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Genetic and epigenetic signatures of breast cancer subtypes

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TABLE OF CONTENTS

1. *Abstract*
2. *Introduction*
3. *Breast Cancer Stem Cells*
4. *Intrinsic Subtypes of Breast Cancer*
    - 4.1. *Tumors arising from luminally-committed cells*
        - 4.1.1. *Luminal A and luminal B Tumors*
        - 4.1.2. *ERBB2/HER2 Tumors*
    - 4.2. *The luminal progenitor ("basal-like") cancers*
    - 4.3. *Tumors arising from transformation of the mammary stem cell*
5. *Genetic Risk Factors for Breast Cancer: BRCA1 and the basal-like phenotype*
6. *Epigenetic Changes in Breast Cancer Subtypes*
    - 6.1. *DNA methylation*
    - 6.2. *Histone modifications*
    - 6.3. *MicroRNAs (miRNAs)*
7. *Final comments*
8. *Acknowledgements*
9. *References*

1. ABSTRACT

Breast cancer is a heterogeneous disease at both the histological and molecular levels. The current model of breast tumorigenesis suggests that the normal mammary stem cell and the various progenitors that arise thereof can be transformed and generate lineage-restricted tumor phenotypes. This model is supported by observations that the different subtypes of breast cancer share transcriptional signatures intrinsic to normal components of the mammary epithelium. Studies have since elaborated these molecular signatures to include recurrent genetic abnormalities, patterns of DNA methylation and dysregulation of microRNAs. Here we aim to review the current state of knowledge concerning the cellular etiology of breast cancer subtypes and the genetic, transcriptional and epigenetic aberrations associated with each subtype.

2. INTRODUCTION

Breast cancer remains the second leading cause of cancer-related death worldwide. The World Health Organization (WHO) estimates that more than one million women are diagnosed with breast cancer annually, and more than 400,000 will die from the disease (1). Though the global incidence of breast cancer appears to be increasing, the five-year relative breast cancer survival rate has increased dramatically in developed countries over the last 50 years due to early detection and treatment of *in situ* and early stage disease and improvements in targeted therapies for specific subtypes of breast cancer.

At both the histological and molecular levels, human breast cancer is a heterogeneous group of diseases. Infiltrating ductal carcinoma (IDC), which comprises 80%

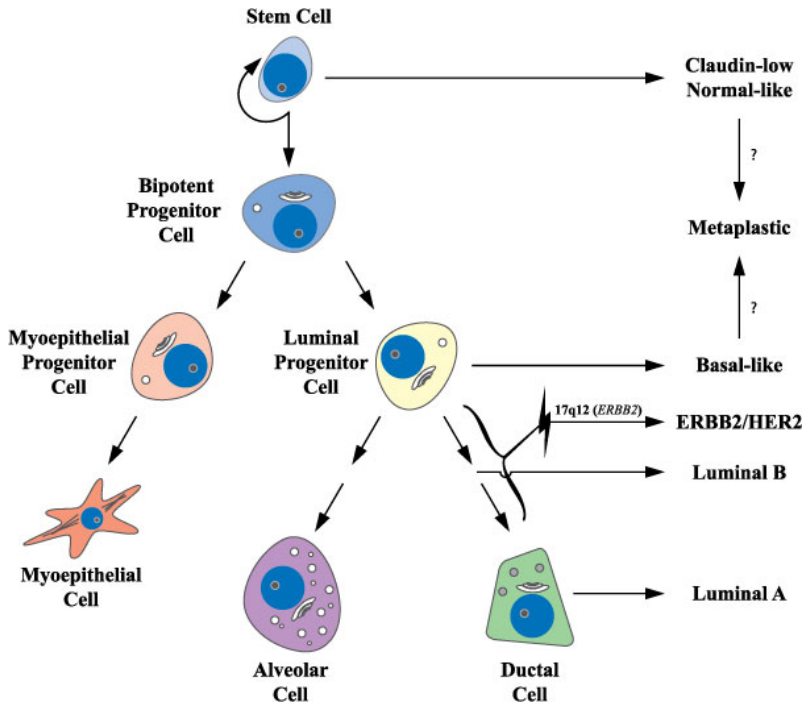

Figure 1. The mammary epithelial hierarchy and its relation to breast cancer subtypes, adapted from the model proposed by Jane Visvader (45).

of all breast cancers, can be divided into more than a dozen histological subtypes and at least six distinct molecular families (2-5). Expression (or absence thereof) of estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (ERBB2/HER2) are widely used to classify tumors clinically and identify patients who will likely benefit from endocrine and HER2-targeted therapies. Despite the predictive and prognostic significance of these markers, clinical responses remain variable within subtypes.

