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Intratumoral heterogeneity in ductal carcinoma in situ: Chaos and consequence

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Abstract

Ductal carcinoma in situ (DCIS) is a non-invasive proliferative growth in the breast that serves as a non-obligate precursor to invasive ductal carcinoma. The widespread adoption of screening mammography has led to a steep increase in the detection of DCIS, which now comprises approximately 20% of new breast cancer diagnoses in the United States. Interestingly, the intratumoral heterogeneity (ITH) that has been observed in invasive breast cancers may have been established early in tumorigenesis, given the vast and varied ITH that has been detected in DCIS. This review will discuss the intratumoral heterogeneity of DCIS, focusing on the phenotypic and genomic heterogeneity of tumor cells, as well as the compositional heterogeneity of the tumor microenvironment. In addition, we will assess the spatial heterogeneity that is now being appreciated in these lesions, and summarize new approaches to evaluate heterogeneity of tumor and stromal cells in the context of their spatial organization. Importantly, we will discuss how a growing understanding of ITH has led to a more holistic appreciation of the complex biology of DCIS, specifically its evolution and natural history. Finally, we will consider ways in which our knowledge of DCIS ITH might be translated in the future to guide clinical care for DCIS patients.

Keywords

Breast cancer; DCIS; heterogeneity; tumor microenvironment

Introduction

Breast tumorigenesis is thought to be a step-wise process in which normal mammary epithelial cells undergo aberrant proliferation to form precancerous lesions [1, 2]. A subset of these progress to form in situ carcinomas, and a further subset escape confinement by the myoepithelial and basement membrane layers to become invasive breast cancer. The current understanding of breast tumorigenesis assumes progression from atypical hyperplasia, to low grade ductal carcinoma in situ (now renamed ductal intraepithelial neoplasia [3], or DIN), to high grade ductal carcinoma in situ (DCIS) that, while itself is non-lethal, serves as a non-obligate precursor to invasive breast cancer. It should be noted, however, that there

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remains significant discord regarding the accuracy and clinical relevance of these subdivisions, including the point in tumor progression at which lesions should be considered malignant [4–10], as well as the generalizability of this progression to all breast cancer subtypes.

Because of our limited understanding of the natural history of in situ lesions, the field's classification of DCIS is grossly imprecise. To date, we remain unable to accurately distinguish breast cancer lesions that will remain indolent from those that will progress to invasive cancer; as a result, all patients diagnosed with DCIS are indiscriminately offered surgical resection, with or without adjuvant radiation or endocrine therapy [11]. Given that in situ carcinomas now constitute approximately 20% of new breast cancer diagnoses in the United States [12], there is an urgent unmet clinical need for detailed and clinically relevant insight into the biology of very early stage breast cancer progression [13]. Of particular interest to the breast cancer field is the emerging concept that the genetic aberrations and intratumoral heterogeneity (ITH) detected in invasive cancer may already be established in advanced DCIS lesions [14–16, 8]. The precise timing with which heterogeneity arises and its functional significance to tumor progression remain unclear. Furthermore, we do not yet know how to incorporate biomarkers of ITH to DCIS to guide its clinical management.

Intratumoral heterogeneity is the presence of non-uniform features within a single tumor. These features can include subclonal populations of tumor cells harboring distinct genomic or non-genomic (phenotypic) properties from one other. In addition, heterogeneity in a single tumor can arise among components of the tumor niche, including tumor-associated stromal cells and the extracellular matrix bed. These three forms of heterogeneity (herein referred to as *genomic*, *phenotypic*, and *compositional* heterogeneity) have been identified in DCIS. Importantly, given the pathological definition of DCIS as a non-invasive lesion restricted by an intact basement membrane, *spatial* heterogeneity (i.e. the non-uniform spatial organization of tumor and stromal compartments relative to one other) is also becoming increasingly recognized as an important feature of DCIS. Intratumoral diversity potentially increases fitness by creating opportunity for adaptation in response to stressful, selective forces, including those encountered in tumor progression and treatment response. Thus, a more comprehensive understanding of heterogeneity (and its functional role) in the progression of DCIS to invasive breast cancer is expected to advance our ability to design effective therapeutic approaches, while also deepening our knowledge of the natural history and evolution of breast cancer.

