Genetic Alterations in Normal and Malignant Breast Tissue

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Chanel E. Smart, Peter T. Simpson, Ana Cristina Vargas, and Sunil R. Lakhani

Abbreviations

ADH	Atypical ductal hyperplasia
ALDH1	Aldehyde dehydrogenase 1
ALH	Atypical lobular hyperplasia
CAF	Cancer associated fibroblasts
CCL	Columnar cell lesion
CGH	Comparative genomic hybridization
DCIS	Ductal carcinoma in situ
ECM	Extracellular matrix
FEA	Flat epithelial atypia
FFPE	Formalin fixed paraffin embedded
G6PD	Glucose-6-phosphate dehydrogenase
HR	Homologous recombination
HUT	Hyperplasia usual type
ILC	Invasive lobular carcinoma
IDC	Invasive ductal carcinoma
LCIS	Lobular carcinoma in situ
LOH	Loss of heterozygosity
NS	Normal stroma
PLC	Pleomorphic lobular carcinoma
ROH	Retention of heterozygosity
SNP	Single nucleotide polymorphism
TDLU	Terminal ductal-lobular unit

S.R. Lakhani (✉)
University of Queensland Centre for Clinical Research, School of Medicine, and Pathology Queensland, The Royal Brisbane & Women's Hospital, Brisbane, Queensland, Australia
e-mail: s.lakhani@uq.edu.au

4.1 Introduction

Breast cancer is a heterogeneous disease with a wide spectrum of morphological subtypes and a range of clinical behaviors. Over a long time period, pathologists have evolved a system of recording cancer-related data that reflects this heterogeneity as well as providing information relevant to prognosis and prediction of response to therapy. It is well established that subtype of breast cancer, grade, and stage (Ellis et al. 1992; Elston and Ellis 1991) provide prognostic information and the use of steroid receptor analysis as well as overexpression and amplification of HER2 provides prognostic and predictive data to manage patients (Oldenhuis et al. 2008). Nonetheless, there are limitations to these data and it is well known clinically that even within the same subtype (e.g., tubular carcinoma) or same stage of disease (e.g., lymph node positive), the behavior can be markedly different. Understanding the molecular abnormalities that drive the biology of each disease will assist our ability to specifically inhibit it.

4.2 The Genetic Basis of Cancer

We currently understand cancer to be a genetic disease: driven by changes in a cell's DNA. Some mutations can be inherited from the germline, thereby being present in every cell of the body and predisposing the individual to cancer development. Otherwise, mutations occur somatically and may be caused by environmental exposure such as chemical carcinogens or radiation or impaired DNA repair mechanisms that become compromised during tumor development. The type and scale of genetic/genomic changes that occur in cancer progression are also numerous and can have profound

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effects on driving the tumor phenotype. These can be (a) gross chromosomal gains and losses, which presumably affect the expression levels of numerous genes; (b) genomic amplifications whereby a specific genomic region is replicated numerous times and these are thought to harbor oncogenes whose expression probably drives tumor growth (e.g., amplification and subsequent overexpression of *ERBB2/HER*2); (c) inactivation of tumor or metastasis suppressor genes due to any combination of hemi/homozygous gene deletion, gene methylation, gene mutation, or transcription repression (e.g., E-cadherin inactivation in lobular breast cancers); (d) genomic rearrangements culminating in the formation of fusion genes (e.g., the *ETV6-NTRK*3 fusion gene in secretory breast cancers, Tognon et al. 2002).

Over the last 2 decades, efforts to sequence the human genome and to study the molecular aspects of disease have led to significant advances in the technology now available for unraveling the genetic basis of diseases, such as cancer. The candidate gene/genomic loci approach of mutation detection or loss of heterozygosity (LOH) analysis are still valid applications for identifying specific alterations. To obtain more comprehensive characterization of somatic mutations (changes in DNA copy number) across the tumor genome, researchers have utilized the whole genome analyses called comparative genomic hybridization (CGH). Traditionally this was a low-resolution analysis providing only patterns of gross chromosomal abnormalities (Reis Fihlo et al. 2005), but nevertheless an important mechanism of identifying important events in tumor development and characterizing molecular relationships between entities. The introduction of microarray-based CGH (aCGH) has further revolutionized this technique, now providing resolution down to the 100 bp level and with the ability to characterize in detail the specific breakpoints of genomic alterations and to precisely map the genes involved. Furthermore, the drive to identify genetic variants associated with disease (genome wide association studies) has led to the development of high-density single nucleotide polymorphism (SNP) arrays that now enable genomic copy number alterations to be defined in an allele-specific manner. Of course, the explosion in genomic profiling has paralleled the boom in gene expression profiling studies that provide the next level of intricate control on the phenotype of the tumor cell. The gold standard in molecular analysis of tumor genomes is now driven by the massively high-throughput genomic and transcriptomic sequencing. This is not yet fully accessible to all researchers, but has the ability to define genomic rearrangements and gene mutations at nucleotide resolution, and obtain unbiased assessment of mRNA and microRNA expression levels (Stratton et al. 2009).

These methods have highlighted the genomic complexity of breast cancer and are fundamentally changing our understanding of the biology of breast disease. These efforts have identified important mutations in disease pathogenesis, led to the development of molecular targets for therapy (e.g., ER and HER2), supported the idea that certain preinvasive lesions are precursors for the development of invasive cancer, and are helping to refine the classification of the disease (Alizadeh et al. 2001; Buerger et al. 1999b; Lakhani 1997; Nishizaki et al. 1997; Pollack et al. 1999; Reis-Filho et al. 2005; Simpson et al. 2005b; Perou et al. 2000; Sorlie et al. 2001).