Though the precise causes of molecular and phenotypic diversity in human breast cancer remains poorly understood, the current working model suggests that the normal mammary stem cell and the various progenitor cells that arise thereof are all potential targets of transforming mutations that lead to the generation and propagation of lineage-specific tumor-initiating cells within the mammary gland (Figure 1). This paradigm is in concert with observations that most human breast malignancies share transcriptional, biochemical and/or morphological characteristics with discrete non-transformed components of the normal mammary gland. Definitive evidence linking each tumor subtype with the correct cell of origin is lacking due to our incomplete understanding of the normal mammary epithelial cell hierarchy. Even without such conclusive evidence, conserved molecular signatures have been delineated in cohorts of breast cancers, stemming from recurrent genetic and epigenetic alterations. Here we aim to review the current state of knowledge regarding the cellular origin(s) of specific breast cancer subtypes as well as the currently identified recurrent genetic and epigenetic aberrations associated with each subtype.

3. BREAST CANCER STEM CELLS

Tumors are composed of heterogeneous populations of cells with differences in morphology, architecture and developmental potential (6, 7). The striking similarity between embryonic tissues and cancer with respect to their enormous capacity for proliferation and differentiation lead to the cancer stem cell (CSC) hypothesis (8, 9). According to the this hypothesis, only a subset of cancer cells within each tumor are long-lived cells with unlimited tumorigenic potential and are responsible for tumor growth, maintenance and relapse (8-13). The CSC model was first established in the leukemia system when it was found that a minority of malignant blood cells could form colonies in the spleen of a mouse (14-16). Pioneering studies by several groups have since demonstrated the existence of CSCs in several additional epithelial and hematologic malignancies by transplanting single tumor cells (17-20). Based on surface marker expression, CSCs in acute myeloid leukemia were identified and they showed tumorigenic potential in SCID mice (16, 21, 22). Later, several other studies demonstrated the presence of CSCs in various solid tumors, including breast tumors, in which the CSC population is characterized by a $CD44^{Hi}/CD24^{-/low}$ phenotype (23-29). Aldehyde dehydrogenase 1 (ALDH1) has since been identified as another potential breast CSC marker (30).

Several mutations are necessary for a cell to become tumorigenic (31, 32) and hence, tissue stem cells are likely candidates to accumulate the requisite number of mutations because of their long life span compared to

restricted progenitors or differentiated cells. In breast cancer, the CSC population displays a mesenchymal phenotype (33). The majority of identified invasive gene signatures (IGS) are overexpressed both in breast CSCs as well as in basal-like breast cancers (34, 35). This similarity indicates that basal-like breast cancers may be enriched in tumorigenic breast CSCs or maintain a similar transcriptional profile. Cheng, *et. al.* found that a population of cells was enriched in patients who underwent chemotherapy or were given drugs that block the action of tumor-promoting sex hormones (36). These cells can resist treatment and cause tumor relapse (36). Moreover, they identified a characteristic gene expression signature that overlapped with the $CD44^{Hi}/CD24^{-/low}$ tumor cells that can be serially passaged for an extended period of time in mammosphere culture (an *in vitro* culture condition to show self-renewal potential) and can readily perpetuate tumors (33, 37). Cells with this gene expression signature are particularly enriched in a relatively uncommon breast cancer subtype called "claudin-low" which displays characteristics of undergoing the epithelial-to-mesenchymal transition (EMT), a latent embryonic developmental program implicated in the spread of breast and other malignancies (33). Breaking down of epithelial cell homeostasis and the acquisition of a migratory mesenchymal phenotype is referred to as EMT and is considered a crucial early event in malignancy (38). Recent studies also indicate that EMT causes increased resistance to chemotherapy and enrichment in breast CSCs (39, 40). These studies suggest the possibility that through EMT, breast epithelial cells can acquire CSC properties by genetic, epigenetic or as yet unknown molecular mechanisms. Consistent with this notion, Villasden *et. al.* functionally identified a stem cell zone in the luminal compartment in breast biopsies as indicated by the presence of cells with a capacity for clonal growth, self-renewal and bipotency (41). They also reported a higher prevalence of progenitor cells in the luminal compartment compared to the basal compartment. Collectively these data suggest that transformation of multiple epithelial components in the mammary gland can generate discrete types of breast cancer (42).

4. INTRINSIC SUBTYPES OF BREAST CANCER

First generation gene expression profiling of human breast cancers established at least six major types of invasive breast cancer: luminal type A, luminal type B, luminal type C, basal-like, ERBB2/HER2-overexpressing, and normal breast-like (2-5). Retrospective analysis of patient outcomes in these studies demonstrated that specific molecular taxonomies are strongly correlated with unfavorable clinical behavior and poor overall survival (4). Refinements in profiling have since identified additional intrinsic subtypes of breast cancer, the most notable of which is the recently described claudin-low subtype (43). The morphological and molecular heterogeneity observed in human breast cancers likely stems from differential mechanisms of transformation in discrete cellular elements of the mammary epithelium (Figure 1). This model is supported by evidence that defined subsets of normal mammary epithelial cells and ductal carcinoma *in situ* (DCIS) lesions, the precursors to invasive ductal carcinoma, can also be classified into the previously described intrinsic subtypes. Thus, the same molecular signatures that exist in advanced invasive breast cancers are present in the earliest stages of neoplastic growth and normal components of the mammary epithelium and consequently represent an indication of cellular ancestry (44). Discrete recurrent molecular abnormalities associated with each of these subtypes and/or cellular origins are emerging and appear to dictate many aspects of the biology and clinical behavior of these subtypes. Given the heterogeneity that currently exists within many of these subtypes, it is likely that additional intrinsic categories of breast cancer will be defined in the relatively near future.