Phenotypic heterogeneity

Decades of traditional histopathology have provided significant evidence of intratumoral phenotypic heterogeneity in DCIS. Various DCIS descriptors and classification systems rely on features such as lesion architecture (e.g. comedo, solid, papillary, micropapillary, cribriform), epithelial apical-basal polarity, degree of differentiation, nuclear grade (e.g. nuclear size, pleomorphism, chromatin arrangement, nucleolar prominence, and mitotic figures), necrosis, and other features to categorize DCIS [17–19, 9, 20–22, 6, 23, 24]. Morphologic heterogeneity is so commonly observed that the absence of homogeneity is a

defining characteristic of some descriptors. For example, nuclear pleomorphism is defined by the irregularity of nuclear appearance from one cell to another within lesions; comedo necrosis tacitly describes spatial heterogeneity of necrosis that occurs at the center (but not edge) of a lesion. Furthermore, DCIS cases commonly display histopathological features heterogeneously [25, 26, 18, 27, 23, 24]. While some cases of DCIS display only a single type of lesion architecture (i.e. architecturally pure), approximately half [25, 28] of cases display multiple architectural features, for example concurrent cribriform and solid features, concurrent cribriform and micropapillary features, concurrent cribriform and solid and micropapillary features, and so on [25, 26, 18, 29, 30, 28]. Nuclear grade, commonly considered in the pathological evaluation of DCIS and used to guide clinical management of DCIS, has also been shown to exhibit heterogeneity across DCIS lesions within the same patient [26, 18, 31, 27, 30].

Intratumoral phenotypic diversity is also evident based on heterogeneity of biomarker expression in DCIS, with the majority of DCIS cases displaying some degree of heterogeneity when evaluated for multiple biomarkers [27, 32]. Routine evaluation of steroid hormone receptor status has shown that only a proportion of cells (~70%) [33] within DCIS lesions express estrogen receptor, a finding that is reflected in ER scoring systems that employ a sliding scale based on proportion and intensity of ER expression [34–36]. Similarly, HER2 is heterogeneously overexpressed in lesions [37, 38, 27, 32], usually in association with concomitant gene amplification [38, 39, 8, 40]; notably, this heterogeneity appears to cluster spatially within lesions, evidenced by HER2-overexpressing regions adjacent to unamplified regions [8]. Ki67, a common biomarker of proliferating/non-G₀ cells that is associated with worse prognosis (particularly when considered with other biomarkers, including p16 and COX-2) [41–43, 27, 44, 33, 45, 32, 46–48], exhibits both phenotypic heterogeneity (with lesions expressing Ki67 in 0.5–61% of cells, depending on molecular subtype) [32, 47] and spatial heterogeneity (i.e. Ki67-positive cells tend to cluster at the edge, but not the center, of a lesion). Expression of p53 protein (increased detection of which is often ascribed to mutant status [49–51]) can also occur heterogeneously in a focal manner [52, 53, 32], usually in association with high grade, high proliferative index, comedo architecture, and other markers of aggressiveness [54, 55, 52, 56]. Finally, a recent study evaluating 14 biomarkers in DCIS revealed heterogeneity between individual ducts within a single case, based on biomarker expression (staining intensity) and co-localization with other biomarkers. Interestingly, HER4 and HER2 were found to be relatively homogeneous across ducts within a single case, whereas other biomarkers exhibiting generally low expression (~5%) such as phospho-mTOR, CD44v6, and CD10, were more heterogeneous. Furthermore, when phenotypes were defined based on biomarker co-expression, the study found that most DCIS were comprised of 1–3 major phenotypes, with additional phenotypes present in the minority [57]. Taken together, ITH in histopathology and expression of biomarkers suggest that phenotypic diversity in DCIS may in fact be the norm, rather than the exception.

Studies of more complex non-genomic features of DCIS, such as metabolomic [58–60], transcriptomic [61–64], and epigenomic [65, 66] phenotypes, have been technically challenging primarily because the limited volume of tissue that can be extracted from DCIS samples typically precludes evaluation of ITH. Nonetheless, although single-cell resolution

of the metabolome remains elusive [67–69], tissue-level metabolic ITH in DCIS has been successfully estimated based on heterogeneous uptake of ^{18}F -fluorodeoxyglucose measured via positron emission tomography, and appears to correlate with a more aggressive lesion based on likelihood of upstaging following surgical resection [70, 60]. In addition, the development of single cell RNA sequencing [71, 72] may soon shed light on the transcriptomic ITH in DCIS. Finally, one group has successfully performed multi-region analysis of promoter hypermethylation using formalin-fixed invasive breast cancer samples [66], suggesting that a similar approach might be adapted to evaluate epigenomic ITH in DCIS. These emerging technologies may soon provide much needed insight into the ITH of these high level -omic phenotypes within DCIS.