4.3 Molecular Analysis of Invasive Breast Cancer

Molecular genetic analyses of invasive breast cancers have defined common genomic alterations as gain of material on chromosome 1q, 8q, 17q, 20q, and losses of material affecting 4q, 5q, 8p, 11q, 13q and 16q. Some common high level genomic amplifications occur at 1q32, 8p12, 8q24, 11q13, 17q12 and 20q13. The pattern of genomic alterations has been shown to closely correlate with histological grade, molecular subtype, and, to a lesser extent, histological type. One of the most important molecular findings has provided fundamental evidence that low-grade breast cancers are different to high-grade breast cancers at the molecular level, and so presumably arise through different pathways of development (Buerger et al. 1999a, b, 2000; Roylance et al. 2006; Stratton et al. 1995). Overall, low-grade ductal carcinomas and tubular carcinomas show a low level of genomic instability with characteristic losses at chromosome 16q, gains on 1q, and few other recurrent alterations, whereas high-grade breast cancers show a greater degree of genetic instability with more complex genomic alterations and more high levels gains (amplifications) on regions such as 17q12, 8q24, and 20q13. These data, initially derived from loss of heterozygosity (LOH) and chromosomal CGH analysis, therefore suggested that

the evolution of low-grade tumors from normal tissue was by a pathway independent to that of high-grade carcinomas. The loss of 16q for instance is frequent in low-grade tumors and involves the whole chromosomal arm, while in high-grade tumors, loss of 16q is less common, and when it does occur it is by a different mechanism (LOH with mitotic recombination) (Roylance et al. 2002, 2006; Cleton-Jansen et al. 2004; Natrajan et al. 2009a).

However, there may be some exceptions to this rule since around 20% of high-grade invasive ductal carcinomas (IDC) harbor loss of the whole of 16q. Grade III IDCs are a heterogeneous group of tumors, both morphologically and molecularly. Recent aCGH analysis of high-grade IDC revealed that the majority of tumors containing loss of the whole arm of 16q were estrogen receptor (ER) positive, suggesting that in these cases there maybe evidence to support progression from the low-grade/ER+ve pathway of tumor development to high-grade/ER+ve breast cancers (Natrajan et al. 2009a). Data in support of this come from the study of pleomorphic lobular carcinomas (PLC). PLC are a recently described variant of classic invasive lobular carcinoma (ILC) (Eusebi et al. 1992; Middleton et al. 2000; Weidner and Semple 1992; Palacios et al. 2003; Sneige et al. 2002; Simpson et al. 2003), with a reported aggressive biological behavior (Orvieto et al. 2008; Buchanan et al. 2008). Briefly, neoplastic cells in pleomorphic lobular carcinoma in situ (LCIS) and ILC show the typical discohesiveness of lobular neoplasms and lack E-cadherin expression; however, PLC are of high grade and show features of apocrine differentiation. Although molecular data on the PLC are scant, these tumors have overlapping genetic changes with both classic ILC and grade III invasive ductal breast carcinomas. Importantly, they harbor loss of 16q and overall a similar genomic profile to ILC. The data suggested that PLC arise from the same pathway as ILC. The sporadic accumulation of genetic alterations more common to high-grade cancers (*HER2*, *p53*, *MYC*) may then contribute to the more aggressive biology (Simpson et al. 2008).

CGH and conventional cytogenetic studies have demonstrated that there is a degree of variation in the pattern of genetic alterations between different histological subtypes of invasive breast cancer. Although differences between histological subtypes do exist, this association is not as strong as with histological grade (Buerger et al. 1999a; Reis-Filho and Lakhani 2003). Comparative analyses between IDCs and ILCs have demonstrated that overall a lower number of genetic changes are found in ILCs relative to IDCs. Although some specific chromosomal abnormalities are found at a significantly different frequency in each histological type, this may only highlight the fact that most ILCs are of lower nuclear grade. Interestingly, several recurrent unbalanced changes, including physical loss of 16q, are common to both types, indicating that ILCs and low-grade IDCs may arise via common tumorigenic pathways.

As a result of this and other molecular data, some authors have questioned whether the boundary between ductal and lobular lesions should be removed and whether the designations "ductal" and "lobular" are appropriate. Since it is clear that the majority of neoplastic breast diseases arise from the terminal duct–lobular unit (TDLU), the terminology of "ductal" and "lobular" is not intended to reflect the microanatomical site of origin, but a difference in cell morphology and biology (Simpson et al. 2003). Hence, it is worth stressing that although loss of 16q is observed in both grade I IDC and ILC, the genes most affected by this deletion probably differ between these two lesions. The likeliest candidate tumor suppressor gene involved in loss of 16q in ILC is *CDH1* (E-cadherin), which maps to 16q22.1 (Cleton-Jansen et al. 2004; Palacios et al. 2003; Simpson et al. 2003). It is accepted that ILC harbor loss of 16q, followed by gene mutation, promoter methylation, or further loss of *CDH1*. Loss of E-cadherin, a critical cell adhesion molecule, is reflected at the morphological level by the characteristic discohesive nature of individual cells and overall growth pattern of lobular carcinomas. However, *CDH1* is almost certainly not the target gene in grade I IDCs as loss of E-cadherin expression and *CDH1* gene mutations are exceedingly rare in these tumors. The hunt for the tumor suppressor gene(s) involved in grade I ductal cancers continues (Cleton-Jansen 2002; Rakha et al. 2004b; Roylance et al. 2003).