4.1. Tumors arising from luminally-committed cells

4.1.1. Luminal A and luminal B tumors

The majority of invasive ductal and lobular carcinomas exhibit evidence of luminal differentiation. These tumors usually express ER and are thus amenable, to varying degrees, to therapies aimed at regulating ER signaling. Based on similarities in gene expression and morphology, it is believed that tumors comprising the luminal A and luminal B subtypes arise from transformation of cells in the terminal stages of luminal fate commitment (45). Specifically, luminal A tumors exhibit robust expression of ER, PR, and other markers of mature luminal epithelial cells including the transcription factor GATA3 and luminal cytokeratins (CK8 and CK18), and likely arise from malignant transformation of the mature luminal ductal or lobular epithelial cell (3, 45). Luminal B tumors commonly express ER, albeit at a lower level than luminal A tumors, and likely stem from transformation of a cell with an intermediate degree of terminal luminal commitment. Accordingly, these tumors usually exhibit lower expression of estrogen-related genes, higher mitotic indices and histological grade and a significantly poorer prognosis than luminal A malignancies (4, 46).

Several studies have confirmed the critical nature of the Notch morphogenetic pathway and the GATA3 transcription factor in the specification and maintenance of the luminal epithelium (47-50). GATA3 likely functions pleiotropically in breast tumorigenesis, simultaneously promoting terminal differentiation of ductal and alveolar epithelial cells and antagonizing the epithelial-to-mesenchymal transition and metastasis (48, 51, 52). In agreement with experimental studies of GATA3 in the mammary gland, high expression of this transcription factor in human breast cancer correlates with lower grade, higher expression of ER and PR and improved survival (53). Among the intrinsic subtypes of breast cancer, tumors with robust luminal phenotypes are associated with significantly better disease-free and overall survival than tumors with less differentiated phenotypes and those with exaggerated expression of ERBB2/HER2 (4). Endocrine therapies aimed at regulating the synthesis and/or cellular responses to estrogen have led to significantly improved outcomes for women with hormone receptor positive breast cancer. Despite these advances, primary and secondary resistance to endocrine therapy remain major clinical obstacles (54). Additional studies are needed to identify factors which can

predict initial responsiveness to anti-estrogen therapies and to understand the biological mechanisms responsible for development of acquired endocrine resistance.

4.1.2. ERBB2/HER2 tumors

Tumors overexpressing the ERBB2/HER2 receptor tyrosine kinase (RTK) appear to originate from a luminally-restricted cell, but have a significantly poorer prognosis than either the luminal A or luminal B subtypes without amplification or overexpression of this molecule. Overexpression of HER2 is observed in approximately 25-30% of human breast cancers, is usually caused by amplification of the 17q12 locus (containing the *ERBB2* gene), and results in exaggerated expression of wild-type HER2 RTK at the membrane (55, 56). Though only a quarter of invasive malignancies exhibit amplification of HER2, this molecular abnormality is observed in nearly half of all ductal carcinoma *in situ* (DCIS) lesions, suggesting that *ERBB2* amplification is an early event in the pathogenesis of this subtype of breast cancer (57, 58) and represents an intrinsic subtype of breast cancer rather than an artifact of advanced disease. Overexpression of HER2 at the cell surface appears to promote dimerization-dependent signaling events that activate numerous signaling nodes and influence proliferation, differentiation and apoptosis (59). ERBB2/HER2 cancers follow a more aggressive clinical course than do luminal tumors without amplification of this gene, are more resistant to chemotherapeutic agents and have an increased risk of distant metastasis (4, 60, 61). The introduction of trastuzumab (Herceptin®) into the treatment paradigm of HER2-positive breast cancer dramatically improved survival for women with this subtype of disease (62). Newer agents like the small-molecule tyrosine kinase inhibitor lapatinib (Tykerb®) can also inhibit HER2-associated signaling events (63, 64). Though *ERBB2* gene copy number remains the best predictive marker of response to HER2-targeted therapies, intrinsic and acquired resistance to agents like trastuzumab and lapatinib remains a clinical problem (65). Accordingly, there exists much interest in identifying factors that can predict responsiveness to HER-targeted therapies. Such advances, coupled with an understanding of both primary and secondary resistance mechanisms will improve quality of life and survival for HER2-positive breast cancer patients.