Genomic heterogeneity

Genomic ITH of DCIS has proven difficult to measure, again primarily due to the often-small size of individual non-invasive lesions that are closely mixed with normal breast tissue. Nonetheless, our understanding of the genomic ITH in DCIS has improved (directly or indirectly) with various approaches, including karyotyping, fluorescence in situ hybridization (FISH), multi-region genomics, and, most recently, next-generation single-cell sequencing.

Early karyotyping studies evaluating DCIS lesions have identified chromosomal changes that existed prior to invasion. In particular, assessment of DCIS suggests that most lesions exhibited some karyotypic abnormalities, of which the majority were notably subclonal [73]. These findings suggest even at relatively low genetic resolution that underlying genomic instability (which presumably drives heterogeneity) may already be in place in DCIS [74]. Assessment of DCIS lesions via FISH suggest heterogeneous alterations in chromosomes [75–77], telomeres [78], and specific genes. FISH for several chromosomes revealed variable numbers of hybridization signals across many nuclei within the same lesion [77, 76], supporting the idea that heterogeneity in chromosomal alterations exists in DCIS, although technical artefacts cannot be ruled out in many cases. Similarly, FISH has identified several genes (such as *HER2*, *C-MYC*, *CCND1*, *COX2*, *CDH1*, *TP53*) that seem to exhibit heterogeneous copy number alterations in DCIS [79, 80]. These findings must be interpreted with care: in many of these studies, heterogeneity was not specifically evaluated but can be inferred through the evaluation of individual cases and through the use of cut-offs to classify cases (for example, categorization as monosomic versus polysomic).

Development of comparative genomic hybridization [81] and, subsequently, next-generation sequencing approaches have permitted genome-wide evaluation of DCIS lesions [82, 14, 83]. However, for these assays and others performed on bulk tumor samples (employed in the majority of recent genomic studies on DCIS), subclonal alterations are difficult to detect and/or distinguish from clonal aberrations. For example, low-level clonal amplifications and high-level subclonal amplifications may yield the same result via bulk analysis. Furthermore, any putative genomic heterogeneity detected is challenging to interpret, given that this heterogeneity could arise from (1) genuine ITH restricted to the tumor compartment, or (2) sample “contamination” by normal tissue [84]. Indeed, these two possibilities have been so challenging to disentangle that genomic heterogeneity detected in

a sample has been previously considered a technical challenge [77, 85]. Although deep sequencing of bulk tumor samples has been successful at identifying genomic ITH and evaluating subclonal architecture in invasive breast cancer [86–93], these approaches are only just now being applied to DCIS.

One tactic to take advantage of genome-wide assays is multi-region sequencing [94–99], which involves microdissection and genomic comparison of multiple regions from individual DCIS cases. This approach typically sacrifices single-cell resolution to gain a detailed, high-resolution view of the genome. In one example of multi-region analysis of DCIS, sequencing of mitochondrial DNA revealed that lesions in unifocal DCIS (involvement of adjacent terminal duct lobular units, or TDLUs) tended to be clonally related, whereas those in multifocal DCIS (involvement of several distant TDLUs) tended to be unrelated [100]. Although only mitochondrial DNA was assessed in this study, the clonal relationships suggest that, at least for multifocal DCIS, a high degree of ITH may exist within a single individual. In an advanced iteration of multi-region sequencing, multiple single cells across different regions of individual cases of DCIS (with synchronous adjacent invasive cancer) were isolated by laser-dissection to identify copy number alterations at single-cell resolution while retaining spatial information [101]. Based on this study, DCIS lesions appear to be comprised of multiple clonally-related (but nonetheless genomically divergent) subclones that are typically maintained in the invasive compartment, albeit at varying fractions. Furthermore, formalized measures of diversity (Shannon index [102]) showed that the ITH detected in DCIS is comparable to that in adjacent invasive breast cancer.