4.4 Molecular Classification of Invasive Breast Cancer

Array-based techniques of CGH and gene expression profiling have led to the development of new molecular-based classification schemes for breast cancer.

These seem to be inter-related, whereby the genomic alterations and subsequent changes in gene expression are controlling tumor phenotype.

More recently, microarray-based expression profiling has added further insight into breast cancer heterogeneity and produced a new taxonomy of breast cancer, dividing tumors into five major molecular subclasses, namely luminal A, luminal B, HER2, basal-like, and normal-like (Perou et al. 2000; Sorlie et al. 2001, 2003). The major distinction is at the level of the ER. The ER positive cluster comprises tumors that have a gene expression signature similar to that seen for the normal luminal epithelial compartment of the breast with expression of low molecular weight keratins such as CK8/18 and ER and related genes. These "luminal" tumors are further divided into subclass A and B with luminal B being higher grade, having higher proliferation index and a poorer prognosis. It is worth noting that although the groups appear distinct on such an analysis, there is a continuum, and further there is at least data from lobular cancers (classic, luminal A; and pleomorphic variant, luminal B) that luminal A cancers can evolve into luminal B cancers through the stochastic acquisition of mutations in genes associated with worse prognosis such as *HER2* and *TP53*.

The ER negative group is more heterogeneous. The normal-like group is the least convincing and may be an artifact of the study, reflecting normal cell contamination in the samples. The HER2 and basal-like were shown to have the worst prognosis in the original studies although it is clear from many studies that the basal-like cancers are an extremely heterogeneous group with prognosis ranging from "good" to "bad." Basal-like cancers are so designated because these tumors express genes usually found in normal basal/myoepithelial cells of the breast, including high molecular weight cytokeratins (CK14, 5/6 and 17) as well as P-cadherin, P63, S100, and epidermal growth factor receptor (EGFR/HER1). The morphological features of these tumors are distinct with central acellular and necrotic zones, pushing borders, high degree of pleomorphism and mitotic index and areas of squamous and spindle cell differentiation (Fulford et al. 2006). These tumors are often but not invariably triple negative (ER, PR, and HER2 negative). HER2 tumors are also high grade and are characterized by overexpression of HER2 and genes associated with the HER2 pathway. In clinical practice, some HER2 over-expressing tumors however fall into the luminal B category. The microarray studies have also identified further ER negative cancer subtypes including an "apocrine" subgroup (Farmer et al. 2005), an "interferon" subgroup, and a "claudin-low" subgroup (Hennessy et al. 2009). The clinical and biological significance of these subgroups remains to be elucidated.

The genomic architecture of invasive tumors, as characterized by array-based CGH analysis, can be classified as "simplex," "complex-firestorm" or "complex-sawtooth" (Hicks et al. 2006; Bergamaschi et al. 2006; Natrajan et al. 2009b), and these show correlation with the molecular subtypes classified by expression profiling. The "simplex" pattern is associated with a good outcome and is typical of low-grade luminal-like cancers, frequently displaying concurrent 1q gain and 16q loss. In contrast, the complex pattern is associated with poor outcome. The "firestorm" pattern involves a region of complex amplification affecting regions such as 11q13, 8p12, 8q, 17q12, and is typically seen in "HER2" and "luminal B" cancers. The "sawtooth" category has many narrow areas of duplication and deletion, affecting all chromosomes and the majority of the genome but with no/few amplifications and is typically seen in "basal-like" cancers. It is possible that the types of copy number profiles seen may be due to the different types of DNA repair defects/instability present in these tumors.

There is of course considerable excitement in the new molecular classification, but it is worth bearing in mind that the systems of classification are still likely to evolve as we get further insights into the biology of breast disease. For instance, the new taxonomy has led some to postulate a histogenetic classification of breast cancer with "luminal" subtypes arising from luminal epithelial cells and "basal-like" cancers arising from the basal/myoepithelial cells or even stem cell since they often express "luminal" and "myoepithelial" keratins. Data emerging from the study of normal cell populations and their progeny suggest that basal-like cancers may arise from luminal progenitors (Lim et al. 2009). There is much work to be done in understanding normal cell lineage differentiation and the plasticity of individual cell types, and we should be cautious in making too many leaps into histogenetic classification of disease.

4.5 Molecular Analysis of Preinvasive Breast Cancer

The frequent association and morphological similarities between invasive carcinomas and many forms of proliferative breast diseases have led pathologists to speculate that certain entities would be biologically related (e.g., LCIS and ILC, ductal carcinoma in situ (DCIS) and IDC) (Reis-Filho and Lakhani 2003). The complexity of these relationships has been thoroughly explored using the advancement in molecular pathology and has largely recapitulated the genotypic/phenotypic patterns observed in invasive ductal and lobular carcinomas in atypical ductal hyperplasia (ADH) DCIS and atypical lobular hyperplasia (ALH) LCIS (Buerger et al. 1999a, b; Lakhani et al. 1995; Lu et al. 1998; O'Connell et al. 1998). The distinct molecular genetic features found in different grades of invasive carcinomas are also mirrored in preinvasive lesions of comparable morphology (Buerger et al. 2000; Reis-Filho and Lakhani 2003).