4.2. The luminal progenitor ("basal-like") cancers

Basal-like breast cancers (BLBCs) were so named because these neoplasms consistently express molecules normally confined to the basal/myoepithelial compartment of the ductal and lobular epithelium, including basal cytokeratins (CK5, CK6, CK14, CK17), α-smooth muscle actin and vimentin (66). BLBCs account for approximately 15% of all invasive breast cancers and are typically of high histological grade, demonstrate high mitotic indices, mutations in the *TP53* tumor suppressor gene, and almost uniformly lack expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 and are thus termed "triple-negative" (TN) (67, 68). Due to the absence of these receptors, BLBCs are not amenable to the targeted anti-estrogen and anti-HER2 therapies that have dramatically improved survival of patients diagnosed with luminal-type or HER2-positive tumors. Because of the aggressive biological features inherent to these tumors as well lack of targeted therapies, the basal-like malignancies are associated with the most aggressive clinical behavior and poorest prognosis among all molecular classifications of breast cancer (4). Interestingly, human breast cancer cell lines derived from basal-like malignancies show exaggerated self-renewal capacity *in vitro* and are almost uniformly composed of $CD44^{Hi}/CD24^{-/low}$ cells, suggesting they may be enriched for cells which possess stem/progenitor-like properties (69). These similarities logically pointed to the mammary stem cell as the likely origin of BLBCs. Unexpectedly, comparison of the BLBC transcriptional profile with the profiles of normal mammary epithelial components revealed great similarity between the BLBC and the $CD49f^{+}/EpCAM^{+}$ luminal progenitor signatures (70). It has also since been demonstrated that deletion of *Brca1* in the luminal-progenitor population of the mouse mammary epithelium generates tumors which phenocopy human BLBCs at both the histological and molecular level, while the identical genetic change in the murine mammary stem cell generates adenomyoepitheliomas, an exceedingly rare form of human breast cancer (71).

Until recently, the therapeutic paradigm for TN breast cancers was limited to traditional cytotoxic chemotherapy. Recent studies have documented two recurrent molecular abnormalities in BLBCs that offer the potential for targeted therapeutic intervention. Overexpression of epidermal growth factor receptor (EGFR) occurs in a number of human malignancies, including certain breast cancers. EGFR plays critical roles in transducing signaling events associated with proliferation, differentiation and survival. Multiple ligands can bind to and stimulate EGFR leading to the activation of several signaling pathways, including RAS/RAF/MAPK, PI3K/AKT/mTOR and SRC/NFκB (72). When EGFR expression is examined with respect to breast cancer subtype, it is significantly more prevalent in TN and basal-like malignancies than in other subtypes (72). Dysregulation of EGFR in malignancy leads to autonomous growth signaling, acquisition of an invasive phenotype, secretion of angiogenic factors, and resistance to apoptosis (72). Monoclonal antibodies and small molecule tyrosine kinase inhibitors which inhibit EGFR signaling are currently in evaluation for treatment of TN and BLBCs, though it is currently unclear whether these agents confer a survival benefit. Thus, while EGFR appears to be a sound marker for the basal-like and/or triple-negative phenotype, clinical data remains mixed concerning the therapeutic benefit of targeting EGFR in breast cancer (72).

BLBCs also commonly demonstrate dysregulation of the breast cancer susceptibility gene 1 (*BRCA1*). As is described later, tumors arising in *BRCA1* mutation carriers nearly always have a TN immunophenotype and basal-like transcriptional signature. Moreover, sporadic BLBCs commonly demonstrate downregulated BRCA1 expression in the absence of mutations at the *BRCA1* locus, a phenotype termed "*BRCA*-ness" (68, 73, 74). Disruption of BRCA1 (or BRCA2) function by genetic or epigenetic mechanisms results in

compromised capacity to repair double-strand DNA breaks (DSBs) by homologous recombination (HR) (75). While this genomic instability likely underlies the proclivity for tumorigenesis observed in heterozygous individuals, it also lends itself to therapeutic exploitation. Agents which induce DSBs (i.e., ionizing radiation and bleomycin) or interstrand crosslinks (i.e., platinum-based alyklating agents) appear to be significantly more toxic in cells with reduced or absent expression of BRCA1 (76-79). Accordingly, human breast cancers arising in *BRCA1* mutation carriers are more likely to achieve clinical responses in response to platinum-based agents than non-*BRCA1/2* tumors (80). Conversely, because of the critical role of BRCA1 in inducing G2/M arrest in response to microtubule poisons, tumors deficient in BRCA1 tend to be relatively resistant to these agents (78, 81-83). Thus, selection of specific cytotoxic agents based on DSB repair capacity may improve responses to traditional chemotherapeutic agents and enable personalized cytotoxic chemotherapy (84).