Taken together, these studies provide abundant evidence of genomic ITH in DCIS, suggesting that these cells are likely undergoing an ongoing cycle of mutation and natural selection posited by the clonal evolution hypothesis of breast cancer tumorigenesis [103]. Continued advancement of genomic sequencing approaches, especially as they apply to technically challenging tissues (such as limited quantities of decades-old formalin-fixed paraffin-embedded DCIS samples) is expected to further expand our knowledge of the nature and degree of intratumoral genomic heterogeneity in DCIS.

Compositional heterogeneity

In addition to genomic and phenotypic ITH, compositional heterogeneity of the tumor microenvironment (that is, heterogeneity of cell types that comprise the tumor microenvironment) is becoming increasingly appreciated as a significant determinant of DCIS biology [104–107]. The tumor niche is a complex, richly-interconnected ecosystem [108] that comprises several cell types and acellular stromal components, including (1) preinvasive epithelial cells that comprise the majority of a DCIS lesion; (2) myoepithelial cells surrounding the lesion that, together with the abutting basement membrane, create a physical barrier between premalignant cells and the surrounding stroma; (3) adipocytes that comprise the majority of the breast and serve as a depot for hormones and other factors; (4) cancer-activated fibroblasts that have been shown (at least for invasive breast and other cancers) to promote tumor progression; (5) a vast range of immune cells (of varying activation statuses) that surround and infiltrate the premalignant lesion; (6) the extracellular

matrix (including the basement membrane, but also more distal matrix), the structure of which may be locally altered; (7) nearby vasculature and lymphatics that potentially service the lesion as it expands; as well as many other tissue components that potentially influence the biology of DCIS [109–115, 24]. The varying cell types that reside in the local lesion microenvironment, comprising the niche in which premalignant cells survive and evolve, represent a level of heterogeneity defined by cell phenotype and functional status. In addition, the composition of the local microenvironment shifts over time and space, thereby presenting another degree of heterogeneity in DCIS.

Rather than being a passive bystander of burgeoning tumors, the associated microenvironment actively co-evolves, shifting in composition and function to constrain or support tumor progression, while also influencing the evolutionary trajectory of tumor cells [116, 117, 114, 118, 111, 119]. This phenomenon has been observed for many solid tumors, including breast cancer, and is likely to be true for in situ lesions as well. Importantly, no element of the tumor microenvironment functions in isolation; rather, each component interacts with others, creating a highly-structured non-random signaling network [120] that maintains the tumor niche. The compositional heterogeneity (or “species” diversity) of the tumor microenvironment is likely to be of particular importance to cancer progression: ecosystems with high biodiversity tend to be highly advantageous by supporting the host tumor (for example, through provision of growth factors) and by promoting niche stability in response to perturbations [121, 120, 122–124], for example, those caused by treatment, tumor progression, nutrient deficit, etc.. Furthermore, given that some mechanisms of evolution may require a minimum number of species [125], a highly diverse tumor microenvironment may promote adaptation and further diversification of malignant cells especially under the shifting fitness landscapes that accompany progression from in situ to invasive cancer, and metastasis from primary to secondary sites [126–130].

To this end, an intense effort is underway to characterize not only the components of the tumor microenvironment but also to understand how interactions between these components can drive cancer progression. Characterization of the tumor microenvironment has greatly improved our appreciation of the complexity of the tumor niche; however, relatively little is known about the impact of microenvironment compositional heterogeneity on breast cancer (including DCIS) tumorigenesis, progression, and response to treatment. Nonetheless, emerging findings suggest that increased heterogeneity in the tumor microenvironment is measurable and corresponds to more aggressive tumor behavior. For example, studies of high-grade serous ovarian adenocarcinoma showed that the tumor microenvironment (particularly T-cell populations) shifts with tumor progression or regression, and that increased diversity is associated with worse prognosis [131, 130]. Heterogeneity within subpopulations of the tumor microenvironment may also drive tumor progression, as seen with the diverse population of tumor-associated macrophages, each of which supports tumor initiation, growth, invasion, and metastasis [132, 126]. Similarly, diversity of immune phenotypes may promote immune escape by tumors [133]. In high-grade breast cancer, increased tumor microenvironmental diversity correlates with poor prognosis [134], suggesting that indices of microenvironmental diversity may serve as novel biomarkers for prognosis, at least for certain subsets of disease. Additional studies are needed to determine the generalizability of these findings to cancer in general and DCIS in particular.