In retrospect, it is clear that there are two major arms in the multi-pathway model of breast cancer progression: one comprising well-differentiated DCIS (low grade) that progress to grade I IDC, and the other encompassing poorly differentiated (high grade) DCIS that progress to grade III IDC. In the "low-grade arm," these in situ and invasive tumors are of low nuclear grade, usually ER and PR positive, negative for Her-2 and basal markers, and harbor low genetic instability and recurrent 16q loss, whereas in the "high-grade arm," the lesions show a higher degree of nuclear atypia, are more frequently hormone receptor–negative, frequently positive for either HER2 or basal markers, and are genetically advanced lesions, showing a combination of recurrent genomic changes including loss of 8p, 11q, 13q, 14q; gain of 1q, 5p, 8q, 17q; and amplifications on 6q22, 8q22, 11q13, 17q12, 17q22–24, and 20q13. Based on their pathological and genetic features, classic LCIS and ILC are remarkably similar to those tumors in the "low-grade arm" (Lu et al. 1998; Simpson et al. 2003). However, in contrast to well-differentiated DCIS/grade I IDC, the vast majority of these tumors lack E-cadherin expression owing to genetic and/or epigenetic changes in the *CDH1* gene (Rakha et al. 2004a; Roylance et al. 2003). On the other hand, the overlapping morphological features of PLCIS and PLC with both classic lobular and grade III carcinomas, and the combination of E-cadherin (16q) loss with occasional HER2 positivity (Eusebi et al. 1992; Palacios et al. 2003; Sneige et al. 2002; Reis-Filho et al. 2005) add another level of complexity to these molecular pathways to breast cancer progression.

Apart from ADH and ALH, which bear stark morphological and molecular resemblance to low-grade DCIS and LCIS, respectively, the other non-obligate/premalignant lesions are more difficult to characterize and to establish their position along the multistep pathways (O'Connell et al. 1994, 1998; Reis-Filho and Lakhani 2003). Interestingly, ADH and low-grade DCIS show identical immunoprofiles with low numbers of chromosomal abnormalities, comprising recurrent loss of 16q. The similarities between ALH and LCIS are also at the morphological, immunohistochemical, and genetic level (Simpson et al. 2003). In fact, differentiating between ALH and LCIS is arbitrary and subjective, being based on subtle quantitative rather than qualitative morphological features. Hence, it is well accepted that both ADH and ALH are non-obligate precursors for the development of low-grade DCIS and LCIS, respectively. Alternatively, one could view them just as small DCIS or LCIS although this is not the view accepted by all.

Clonal diversity, evidenced by morphological and molecular intra-tumoral heterogeneity, adds further complexity to this model and probably accounts for some of the considerable diversity in the clinical nature of the disease. This diversity might be explained by ensuing genetic instability leading to the development of multiple neoplastic clones within the same tumor. Clonal diversity has been reported in DCIS, where up to 50% of cases studied showed heterogeneity in grade, with 9% of cases of low-grade DCIS also showing areas of intermediate and high-grade DCIS. Such cases exhibited heterogeneous expression of immunohistochemical biomarkers and, in particular this correlated with p53 positivity (Allred et al. 2008). The authors speculated that in some cases, ADH (the precursor to low-grade DCIS) could therefore be the precursor to high-grade DCIS and hence grade III IDC. The molecular data that low-grade disease is different to high-grade disease also raise the possibility of coexistence of independent clones of differing grades rather than one arising from the other.

For many years, hyperplasia of usual type (HUT) has been seen as the precursor of ADH and DCIS.

However, its role in the multistep model of breast carcinogenesis has been questioned (Aubele et al. 2000; Jones et al. 2003; Lakhani et al. 1996). The morphological features and immunoprofile of HUTs are different to those of the accepted precursors since they are composed of a mixed population of cell types with a variable proportion of ER, PR-positive luminal cells, and myoepithelial/basal marker-positive cells. At the molecular level, few fairly random chromosomal changes are observed (Aubele et al. 2000; Jones et al. 2003; Lakhani et al. 1996). Nonetheless, there is evidence to suggest that a small proportion of HUTs may be clonal, neoplastic proliferations (equivalent to colonic adenomas) that may putatively progress to ADH or DCIS, whereas the majority of them fail to show any evidence of a neoplastic/monoclonal nature using existing technology. Currently, most authors do not regard these lesions as playing any significant role in tumorigenesis and see these lesions as "dead-end."

A more likely candidate for precursor to ADH and low-grade DCIS is columnar cell lesion (CCL) (Fraser et al. 1998; Schnitt and Vincent-Salomon 2003; Simpson et al. 2005a). These comprise a spectrum of lesions with varying degrees of architectural and nuclear atypia. At the lower end of the spectrum are lesions referred to as columnar cell change and hyperplasia, and at the worst end, lesions that have atypia sufficient to be designated "flat epithelial atypia" (FEA) to fully developed ADH lesions. Throughout the spectrum, CCLs show an immunoprofile similar to that of ADH/low-grade DCIS (Schnitt and Vincent-Salomon 2003). However, the degree of proliferation, architectural, and cytological atypia are mirrored at the genetic level, with a stepwise increase in the number and complexity of chromosomal copy number changes as defined by CGH (Simpson et al. 2005a). Moreover, the hallmark genetic feature of "low-grade" lesions, loss of 16q, is the most frequently detected recurrent change and in addition, there is some degree of overlap in the molecular genetic profile of CCL and associated more advanced lesions (Moinfar et al. 2000b; Simpson et al. 2005a). Interestingly, it is not infrequent to observe ALH/LCIS in the context of multifocal CCLs. Hence CCLs may be the link between normal breast and ADH, as well as between "ductal" and "lobular" neoplasia (Abdel-Fatah et al. 2007, 2008). The precursor of poorly differentiated DCIS has been elusive. Based on morphological, immunohistochemical, and molecular findings, CCL, ADH, and low-grade DCIS would be unlikely candidates.