The most promising recent advancement in the treatment of both *BRCA1*-associated and sporadic TN and BLBCs is based on the concept of "synthetic lethality". Synthetic lethality is a biological concept describing cell death resulting from inactivation of two pathways, neither of which is cytotoxic alone. Because *BRCA1*-associated, TN and basal-like malignancies have intrinsic defects in HR-mediated DNA damage repair, ancillary DNA repair pathways dependent on poly(ADP-ribose)polymerase (PARP) become critical (85). Investigators proposed that inactivation of PARP in tumors which have lost functional BRCA1 or BRCA2 would induce a synthetic lethal state. This hypothesis has proven correct in breast, ovarian and other malignancies with impaired DSB repair (86). Numerous PARP inhibitors are currently in evaluation, and the two frontrunners AZD2281 (olaparib) and BSI-201 have shown clinical efficacy in the treatment of TN and BLBCs (87).

4.3. Tumors arising from transformation of the mammary stem cell – Claudin-low, metaplastic and normal-breast like tumors

The claudin-low subtype of breast cancer was identified in 2007 by examining similarities between mouse and human mammary tumors (88). This molecular subtype is characterized by low expression of components of the tight and adherens junctions, including claudins 3, 4, 7, and E-cadherin (43, 88). When compared to all breast tumors, those classified as claudin-low also were enriched for expression of genes involved in immunological responses, cellular communication, extracellular matrix, migration and angiogenesis and showed recurrent copy-number amplification of the *KRAS2* locus (43). Further studies revealed that claudin-low tumors display molecular features consistent with the mammary stem cell and exhibit transcriptional evidence of epithelial-to-mesenchymal transition, including high proportions of $CD44^{Hi}/CD24^{-/Low}$ cells, high expression of *TWIST1* and *SNAI3* and repression of E-cadherin (2). Comparing the transcriptional profiles of claudin-low tumors with components of the normal mammary epithelium revealed that the gene expression patterns of these tumors closely mirrored those observed in the mammary stem cell-enriched population (70).

Metaplastic breast cancers (MBCs) are a morphologically diverse group of mostly TN malignancies that exhibit mesenchymal, sarcomatoid and/or squamous metaplasia (89-92). Transcriptional profiling of these tumors originally classified them as basal-like malignancies (92). By refining the criteria used for classification and including the recently-identified claudin-low subtype, MBCs were shown to be molecularly heterogeneous and may cluster with the basal-like, claudin-low or normal breast-like subtypes (2, 43). Whereas BLBCs commonly have high pathologic complete responses to neoadjuvant chemotherapy, both claudin-low and metaplastic breast cancers are usually resistant, providing further rationale for distinguishing these lesions from BLBCs (90, 93).

5. GENETIC RISK FACTORS FOR BREAST CANCER: BRCA1 AND THE BASAL-LIKE PHENOTYPE

Approximately 90% of all breast cancers are sporadic in nature. Of the remaining 10% which appear to be associated with inheritance of dominantly-acting genes, 20-40% are due to mutations in the *BRCA1* gene. While BRCA1 dysfunction is notably associated with inherited breast cancers, several studies have reported loss of BRCA1 expression by non-mutational means in 30-40% of sporadic malignancies (74, 94). Transcriptional profiling of *BRCA1*-mutated breast cancers has revealed that these tumors, almost without exception, cluster in the basal-like subtype (61, 95, 96). Moreover, immunohistochemistry studies reveal that nearly 70% of *BRCA1*-mutated breast cancers express basal cytokeratins and lack expression of ER, whereas this immunophenotype is present in less than 9% of matched control tumors (96). Sporadic breast cancers which exhibit loss of BRCA1 expression also have a strong tendency to be of the basal-like phenotype (68, 74). Conversely, tumors which maintain expression of functional BRCA1 are almost uniformly luminal type cancers and are accordingly associated with more indolent clinical courses, responsiveness to endocrine therapies, and improved survival (97, 98). Taken together, these findings suggest that loss of BRCA1 expression and/or function has a causal role in the development of the basal-like phenotype. Though this association is now well-supported, the molecular consequences of BRCA1 deficiency that result in the generation of BLBCs remain undefined.

Recent studies support a model in which BRCA1 is necessary for the normal luminal differentiation program within the mammary gland. *In vitro* and *in vivo* studies have revealed that loss of BRCA1 expression in mammary epithelial cells leads to marked dysplasia and failure of terminal luminal epithelial cell differentiation (70, 99, 100). These morphological abnormalities are associated with exaggerated expression of basal/myoepithelial antigens and enrichment of cells with expression of the putative stem/progenitor cell marker ALDH1A1 (70, 100, 101). These findings would suggest that BRCA1 deficiency may induce BLBCs by causing expansion and transformation of

the basal/myoepithelial population. As previously discussed, recent studies have unexpectedly documented expansion of the $CD49f^{+}/EpCAM^{+}$ luminal progenitor population in the pre-malignant breast tissue of *BRCA1*-mutation carriers and demonstrated that loss of *Brca1* in the luminal progenitor population generates murine mammary tumors which phenocopy human BLBCs at both the histological and molecular level (70, 71).

A mechanistic understanding of how BRCA1 dysfunction contributes to the pathogenesis of BLBCs is currently lacking. Studies detailing the role(s) of this protein in the normal luminal differentiation program of the mammary gland are needed and may illuminate the molecular events that initiate basal-like malignancies.