Spatial heterogeneity

Because DCIS is defined pathologically based on its physical relationship with the stroma (that is, non-invasion through the epithelial basement membrane), attentive evaluation of ITH would preserve and account for this characteristic architecture of a lesion within its microenvironment. ITH of the DCIS microenvironment has long been evident via standard pathological evaluation, such as the common observations of necrotic centers and proliferative edges within individual lesions, as described above. Akin to allopatric speciation, distinct lesion microenvironments could, presumably, generate distinct selective forces that drive divergent evolution of premalignant cells, resulting in genomic and phenotypic ITH defined spatially by niche. In support of this hypothesis, evaluation of spatially-separated DCIS cells via single-nucleus sequencing and genome-wide copy number profiling revealed that different regions within DCIS (or DCIS and adjacent invasive lesions) were comprised of distinct but genomically-related subpopulations inferred to share a common genetic ancestor. These findings suggest that, following migration away from this common ancestor, daughter cells underwent continued evolution and expansion to form spatially-distinct subpopulations [101]. Presumably, such diversity could be driven by divergent selective forces imposed by distinct microenvironments. Furthermore, tumor cell phenotypes (defined based on multiple biomarker expression) have been reported to cluster in a spatially heterogeneous manner both within ducts and across ducts of the same individual. Importantly, the distribution of these phenotypic clusters appeared to be associated with local immune infiltration: for example, regions of DCIS harboring tumor cells with high EGFR and CD10 were significantly associated with a high T-to-B cell ratio, while those harboring tumor cells high in HER2 were associated with a heavy B-cell infiltrate [57]. While the molecular mechanisms underlying these associations remains unknown, these findings suggest that interaction with the microenvironment may be an important determinant of spatial heterogeneity in DCIS [57, 105]. Additionally, recent studies on the metabolism of mouse mammary and pancreatic tumors suggest that metabolic gradients shape the spatial organization of tumor cells and accompanying stromal cells (such as macrophages), and promote functional adaptation to shifting tumor needs [135]. Collectively, these studies suggest that unique neighborhoods of malignant epithelial and stromal cells may function and evolve in a spatially-dependent manner to maintain the ecosystem of DCIS lesions, potentially determining whether any given lesion harbors cells capable of invasion, metastasis, dormancy, or other biological functions.

Documentation of phenotypic heterogeneity across spatially distinct regions within DCIS has been particularly challenging due to the limited availability of tissues and minimal quantity and quality of protein that can be isolated from them. Traditional immunostaining approaches are powerful and accessible assays to evaluate DCIS ITH since biomarker heterogeneity can be evaluated while maintaining the tissue architecture. However, given that these approaches can typically report only a few features or biomarkers on any one section of tissue, they are of limited utility (and/or cumbersome to employ) when attempting to evaluate phenotypic ITH on a more complex scale (that is, when evaluating the concurrent spatial heterogeneity of multiple phenotypic parameters across many cell types).

To circumvent these technical limitations, several groups have undertaken histopathological evaluation of serial tissue sections, or serial staining and imaging of the same section, to assess the expression of multiple biomarkers while preserving spatial information [57]. However, despite advances in image registration (tissue alignment) approaches, it can be challenging to guarantee that the biomarker statuses acquired from different sections truly apply to the same cell. This is particularly limiting when multiple biomarkers are required to accurately identify cell phenotype and activation status, as is frequently required for immune cells [136, 137] and malignant epithelial cells of varying lineages [138–145].

A number of new approaches have recently been developed that allow assessment of DCIS phenotypic, spatial, and compositional heterogeneity while preserving the spatial architecture of a lesion. Here, we summarize two approaches to improve phenotyping of DCIS lesions and their microenvironment using formalin-fixed paraffin embedded tissues, allowing evaluation of lesions that have been preserved in their native environment (i.e. *in situ*) with essentially minimal manipulation beyond fixation. These approaches not only permit phenotyping using a very small amount of tissue (3–5 μ m sections), they also permit evaluation of clinical tissues that have been archived as FFPE blocks over the past decades.

