohistochemically (IHC). In this analysis 80% of cases with amplification also had higher protein expression. This

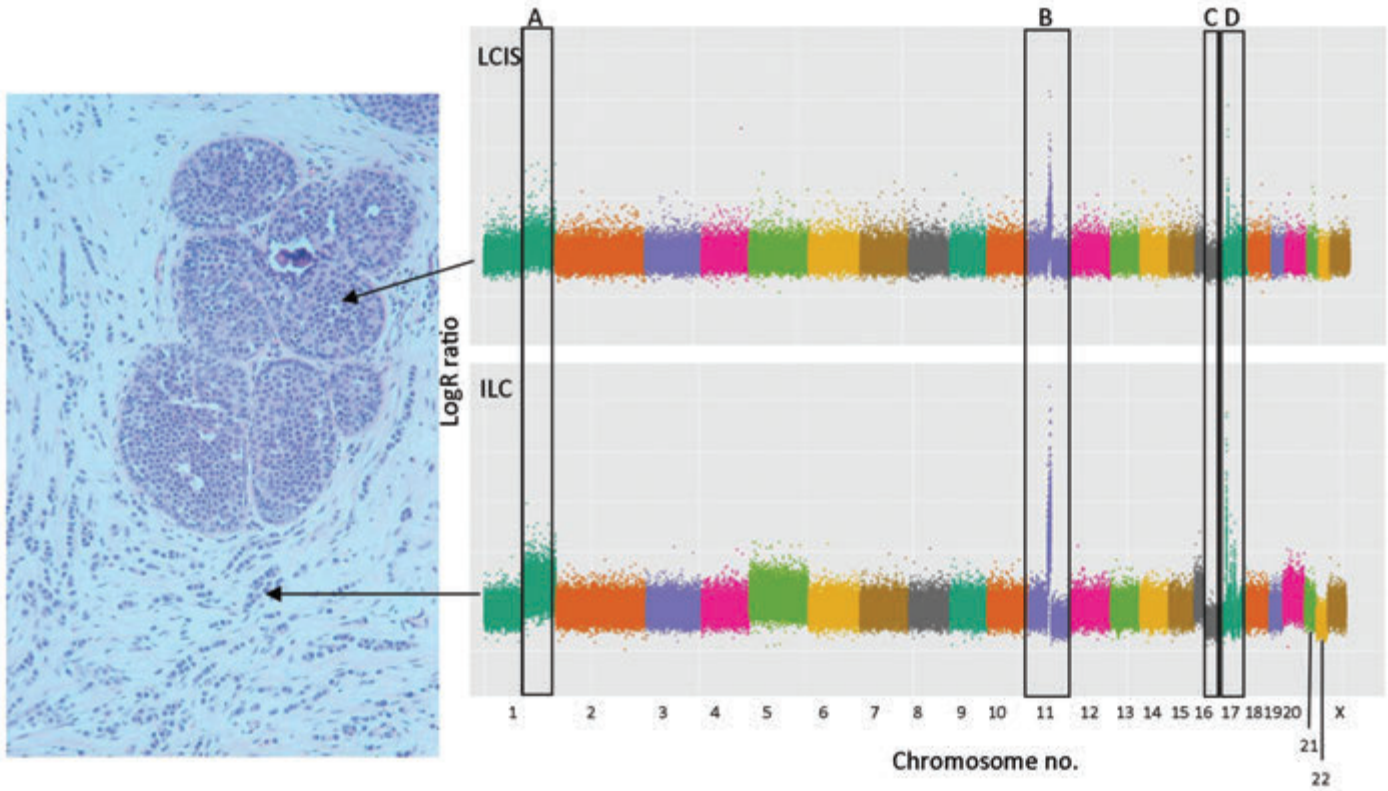

Figure 2.
A) 1q gain, characteristic of lobular lesions
B)Amplification in region containig *CCND1*, codes for cyclinD1
C) 16q loss, characteristic of lobular lesions
D) *HER2* amplification
Copy number profile (OncoScan™ array, Affymetrix) obtained from microdissected LCIS and adjacent ILC shows similar genetic changes: gain 1q, amplification of cyclin D1 and her 2 gene, 16q loss.

is an interesting cell cycle regulatory gene which has been identified in several studies which suggests it may have a role in disease progression [9].