6. EPIGENETIC CHANGES IN BREAST CANCER SUBTYPES

In addition to sequence alterations in the genome, there are also changes in the epigenetic information in malignant disease. However, unlike genetic changes, epigenetic changes may be reversible. In breast cancer, the epigenetic regulation of critical tumor suppressor and growth regulatory genes are extremely important because of their well-documented role in breast cancer progression, diagnosis, prognosis and individualized therapy. Cellular epigenetic changes generally are classified into two main categories: DNA methylation and histone modifications (102, 103). These processes covalently attach small chemical moieties to DNA or histones and increase the capacity of the genome to store and transmit biological information beyond that encoded in the DNA sequence.

6.1. DNA methylation

The most widely studied epigenetic modification in humans is cytosine methylation of DNA within the dinucleotide CpG. Nearly 3–6% of all cytosines are methylated in normal human DNA (104, 105). The enzymes which catalyze these reactions, the DNA-cytosine methyltransferases (DNMTs), transfer a methyl group from the methyl donor S-adenosylmethionine to nascent DNA using a hemimethylated DNA template to maintain DNA methylation patterns during cell division in mammals.

Like all cancers, breast cancer is also considered as the result of, in part, accumulation of epigenetic alterations leading to oncogene overexpression and loss of tumor suppressors. Normally, CpG dinucleotides within promoters tend to be protected from methylation, whereas both benign and malignant tumors have shown global reduction of DNA methylation (106). This property is now considered a universal feature of cancer (106-108). However, epigenetic changes, specifically silencing of tumor suppressor genes via DNA hypermethylation, plays a critical role in the initiation and progression phases in many human cancer types, including breast cancer. Methylation of CpG islands in the promoters of various important genes such as *CDKN2A* (p16), *SFN* (14-3-3σ), steroid receptors, *RARB* (RARβ*2*), *GSTP1* and *BRCA1* have been reported to be inactivated in breast cancer. These data are reviewed extensively by Yang *et. al.* (109).

It has recently been reported that the gene expression profiles of progenitor and differentiated populations (defined as $CD44^{Hi}/CD24^{-/Low}$ (CD44+) and $CD44^{Low}/CD24^{+}$ (CD24+), respectively) in both normal and neoplastic breast tissue are highly similar between analogous cell types (110). However, follow-up studies using MSDK (methylation-specific digital karyotyping) and SAGE (serial analysis of gene expression) have identified well-conserved epigenetic programs that define the progenitor characteristics regardless of tissue type (111). The methylation pattern of luminal tumors were found to be similar to that of normal CD24+ cells, whereas HER2+ and basal-like tumors were more hypomethylated and similar to CD44+ cells (111). This suggests that the epigenetic profiles of progenitor-like cells in different subtypes of breast tumors are distinct, probably due to subtype-specific developmental processes. Consistent with these observations, very recently Holm *et al.,* reported methylation analysis of 807 cancer related genes in 189 primary breast tumors (112). They clustered the tumors into three groups with characteristic methylation patterns, which were associated with basal-like, luminal A and luminal B molecular subtypes. Their study revealed that the methylation frequency in basal-like tumors was significantly reduced compared to the luminal B types. In normal stem/progenitor cells, Polycomb repressive complex 2 (PRC2) mediated gene silencing through trimethylation of H3K27 is common and characterized by high EZH2 expression as well as low expression of PRC2 targets with unmethylated CpG sites. Their study observed similar characteristics with basal-like tumors. High expression of PRC2 targets with low methylation in CpG sites and low EZH2 expression promote differentiation in normal ES cells, which they found to be similar in luminal A breast cancers. Additionally, they found an aberrant state for luminal B types where both high expression of PRC2 targets and higher methylation of CpG sites were observed. A parallel study by Bediaga *et al.,* also analyzed more than 800 genes in 28 breast cancer paired samples and identified 15 individual CpG loci differentially methylated in breast cancer molecular subtypes (113). They found that basal-like tumors showed hypomethylation at the *NPY*, *FGF2*, *HS3ST2*, *RASSF1*, and *Let-7a* loci compared to HER2-overexpressing tumors, different methylation levels in *SOX1* and *SOX17* between luminal B and luminal A tumors, and the *HS3ST2, DBC1, FGF2, CD40, JAK3, Mir-93*, and *Mir-10a* loci displayed higher methylation levels in luminal B and HER2+ subtypes than in the basal-like and the luminal A tumors (113). The precise role(s) of DNA methylation in the pathogenesis of breast cancer subtypes remains unclear. Small molecule DNA methyltransferase inhibitors have been developed with the hope of re-expressing tumor suppressor genes that have been epigenetically silenced through methylation of their promoters. Several of these agents, including 5-azacytidine and 5-aza-2'-deoxycytidine demonstrate safety and efficacy in clinical trials and are in ongoing trials for the treatment of human neoplasms (114).