Multiplex immunofluorescence allows staining of a single tissue with up to 7–10 fluorophores using a modified tyramide signal amplification protocol. Tissues are stained one marker at a time with a primary antibody of choice, an HRP-conjugated secondary antibody, and finally a tyramide-bound fluorophore that becomes covalently bound to tyrosine residues adjacent to the bound epitope. Following heat-mediated antigen retrieval that also serves to strip the tissue of bound antibodies (while maintaining the covalently-bound fluorophore), tissues are stained again with another primary antibody, HRP-secondary antibody, and tyramide-fluorophore. This process can be repeated several times to stain tissue with up to 7–10 fluorophores, which are then imaged multispectrally and visualized following spectral unmixing. This approach has been successfully used to efficiently detect several biomarkers across an entire tissue section (e.g. ER/PR/Ki67/HER2 [146]), and to begin delineating specific cell types based on the expression of multiple markers, as is required most notably for (though not limited to) immune phenotyping [147, 148]. Multiplex immunofluorescence has greatly advanced our ability to simultaneously detect the heterogeneous expression of at least a handful of biomarkers at a very high subcellular resolution, allowing the identification of unique cell subtypes and stromal features that exist both within DCIS lesions and in their microenvironment.

Highly multiparametric mass-tagged imaging allows simultaneous evaluation of >30 biomarkers on a single tissue section at micron to sub-micron resolution [149]. Tissues are labeled via conventional immunohistochemistry methods with a cocktail of several different primary antibodies conjugated to lanthanide heavy metals of different masses. In imaging mass cytometry (IMC), the labeled tissue is rasterized via spot-by-spot ablation by a UV laser; ablation results in a heavy metal-containing aerosol that passes into a mass cytometer, allowing detection of the type and quantity of heavy metal present in the tissue spot. Information from hundreds of spots is compiled to render an image representation of the heavy metals present on the tissue section [150]. A similar alternative approach, multiplexed ion beam imaging (MIBI), also uses mass-tagged antibodies but acquires metal ions using an

oxygen primary ion beam, detected seven at a time by a multi-detector mass spectrometer [151]. These approaches potentially permit more thorough phenotyping of various cell types and assessment of functional status (e.g. activation of checkpoint blockade in immune cells, activation of signaling pathways in premalignant cells, etc.), while ascertaining the spatial orientation of each cell within the DCIS niche. Importantly, we can begin to scrutinize neighborhoods of adjacent cells that comprise mini-niches within a cancer ecosystem [152]. Ongoing and future studies using both multispectral immunofluorescence and imaging mass cytometry are aimed at investigating how these neighborhoods might impact DCIS biology, promote or prevent invasion, and correlate with long-term outcome. Table 1 summarizes key techniques that have provided insight into the ITH of DCIS.

Lessons from Heterogeneity: Natural History of Breast Tumorigenesis

Despite decades of investigation, our knowledge of the natural history of breast cancer remains incomplete; however, a deepening understanding of the heterogeneity that exists in DCIS (either pure or synchronous with invasive ductal carcinoma) have begun to inform some of our models of breast cancer progression, especially the evolution of breast lesions over time.

In one commonly accepted model of breast tumorigenesis, breast cancer initiation is thought to arise from a single transformed cell amidst a field of normal mammary epithelial cells to form precancerous intraductal lesions [153–156, 101]. Indeed, data showing that spatially distant cells in DCIS (and adjacent invasive cancer) are nonetheless clonally-related suggest that a common ancestor gave rise to the resulting lesions [157, 158, 79, 101]. However, contrasting data from multi-region sequencing of multifocal DCIS have revealed that genetically unrelated lesions can be found in the same individual, suggesting that, at least in some cases, field cancerization may have contributed to tumor initiation [157, 159, 160, 100].

Following the formation of carcinoma in situ, a subset escape confinement to become invasive breast cancer. The point in this progression at which ITH arises still remains undefined; however, it must occur prior to or during the establishment of DCIS given the high degree of heterogeneity that already exists at this stage. The long-standing observations of phenotypic intratumoral diversity in DCIS have led to several explanative hypotheses, including the cancer stem cell hypothesis and the clonal evolution hypothesis [103, 161–163, 156]. Briefly, the cancer stem cell hypothesis proposes that ITH arises due to the propagation of tumors by cancer stem-like cells with the capacity to differentiate into a phenotypically diverse hierarchy of tumor cells (which themselves possess limited tumor propagating potential) [164]. The clonal evolution hypothesis proposes that cancer cells evolve as a product of ongoing mutation and natural selection, resulting in genetic (and, subsequently, phenotypic) diversification of tumor cells [165]. In the simplest sense, while the cancer stem cell hypothesis would predict that phenotypically diverse tumor cells would nonetheless be genetically homogeneous, the clonal evolution hypothesis, in contrast, would predict that phenotypically diverse tumor cells would also be genetically heterogeneous. Detailed evidence of genomic ITH in DCIS, coupled with the finding that heterogeneous subpopulation of cells within DCIS probably diverged from a common genetic ancestor

[157, 158, 79, 101], suggests that clonal evolution plays a key role in generating genomic diversity (possibly driven by underlying increased genomic instability already present in these non-invasive lesions [166]). Most likely, both cancer stem cells [167, 156] and clonal evolution drive genomic and phenotypic ITH in DCIS.