Although apocrine change has long been considered a metaplastic process in breast tissues, usually associated with aging, this concept has come into question with the application of molecular findings (Jones et al. 2001; Selim et al. 2000, 2002). At least a subset of lesions with apocrine morphology show molecular changes, including LOH/allelic imbalance at 1p (*MYCL1*), 11q (*INT2*), 13q, 16q and 17q, and recurrent chromosomal changes as defined by CGH, including loss of 1p, 2p, 10q, 16q, 17q and 22q, and gain of 1p, 2q and 13q. These findings are more frequently observed in apocrine adenosis, and apocrine hyperplasia compared with apocrine cysts. For the large part, these observations have been ignored. Whether this prejudice is justified should be questioned. It may turn out to be wrong, but we would suggest that there is compelling molecular data that at least some of these lesions may be the precursors of high-grade DCIS and invasive cancer.

4.6 Molecular Alterations in Normal Breast

Since molecular alterations at genetic loci have been identified in many putative precursor lesions, the attention also shifted to whether "normal" tissues in the vicinity of preinvasive and invasive carcinomas may harbor mutations. With developments in microdissection in the early 1990s, Deng et al. (1996) reported that LOH identified in invasive carcinoma is indeed present in morphologically normal breast lobules adjacent to carcinomas, but not away from the tumor. Since their studies were carried out on microdissected tissues from paraffin-embedded sections, the possibility that the LOH was a result of tumor cells migrating to the lobular units via pagetoid spread could not be entirely excluded. Their findings could therefore be accounted for by one of three hypotheses: first, that the LOH identified is actually due to the presence of tumor cells, which have migrated into the normal lobule from the nearby invasive carcinoma; second, that the LOH is indeed present in the morphologically normal cells analyzed. The second hypothesis could imply that a "normal" area of the breast harbored genetic change preceding the development of the invasive carcinoma. Furthermore, that the carcinoma may arise as a result of additional changes to these "normal"

cells. How this preliminary change arose is a major tenet of the sick lobe hypothesis (Tot 2005).

The sick lobe hypothesis suggests that changes to the breast epithelium occurring during its development can result in entire lobes or segments of the breast that are in someway predisposed to further changes (in adulthood) that result in the onset of cancer (see Fig. 4.1a, b). It is possible that this initial change in one cell, then passed on to all other cells derived from it to comprise a lobe of the breast, occurs at the genetic level by DNA mutation. This would suggest that the normal lobule is clonal, which would raise questions about the existence or otherwise of a common progenitor or stem cell from which all the epithelial cells