6.2. Histone modifications

In eukaryotes, histone proteins organize DNA into nucleosomes, which are regular repeating structures of

chromatin. The nucleosomes are composed of an octamer of histones H2A, H2B, H3 and H4 wrapped by 147 bp of DNA. Certain configurations of chromatin inhibit gene transcription by restricting DNA-binding transcriptional regulators from accessing promoter regions of genes (115). However, chromatin structure is plastic and chromatin remodeling can lead to activation or repression of transcription. Remodeling of chromatin can happen when histone proteins undergo a variety of post-translational modifications, especially on their N terminus, including acetylation of lysines (K), methylation of lysines and arginines (R), as well as phosphorylation, ubiquitylation, glycosylation, sumoylation, ADP-ribosylation and carbonylation (103). The combination of these post-translational modifications of histones are crucial and create a regulatory epigenetic 'code', which is read by the non-nucleosomal DNA binding multiprotein complexes that form the transcription-activating and transcription-repressing machinery to modulate gene expression (116). Although elucidation of the histone code is in its infancy, specific histone marks such as lysine acetylation (H3K9ac, H3K18ac, and H4K12ac), lysine methylation (H3K9me2 or H3K9me3 and H4K20me3), lysine trimethylation (H3K4me3), and arginine dimethylation (H4R3me2), are characterized and associated with transcriptionally active and repressed chromatin structure (117-121). In fact, global loss of acetylation (K16) and trimethylation (K20) of histone H4 have been shown to be hallmarks of human cancer (122).

Acetylation is the most studied histone mark of chromatin structure in cancer development and this post-translational modification is mediated by histone acetyl transferases (HATs) and removed by histone deacetylases (HDACs) (123, 124). In addition to acetylation of histones, HATs can also acetylate several transcription factors, including GATA1, E2F1, pRB or p53 to modulate their DNA binding affinities (125). Hbo1, a histone acetyltransferase of the MYST family, and hMOF, a CBP-p300 HAT, have already been linked to breast cancer progression (126-128). Similarly, expression levels of several HDACs such as HDAC1, -2, -3, and -6 have also been shown to be aberrant in breast cancer (129-134). A number of HDAC inhibitors have been shown to inhibit tumor growth *in vitro* and *in vivo* and several of these are now in clinical trials (116). Besides acetylation, methylation and demethylation of histones as well as the expression levels of methyl transferases and demethylases are also altered in breast cancer and has been reviewed extensively by Dalvai *et al.,* (135). Recently, Elsheikh *et al.,* studied the relative levels of seven modified histones, including H3K18ac, H3K9ac, H4R3me2, H3K4me2, H4K12ac, H4K16ac and H4K20me3 in a series of 880 invasive breast carcinoma cases and identified distinct histone marks which have distinct relationships to known prognostic factors and clinical outcomes (136). In their study, medullary carcinomas showed low-level detection of all histone marks compared to the lobular, mucinous, tubular and mixed tubular carcinomas. In addition, complementary biomarker analysis showed higher level of histone modifications in tumors with steroid receptor-positive subtypes, luminal subtypes, high E-cadherin and BRCA1 expression, whereas lower global levels of histone modifications were observed in tumors which expressed basal cytokeratins (CK5/6 and CK14), p53 and HER-2. These results indicate that low levels of these histone marks are associated with adverse patient outcome, whereas high levels of H3K18ac, H4R3me2, and H3K9ac were significantly associated with a more favorable clinical course and longer disease-free survival.

6.3. MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are a novel class of small non-coding RNAs of 20 to 25 nucleotides in length that can hybridize to the 3' untranslated region (UTR) of messenger RNAs (mRNAs) and either block translation or direct degradation of their specific target mRNAs by the RNA-induced silencing complex (RISC) (137). MiRNA was first discovered in *C. elegans* in 1993 by the laboratory of Dr. Victor Ambrose (138) and have since been identified in almost every species, including humans (137, 139-141). The 5' region of a miRNA is critical for targeting and miRNA function and is known as the "seed" region (nucleotides 2 through 8) (137, 142, 143). The base pairing of miRNAs with their target sites in the 3' UTR are often imperfect and thus, a single miRNA molecule can have multiple target mRNAs (144-146). As a result, miRNAs have the ability to alter the proteome as well as the phenotype of a single cell (137).