Such a mixed model might suggest the following course of breast tumorigenesis: very early hyperplasias arise from a common ancestor, experience slightly dissimilar selection forces imposed by (small) differences in spatial and microenvironmental conditions, and subsequently undergo divergent evolution over time. At any time in the evolutionary history of these cells, a subclone may acquire self-renewal capacities (if not already in possession of them) and contribute to phenotypic diversity through epigenetic plasticity and cell-fate reprogramming. Importantly, the inexorable selective force generated by a heterogeneous local microenvironment may continue to act on these diverse cell phenotypes (including cancer stem-like cells) to drive ongoing clonal evolution [161, 163, 109, 134, 106, 156]. While this mixed model has gained some traction, it remains unclear whether one or both (or other) forces predominate the generation of ITH in DCIS.

An understanding of DCIS ITH has also provided some insight into the potential mechanism by which in situ lesions become invasive. Evaluations of synchronous DCIS and invasive ductal carcinoma have revealed a surprising level of phenotypic and genomic agreement between these stages of breast cancer progression [168–170, 15, 171–175, 16, 176–181, 51, 182], arguably [79, 183] suggesting that there may not be a single (or small handful of) fixed cell-intrinsic alterations driving invasion. Consequently, an alternative hypothesis to explain the transition from in situ to invasive carcinoma emphasizes a determinant role for the local microenvironment, including, to name a few examples, modifications in extracellular matrix components and stiffness that allow epithelial cells to breach the basement membrane, functional switching of stromal cells (including myoepithelial cells and fibroblasts) resulting in local secretion of matrix metalloproteases, chemokines, and other tumor-promoting factors, and the infiltration of immune cells that modulate anti-tumor immunity [184–187, 175, 188, 109, 189, 190]. The transition from in situ to invasive carcinoma (which most likely represents the culmination of shifting physical and biochemical interactions between the evolving tumor cells and their inconstant microenvironment) remains incompletely understood and continues to be an area of intense investigation.

Clinical implications of ITH in DCIS

Heterogeneity between and within patients poses several important clinical challenges in the diagnosis, treatment, and prognosis of DCIS. For invasive breast cancers, ITH is proposed to be at least partially responsible for inaccurate biomarker classification [191]. Discordance in the diagnosis of DCIS has also been reported, in which cases of DCIS were misdiagnosed as less advanced (benign or atypia, ~13–18%) or more advanced (invasive carcinoma, ~1–3%), versus a reference consensus diagnosis [192, 193]. Notably, the cases selected for study were considered “difficult” cases that typically would require additional slides or biomarker assessment for diagnosis [194]. ITH in DCIS, combined with the biological continuum often present synchronously within each case, is likely to contribute to discordance, highlighting the need for standardized multi-level evaluation. This may be particularly relevant for cases

in which a preoperative diagnosis of DCIS is made via biopsy alone, given that biopsy is unlikely to capture the full extent or heterogeneity of disease as evidenced by the identification of invasive carcinoma and subsequent upstaging of ~25% of DCIS cases following surgical excision [195–198]. Even in cases when the diagnosis of DCIS is upheld, treatment options following surgical excision (based on a number of criteria, including histopathologic features of disease) may be further complicated in cases of high ITH and in the absence of strict standardized criteria to define recurrence-risk in DCIS. Additionally, in cases where anti-hormonal therapy follows surgical excision, ITH in ER expression and/or dependence may result in incomplete response, as has been observed, for example, with anti-HER2 therapies in invasive breast cancer [199–201].