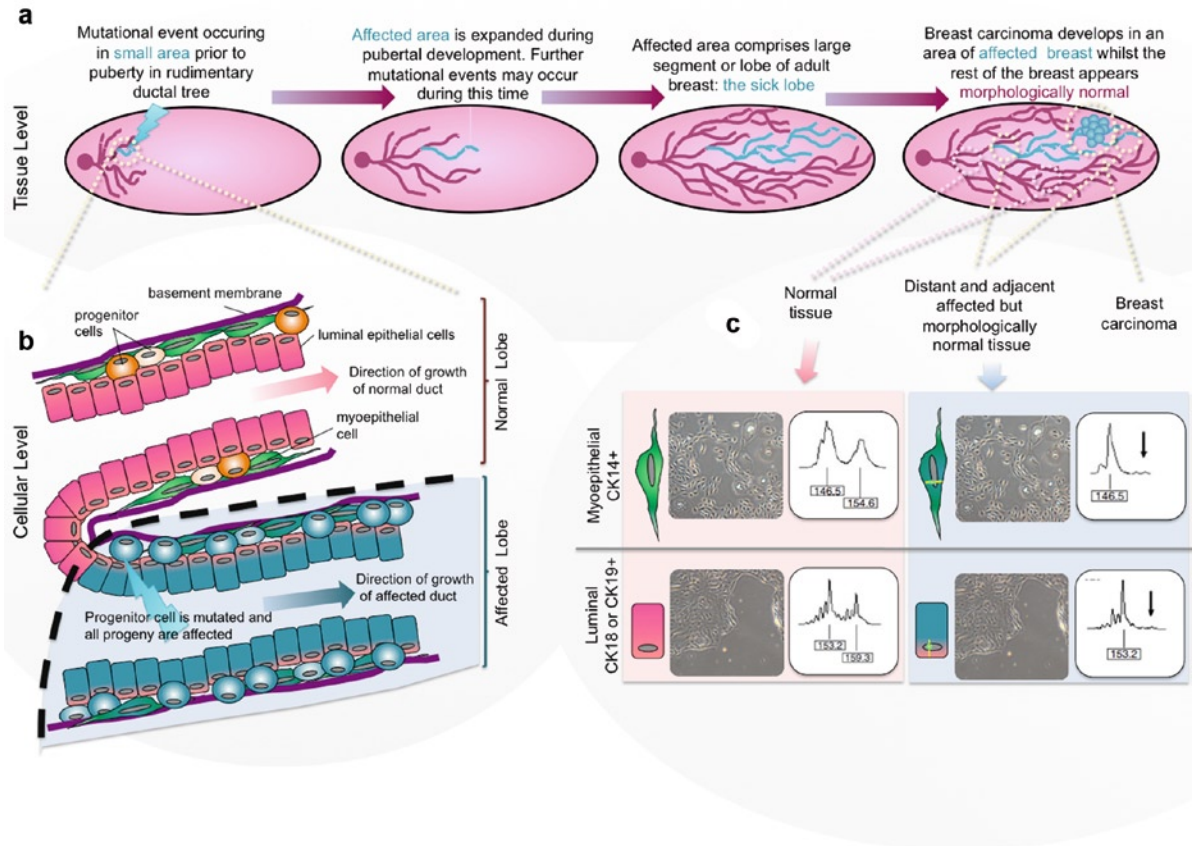

Fig. 4.1 Possible mechanisms of genetic alteration affecting the sick lobe. (**a**) Simplified diagram demonstrating how genetic change in the glandular epithelium of the developing breast may result in a "sick lobe" by passing this change onto progeny. Normal glandular epithelium is illustrated in red, while affected epithelium comprising the sick lobe is shown in blue. (**b**) Diagram illustrating possible cellular composition of bifurcating region of normal and sick lobes. Should a mutational event occur in a breast stem or progenitor cell, this change might be later observed in all cell types derived from it during expansion and differentiation of the ductal tree as it grows into the mammary fat pad, including both luminal and myoepithelial cells as well as other progenitors cells. It is also possible that the frequency of cell types, their function, and the molecular profiles of these affected areas may be altered (shown in blue), although it is possible that such change may not be detectable by traditional histomorphological examination. (**c**) The adult breast may therefore be comprised of normal lobes and sick lobes, the latter harboring genetic alteration that may predispose to development of carcinoma. This model is supported by the findings of Clarke et al. Cell clones derived from fresh dissociated mastectomy samples from *BRCA1/BRCA2*-mutation carriers were identified as either luminal (CK18/19 positive) or myoepithelial (CK14 positive) features. While DNA derived from most clones demonstrated retention of heterozygosity (ROH) and were therefore considered normal, some rare clones of both luminal and myoepithelial phenotypes showed loss of heterozygosity (LOH) at *BRCA1* or *BRCA2* loci. Where these morphologically normal cells harbored the same mutation as the tumor residing in the breast, it could suggest that this change has predisposed to the development of the cancer. Indeed, LOH (particularly of tumor suppressor genes such as *BRCA1/2*) is considered one of the initial steps of tumor formation. Furthermore (not shown here), where both myoepithelial and luminal cell clones are shown to harbor the same genetic alteration, this could be interpreted to suggest that the initial change occurred in a progenitor cell, which is common to both cell types

comprising the lobe were derived, there are several studies whose findings suggest this to be true.

Using an in vitro cell cloning technique, Lakhani et al. (1999) addressed these issues by looking for LOH in breast samples free of contamination from tumor cells and examined LOH independently in both the luminal and myoepithelial cells of the breast. Chromosomal loci exhibiting LOH at high-frequency in invasive breast cancer were investigated in "normal" breast tissue from patients with carcinoma and from reduction mammoplasty specimens. Ductal-lobular units dissected from paraffin-embedded tissues and 485 "normal" luminal and myoepithelial cell clones cultured from a fresh dissociation were studied. The ability to distinguish between different epithelial types is important in determining whether the change occurred in a common progenitor or stem cell whose progeny then differentiated into luminal and myoepithelial cells. Overall, LOH was found in normal cells in five of ten breast cancer cases and one of three reduction mammoplasty specimens. LOH was identified in normal cells adjacent to and distant from the tumor. One of 93 clones from three reduction mammoplasties also showed allele loss at a locus on chromosome 13q. In one of the cases, all luminal and myoepithelial samples exhibited loss of the same allele on chromosome 13q. These data confirmed the presence of LOH in normal tissues as well as demonstrated an independent loss in the luminal and myoepithelial component, suggesting that the alteration may have occurred in progenitor/stem cells prior to lineage differentiation. The fact that alterations are in both cell types and can also be identified using microdissection also provided evidence that the clonal patch derived from stem cells was likely to be large within the breast.

The ability to identify clonal patches is difficult in human samples but important, not just for normal biology but because there has been a huge body of literature suggesting clonal nature of lesions in the breast using X-linked inactivation methodologies. Many authors failed to realize that without knowledge of clonal patch size, these data were not meaningful. If the patch size is large, it is possible to get a proliferation from multiple cells but yet would appear clonal using X-linked methods. In order to examine the clonal architecture of normal tissue, it is necessary to have a cellular marker that can be used to identify a subset of germ-line cells. Experimentally using chimeric or mosaic animals can achieve this.