miRNAs have been demonstrated to be important in many biological processes, including cell proliferation, differentiation and apoptosis (147-149). Since all of these processes are often altered in cancer cells, it is not surprising that miRNAs have a distinct role in cancer progression. In fact, the majority of human miRNA genes are located in fragile chromosomal regions that are susceptible to amplification, deletion or translocation during tumor development (150, 151). Expression of miRNAs are altered in tumors compared with normal tissue, supporting the diverse functions of these biomolecules as oncogenes or tumour suppressors (152-155). For example, miRNA-21 is overexpressed in nearly every cancer examined, and thus has been labeled an "oncomir" (156). The targets of miRNA-21 include the tumor suppressor genes programmed cell death 4 (PDCD4) and phosphatase and tensin homologue (PTEN) (157, 158). This miRNA also pleiotropically regulates numerous cell functions, including protein translation, apoptosis, cell proliferation and migration (157, 159, 160). Using miRNA profiling, Iorio *et al.,* (161) have identified 29 miRNAs that are differentially expressed in breast cancer tissue compared to normal mammary tissue. Of these, 15 were able to distinguish tumor versus normal breast tissue with 100% accuracy. Their study also showed that miRNA expression correlated with ER and PR expression (miRNA-30) as well as tumor stage (miRNA-213 and miRNA-203). The differential expression of miRNA let-7 isoforms was related to PR status (let-7c), lymph node metastasis (let-7f-1, let-7a-3, let-7a-2), or high proliferation index (let-7c, let-7d). Recently, it has been also reported that miRNA let-7 is a tumor suppressor controlling breast CSC self-renewal and is downregulated in breast CSCs (162, 163). Another study identified unique sets of miRNAs associated with the

ERBB2/HER2 or ER/PR status of breast cancers (164). Ma *et al.,* identified that miRNA-10b was highly expressed only in metastatic cancer cells (165). In breast cancer cells, the transcription factor TWIST regulates the expression of miRNA-10b, which then inhibits translation of the homeobox D10 (HOXD10) mRNA, resulting in increased expression of a well-characterized pro-metastatic gene, RHOC. Another study reported that miRNA-373 and miRNA-520c could also promote breast tumor invasion and metastasis, at least in part by regulating the gene *CD44* (166). Tavazoie *et al.,* identified that miRNA-335, miRNA-206, and miRNA-126 are metastasis suppressors of breast cancer (167).

Many miRNAs are differentially expressed in breast cancer stem cells and several appear to be important in maintaining their tumorigenic potential (162, 168, 169). In a study published by Shimono *et al.*, miRNA expression in human breast CSCs ($lin^-CD44^{Hi}/CD24^{-/Low}$) was compared with differentiated progeny and discovered that members of the miRNA-200 family (a, b, and c) were significantly downregulated in breast CSCs (168). Targets of miRNA-200 family miRNAs include stem cell self-renewal factor BMI1 as well as the transcriptional repressors of E-cadherin, ZEB1/ZEB2 (168, 170). Consistently, overexpression of miRNA-200c in breast CSCs resulted in suppression of tumorigenic potential (168). Another study demonstrated a feedback loop involving Lin28-mediated downregulation of tumor suppressor miRNA let-7 (163). Lin28 is an RNA-binding protein that induces uridylation of specific miRNAs to block miRNA processing by Dicer (171). Additionally, miRNA-200c and another miRNA, miRNA-107, which is also downregulated in breast CSCs, are also regulated by Lin28 (171), suggesting a broader role of Lin28 in regulating the expression of multiple miRNAs to promote a stem cell phenotype. Recently, a group led by Stefano Piccolo has reported that the miRNA-103/107 family can also downregulate Dicer, and that its overexpression is associated with an increased risk of developing metastases in breast cancer patients (172). Overexpression of miRNA-103/107 induced epithelial–mesenchymal transition (EMT) and treatment of mice with antagomiR-103/107 reduced metastatic colonization as well as restored levels of mature miRNAs including miRNA-200 expression (172).

The delineation of specific alterations in miRNA expression in different subtypes of breast malignancy highlights the importance of these biomolecules in specifying and maintaining discrete tumor phenotypes. Definitive patterns of miRNA expression may provide novel tools in the diagnosis, classification and prognostic stratification of human breast cancers. Moreover, targeting miRNAs may provide a promising new approach to cancer therapy, since miRNA-based therapies have the potential to treat chemo-resistant CSCs that are responsible for relapse (173). While specific miRNAs may be dysregulated in cancer, the same miRNAs likely play essential roles in normal cells (169). Thus, it is necessary to deliver miRNA effectors specifically to the tumor. In this regard, several approaches have been used to deliver miRNA effectors to cancer cells (169, 174-176).

7. FINAL COMMENTS

The past decade of breast cancer research has transformed our understanding of this disease. Though the histological heterogeneity of human breast cancer has been appreciated for some time, profiling breast cancers at the genetic, epigenetic and transcriptional levels has revealed immense diversity that is not apparent at the morphological level. These advances have enhanced our ability to predict the biological behavior and even clinical course of specific human breast cancers, though we still have much work to do in this regard. Given the molecular heterogeneity within a given subtype in our current classification scheme, it is apparent that additional types or subtypes of breast cancer will likely emerge in the relatively near future. By utilizing our ever-growing base of knowledge concerning transcriptional, genetic and epigenetic changes associated with the subtypes of breast cancer, we will undoubtedly identify exploitable targets that can be used to rationally treat breast cancer patients on the basis of the biology of their individual tumor. Precisely defining the myriad molecular alterations present in cancer offers the opportunity to specifically define rational targeted therapeutic approaches that are individualized and optimized for each patient. This approach will define the development of personalized medicine in the 21st century.

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