Due to the limitations surrounding the molecular profiling of LCIS, obtaining a complete gene expression profile has been difficult. In 2008 the first global gene expression profile of LCIS was created. Analysis was carried out to identify genes which were differentially expressed between LCIS and normal breast epithelial cells in order to uncover dysregulated pathways. As only one complete RNA profile was obtained, subsequent validation of findings was carried out at the protein level using IHC on a larger number of LCIS cases. Proof of validity was achieved by the observed characteristic down regulation of E-cadherin. The two main findings of this work showed down regulation of claudin 4, a tight junction protein, which according to the functional biology of this molecule would correlate with E-cadherin dysregulation and interestingly, over expression of matrix metalloproteinase 9 (MMP9). MMP9 is thought to be essential for the process of cancer cell invasion and could therefore play a role in the development of invasive disease [10].

The advent of next generation sequencing means that whole exome, genome and transcriptome analysis of tumours is now feasible. As well as identifying genes or pathways which may be important in progression of DCIS/LCIS, these techniques also allow more global analyses, looking at genetic diversity and clonal structure. These may also prove to be biomarkers of progression as they measure the carcinogenic process as a whole. These techniques require good quality DNA/RNA and have not yet been widely applied with success to FFPE material. Next generation sequencing has the added complication for in situ disease of requiring relatively large amounts of DNA/RNA that are often not easy to obtain from the limited tissue available. However the field is rapidly evolving and massively parallel targeted sequencing is now an option for small quantities of FFPE material although paired germline DNA is essential in order to differentiate between germline and somatic mutations. A recent comparison of library preparation methods, from both low quality and quantity RNA for sequencing has shown that from 100 ng of fragmented RNA, it was possible to achieve over 60% 5' and 3' end coverage with a low duplication rate and percentage of ribosomal RNA, factors which indicate good library performance [11].

As LCIS is more commonly bilateral than DCIS and is more common in women who have a first degree relative

diagnosed with breast cancer it has been suggested there may be an inherited component to LCIS development. A recent study has shown that 8% of the cases with bilateral LCIS harbour rare truncating germline CDH1 mutations [12].

In addition a genome wide association study has focused specifically on finding common low risk genetic variants associated with ILC and /or LCIS. The results showed many of the genetic variations predisposing to ILC also predispose to LCIS, with some having a stronger effect on LCIS than ILC, given further support to the hypothesis that LCIS is a precursor of ILC. Once genetic predisposition to ILC and LCIS is fully understood it may be possible to identify those patients most at risk of developing invasive disease by genotyping germline variants using DNA extracted from a blood sample [13].

Similar issues exist with DCIS with one of the main barriers to identifying biomarkers being the lack of large patient cohorts. 90% of published DCIS biomarker studies have used less than 50 cases. More recently, the Genomic Health DCIS score, based on the expression of a commercially-protected unknown set of genes, has been shown to predict recurrence of pure DCIS, but this awaits validation [14] Two large DCIS resources exist in the UK (Sloane Project and ICICLE study) but again only FFPE material. As with LCIS, DCIS can also be divided into different subtypes based on grade and architecture.

Although profiling has provided no firm changes in the treatment of LCIS / DCIS to benefit patient outcome to date, this is a rapidly changing field particularly with the development of RNA seq and genome/exome sequencing. Identification of biomarkers that identify those women most likely to develop invasive disease following DCIS/LCIS would dramatically impact on the clinical care of these women. This area has been highlighted as a critical gap in breast cancer research internationally. There is concern regarding over-treatment of DCIS and this is reflected in new clinical trials of DCIS offering observation alone following biopsy for low and intermediate grade DCIS (LORIS trial, UK and LORD trial, the Netherlands). However, for some women omitting radiotherapy is associated with a high risk of development of invasive disease. For observation-only to be a viable treatment option, it is imperative that biomarkers are identified in order to avoid under treatment of those most at risk following a diagnosis of DCIS or LCIS. ●

"Identification of biomarkers that identify those women most likely to develop invasive disease following DCIS/LCIS has been highlighted as a critical gap in breast cancer research internationally and would dramatically impact on the clinical care of women with DCIS/LCIS"

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