As a result of the process of X inactivation, females heterozygous for X-linked polymorphisms are functionally mosaic at the mRNA and protein levels. Previous studies have used X-linked genes such as glucose-6-phosphate dehydrogenase (*G6PD*) (Fialkow 1976) or restriction fragment length polymorphisms (Vogelstein et al. 1985) without reference to patch size. In female mammals, the process of X inactivation occurs early during embryogenesis (day 16 in the human female). This process involves random inactivation of most of the genes on one or the other of the two X chromosomes by methylation of CpG islands (Lyon 1972). The pattern of methylation is stable and inheritable so that it is passed on to all cellular progeny. The pattern of X inactivation is also widely believed to be stable during tumorigenesis (Jones 1996). As X inactivation occurs at a relatively early stage of embryogenesis, although there is inevitably some mixing of cells during further development, in the adult mammal, many of the progenies of a single X-inactivated embryonic cell are arranged together. In epithelia, these groups of cells sharing a common X-inactivation pattern are termed patches. A single patch may be formed of the progeny of one cell or several cells all showing the same X-inactivation pattern. Thus, cells in a single patch are monophenotypic but may be clonal or polyclonal in derivation. Novelli et al. (2003) collected surgical resection specimens of Sardinian females heterozygous for the *G6PD* Mediterranean mutation (563 C → T). All patients had been previously shown to have reduced G6PD enzyme activity, and heterozygosity for the *G6PD* Mediterranean mutation was confirmed by PCR analysis of genomic DNA followed by *Mbo*II restriction endonuclease digestion. Using histochemical method on tissue sections, they confirmed that the clonal patch within the female breast was large, involving whole ducts and lobular units, providing further evidence to support the hypothesis generated from the LOH data.

In their original paper, Lakhani et al. (1999) had one case in which the patient had a germ-line truncating mutation in the *BRCA1* gene and they found LOH on 17q in 3 of 33 normal clones. One of these clones showed loss of wild-type allele, indicating gene inactivation. This sample also had LOH at markers on chromosomes 11p and 13q, suggesting that further alterations may have occurred as a result

of genomic instability due to loss of homologous recombination (HR). This work was followed by further analysis of cases with germ-line *BRCA1/2* mutations. Clarke et al. (2006) studied LOH at the *BRCA1* and *BRCA2* loci in 992 normal cell clones derived from topographically defined areas of normal tissue in 4 samples from *BRCA1/BRCA2* mutation carriers. The frequency of LOH in the clones was low (1.01%), but it was found in all 4 samples, whether or not a tumor was present. Again, LOH could be detected in both luminal and myoepithelial clones, which indicates not only that both cell types can harbor such genetic changes (see Fig. 4.1c), but where those changes are identical it suggests they have been derived from a common progenitor cell. It is also possible the cell clones are themselves derived from committed progenitors by virtue of the fact that they can grow in in vitro culture. Interestingly, topographical mapping revealed that the genetic changes were clustered in a segmental distribution in some of the breast samples. The study provided further evidence that a field of genetic instability can exist around a tumor and that this size was greater than one TDLU. Although there are little additional data to confirm these findings, at face value, it does suggest that sufficient tissue must be removed at surgery to avoid local recurrence and raises questions about whether such alterations could account for some cases of local recurrence after apparent "complete excision" of the tumor.

Two other more recent studies provide further evidence that the normal breast of *BRCA*-mutation carriers is altered and shows early changes similar to those found in the subsequent carcinoma. Max Wicha's group not only reproduced the observation of *BRCA1* LOH in morphologically normal areas of the breast in *BRCA1*-mutation carriers (Liu et al. 2008), they also discovered that ALDH1 – a putative stem cell marker – could be used to identify those lobules which contained this genetic alteration. While ALDH1 positive cells appear to be extremely rare in the normal breast of healthy donors, *BRCA1*-mutation carriers have a higher frequency of ALDH1 positive cells that appear to comprise entire acini in breast lobules of these patients. This is significant because it provides evidence that entire areas of the *BRCA1*-breast can show both genetic and molecular (in this cases protein) differences despite appearing morphologically normal. In this way it is possible that the color used to delineate the sick lobe in Fig. 4.1a could be representing ALDH1 positivity in the breast of *BRCA1*-carriers, depending on how early these changes occur. Furthermore, as a putative stem cell marker, the expression of ALDH1 at high frequency in the lobules of *BRCA1*-mutation carriers may suggest that the initial genetic change that occurs in a breast stem cell, which although has then expanded to form the acini, may in someway be defective, unable to lose expression of this marker, and somehow blocked in its ability to differentiate. Another study that revealed an expanded and abnormal luminal progenitor population in mastectomy samples of *BRCA1*-mutation carriers adds weight to this hypothesis (Lim et al. 2009) although they did not investigate the presence of genetic differences in this population.

4.7 The Sick Stroma

LOH in the mammary stroma of patients with breast cancer has also been demonstrated by Moinfar et al. (2000a). By using 11 DNA markers on FFPE tissue, LOH was reported in the morphologically normal stroma in 11–57% of cases. A comparison of LOH frequency in the epithelial/stromal cells revealed that 73% of cases were associated with at least one identical LOH in both the epithelial and stromal components. This intriguing observation suggests that there may be common precursors for epithelial and stromal cells; however, these data need validation and are certainly contrary to other more recent analysis. Kurose et al. (2001) identified that genetic alterations occurred in the epithelial compartment, followed by LOH in the stromal compartments, indicating that genetic alterations in the epithelia precede the ones in the stroma.