In addition, ITH itself may be associated with more aggressive lesions. The use of ITH as a biomarker for prognosis has been suggested for other cancers, including invasive breast cancer. For example, an elevated Shannon index in invasive breast cancer has been reported to correlate with decreased disease-free survival [202]. In support of a putative link between heterogeneity and more aggressive in situ lesions, DCIS cases with elevated ITH are associated with markers of worse prognosis, including high proliferative index, poorly differentiated tumors [27], necrosis, and increased extent of disease [203]. In addition, increased DCIS ITH has been associated with increased immunostaining for (assumed mutant) p53, a link explained arguably by the genomic instability that accompanies p53-deficiency [27]. Moreover, in a group of patients with a preoperative diagnosis of DCIS, a high level of intratumoral metabolic heterogeneity was significantly associated with upstaging to either DCIS with microinvasion or invasive carcinoma, suggesting that metabolic heterogeneity may be associated with an increased propensity to become invasive [60].

Additional studies further support (albeit indirectly) a link between ITH and aggressive DCIS. Given the finding that cells from an in situ lesion invade in a multi-clonal fashion [101], one might hypothesize that multiple clones may be required to facilitate the transition from in situ to invasive breast cancer. In addition, ITH may shelter occult invasive subclones that may be responsible for disease recurrence [204–207]. In agreement with these findings, preliminary data evaluating a small number of cases revealed a potential (though not statistically significant) association between high ITH and nodal involvement [32]. Furthermore, one mechanism by which early dissemination occurs involves cooperation between epithelial tumor cells, myeloid cells, and macrophages [206], suggesting that compositional heterogeneity may be a key requirement for aggressive tumor behavior. Although these studies have begun to suggest a tentative relationship between ITH and aggressive DCIS, more studies utilizing large sample size and long-term outcome are needed to measure the degree of ITH in DCIS using standard indices, and to determine more definitively whether this heterogeneity can indeed predict long-term outcomes such as recurrence or survival.

Summary

Heterogeneity in breast cancer has been recognized for many decades. Indeed, heterogeneity across patients has led to the classification of invasive breast cancer as multiple subtypes,

varying in natural history and response to treatment. Interestingly, ITH has also been observed in DCIS. Traditional routine histopathological evaluation of DCIS has provided incontrovertible evidence of intratumoral phenotypic diversity. Development of cytogenetic assays to evaluate chromosomal aberrations provided insights into intratumoral genomic heterogeneity in DCIS and invasive breast cancer; even higher resolution insights have been achieved with the advent of single-cell next-generation sequencing. Appreciation of the active tumor microenvironment, its role in shaping tumor ITH, and its own compositional and functional heterogeneity have also emerged. Importantly, new technologies are allowing us to link phenotypic and genomic heterogeneities with their spatial and functional contexts. Ongoing studies utilizing novel technologies have led to increasingly detailed documentation of ITH in DCIS, particularly the role it plays in shaping the natural history of breast cancer and its potential as a biomarker to inform clinical management. A comprehensive assessment of the diversity present both within and around early breast cancer lesions will be required before we are able to confidently distinguish those lesions that will progress to invasive disease from those that will not.

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Table 1:

Utility of various assays in evaluating ITH

Technique	Phenotypic	Genomic	Compositional	Spatial	Single-cell resolution?
Hematoxylin and Eosin	Yes, architecture and morphology	No	Yes, can identify broad microenvironment features (tumor, stroma)	Yes, but only if morphologically evident	Yes
Single or double IHC/IF	Yes, architecture, morphology, a small number of biomarkers.	No	Yes, can identify cell types based on broad cell-specific markers (cytokeratin, vimentin)	Yes, if morphological evident and/or biomarker-specific	Yes
Multiplex immunostaining	Yes, architecture, morphology, 7–10 biomarkers.	No	Yes, can identify some specific cell types based on a handful of biomarkers markers	Yes, if morphological evident and/or biomarker-specific	Yes
Mass-tagged imaging	Yes, architecture, morphology, up to 100 biomarkers.	No	Yes, can identify many novel cell phenotypes	Yes, if morphological evident and/or biomarker-specific	Yes
Karyotyping	No	Yes, chromosomal aberrations only (usually chromosome level)	No	No	Yes, but usually only a small number of cells
FISH	No	Yes, chromosomal aberrations, gene locus aberrations	No	Yes, if performed on embedded tissues	Yes, but usually only a small number of cells
NGS (bulk tumor)	No	No, but may infer using computational tools	No	No	No, but may infer using computational tools
NGS (multi-region bulk)	No	Yes, by region sampled	Yes, if sampling microenvironment	Yes, by region sampled	No
NGS (multi-region single cell)	No	Yes	Yes, if isolating single cells from both tumor and microenvironment	Yes, by spatial distribution of single cells sampled (if sampling intact tissue section)	Yes