Most of studies based on cDNA microarrays have focused mainly on the neoplastic transformation of the breast epithelial compartment. Recently attention has focused on the role of breast tumor stroma in breast cancer progression demonstrated by gene expression profile. In a series of 14 patients with matched DCIS, IDC, normal epithelium, IDC-associated stroma (IDC-S), DCIS-associated stroma (DCIS-S), and normal stroma (NS), Ma et al. (2003) provided evidence that gene expression changes occurred in the stroma

during breast cancer progression, suggesting that tumor stroma may co-evolve along with the malignant epithelium even before epithelial transformation occurs. Genetic alterations mainly involved components of the extra cellular matrix (ECM) and ECM-remodeling matrix metalloproteases (MMP). While cytoplasmic ribosomal proteins were decreased in both compartments, mitochondrial ribosomal protein genes were increased. Differentially expressed genes between IDC-S and NS included antagonists of the WNT receptor signaling which were downregulated and upregulation of TGFb family members. The stroma showed genes enriched for extracellular matrix, MMP, and cell cycle–associated genes, indicating that increased proliferation is a feature of stroma. Particularly, the stroma showed significant expression of MMP11, MMP2, MMP14, and MMP13 in IDC-S compared to DCIS-S, suggesting that MMPs may play a role in the transition from in situ to invasive carcinoma (Fleming et al. 2008; Ma et al. 2009).

Allinen et al. (2004) developed a purification procedure that allows the isolation of pure cell populations from breast tissue. This method is based on cell type–specific cell surface markers and magnetic beads. BerEP4 sorted epithelial cells, CD45 was used for leukocytes, P1H12 for endothelial cells, and CD10 for sequential isolation of myoepithelial cells and myofibroblasts. The unbound fraction following the removal of all other cell types was regarded as the fibroblast-enriched stromal fraction. However, further differentiation between myoepithelial cells and myofibroblasts was not possible. As a result, these cell types were regarded as a single group. By aCGH analysis, no genetic changes were detected in myoepithelial cells/myofibroblasts isolated from DCIS/IDC. By contrast, numerous genetic changes were observed in tumor epithelial cells. Normal tissue adjacent to tumors did not express any genetic alterations. SNP arrays and LOH methodology were also applied to the same purified cell types and showed no evidence of LOH in any stromal cell from DCIS/IDC samples. Therefore, unlike the studies of Lakhani et al. and Clarke et al., genetic changes using these methodologies were only detected in luminal epithelial cells (Allinen et al. 2004).

Genomic alterations of breast cancer–associated fibroblasts/myofibroblasts are not consistent among the studies. Qiu et al. (2008) by using SNP array–based technologies studied cancer-associated fibroblasts (CAFs) microdissected from fresh frozen primary human ovarian and breast cancers as well as some specimens derived from primary culture. None of the 10 CAFs from breast tumors harbored any evidence of copy number alteration or LOH on any chromosome. However, one fibroblast culture showed gains on chromosomes 7 and 10. Interestingly, when CAF cultures without any detectable somatic changes were injected in xenografts, tumor growth occurred more efficiently compared to normal breast stromal fibroblasts.

Although genomic alterations could not be demonstrated, epigenetic alterations cannot be ruled out. Qiu et al. (2008) showed in the same study that CAFs from primary cultures showed different methylation and expression patterns compared to normal counterparts, implying an abnormal phenotype of CAF cultures in the absence of somatic genetic changes (Qiu et al. 2008). Furthermore, genomic methylation profiling, bisulfite sequencing, and anti-5-methyl-C immunohistochemistry have been used to show global hypomethylation in myofibroblasts. Studies in a transgenic mouse model from gastric carcinoma indicate its early occurrence in cancer progression; however, the cause of genomic demethylation in cancer cells remains unknown (Jiang et al. 2008).

Hence overall, there is little doubt that normal tissue harbors molecular alterations and that epithelial cells do show genetic mutations; but whether stromal cells show mutations or whether they show changes in expression without DNA alterations remains to be clarified. What is clear is that changes in the normal cell compartments play an important and interesting role in breast cancer development.

4.8 Hypothesis

Taking together the totality of evidence relating to molecular alterations involved in the multistep model of breast cancer and the finding of changes with normal luminal and myoepithelial cells of the breast, it is plausible that the first alterations giving rise to tumorigenesis may occur in stem/progenitor cell populations. Since most mutations occur during the process of cell division when DNA replication occurs, it is also likely that mutations in these progenitor populations are occurring at a time of greatest cell division, i.e., at puberty when there is prolific cell division to

produce the adult breast. Alterations in the stem/progenitor cells at that time could conceivably give rise to large clonal patches with genetic alterations, which subsequently predispose the tissues to further changes and hence begin the journey toward tumor formation. Such a hypothesis would certainly explain the segmental distribution of many forms of breast diseases, e.g., DCIS, although it would not by itself explain multifocal disease such as seen with LCIS. Presumably, in these cases, there is a combination of germ-line predisposition and a somatic predisposition coming together to produce the risk and disease distribution seen in clinical practice. Current data on molecular alterations within the stroma and the increasing recognition of the importance of stromal–epithelial interactions raise the possibility that the stromal component as well as the epithelial component may be abnormal within the "sick lobe."

4.9 Conclusion: Is There Any Relevance to Clinical Practice?

This is a difficult question to answer other than that if the hypotheses are correct, it would suggest that recurrences may be new tumors arising from the already unstable clonal patch that has been left behind since the excision is currently done to excise the cancer without knowledge of the topography of the abnormal patch. It is not possible at present to excise in such an anatomically precise manner. Perhaps in the future, if these abnormal patches could be identified *in vivo*, intraductal or external methods to ablate the ductal tree may be feasible. This of course does not take into account the role that the stroma may play and hence in reality, the management could be a lot more complicated than we envisage.

The next decade will be critical as new technology combined with traditional pathology comes together to unravel the biology of breast tumorigenesis and hence provide insights into how we should manage our patients with breast disease.

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