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Unraveling Breast Cancer Heterogeneity Through Transcriptomic and Epigenomic Analysis

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Abstract

Breast cancer diversity is histologically evident as various proliferative benign lesions, in situ carcinomas, and invasive carcinomas that may develop into distant metastases. Breast tumor molecular subtypes have been defined by genome-wide expression microarray technology and reveal associations between genetic alterations and the malignant phenotype. Early work has been conducted to use subtype-specific biomarkers to elucidate targeted treatment options early in the course of breast cancer progression. Additionally, DNA methylation is an epigenetic modification that contributes to breast cancer progression by transcriptionally silencing certain tumor suppressor genes. Among the genes characterized as targets for silencing are well-established tumor suppressors such as *RASSF1A*, *RARB*, *SFN*, and *TGM2*. Measuring elevated gene copy number and aberrant gene promoter methylation can further facilitate characterization of breast tumor molecular subtype; however, profiling of breast tumors based on epigenetic criteria has yet to be established. Epigenomic analysis has been investigated for clinical applicability, and it has great promise when used in combination with minimally invasive techniques for both diagnostic and prognostic purposes.

Breast cancer is a clinically and morphologically heterogeneous disease, and the current standard classification system by the World Health Organization (WHO) defines 18 different histologic types.[1] However, breast cancer patients with apparently identical clinicopathologic characteristics may have variable prognostic outcomes, and a tumor's gene expression signature contributes to differences in prognosis and response to chemotherapy.[2] Molecular techniques such as expression microarray, comparative genomic hybridization (CGH), and epigenomic analysis provide approaches for accurate tumor subtyping based on a defined "molecular signature." In this way, breast tumor subtyping promises to provide clinicians with a better understanding of individual tumor biology and an opportunity to personalize patient treatment strategies.

TRANSCRIPTOMIC SUBTYPING IN BREAST CANCER

Genome-wide gene expression profiling by complementary DNA (cDNA) microarray has revealed several distinct molecular subtypes of breast cancer.[3–6] Cluster analysis using an intrinsic gene subset has consistently identified five distinct subtypes: luminal A, luminal B,

HER2 + (ERBB2 +), basal-like breast cancer (BLBC), and normal-breast-like.[5,6] Quantitative polymerase chain reaction (qPCR) has independently validated microarray data, revealing 54 genes that accurately discriminate luminal A tumors from basal-like tumors.[6] Of note, invasive ductal carcinoma (IDC) not otherwise specified and invasive lobular carcinoma (ILC) encompass all molecular subtypes (i.e., luminal, HER2 +, and BLBC), while the histologic "special types" other than ILC and apocrine carcinoma are less heterogeneous and only belong to one molecular subtype.[7] For example, the luminal subtype encompasses all neuroendocrine, mucinous A, and mucinous B tumors, while BLBC includes all metaplastic, medullary, and adenoid cystic carcinomas.

The clinical implication of molecular subtyping is evident foremost in the significant differences in long-term survival between tumor subtypes. HER2 + tumors and BLBC are both associated with shorter disease-specific survival, relapse-free survival, and time to metastasis compared with luminal A, luminal B, and normal-breast-like tumors, which have a relatively favorable prognosis.[4,5,8] Paradoxically, medullary and adenoid cystic carcinomas are histologic special types associated with a favorable prognosis despite their general subtyping as BLBC.[7] Moreover, these findings suggest that BLBC constitutes a pathohistologically heterogeneous subtype of breast tumors.

Determining the specific subtype of a breast tumor can help guide clinical decisions by focusing attention on tumor phenotype and interaction with the endocrine system. Relatively high estrogen receptor (ER) expression is a defining characteristic of the luminal subtype, and luminal tumors are clinically ER +.[3] HER2 +, BLBC, and normal-breast-like tumors either express low levels of ER or lack ER expression altogether.[3] Luminal A tumors demonstrate the highest expression of ER and the luminal epithelial gene cluster, while luminal B tumors have low/moderate expression of this gene cluster.[4,6] Sorlie et al. defined an additional low/moderate-ER subtype, luminal C, which is divergent from luminal A and B tumors because of shared traits with HER2 + tumors and BLBC, including expression of a unique gene cluster, prevalence of *TP53* mutations, and poorer overall survival.[4] Normal breast specimens and fibroadenomas cluster with the normal-breast-like tumors, which have low luminal epithelial gene expression and high basal epithelial and adipocyte gene expression patterns.[3,4]

Women with localized luminal breast cancer have the prognostic benefit of therapies utilizing selective estrogen antagonists and aromatase inhibitors such as tamoxifen and anastrozole, respectively.[9,10] Despite these therapeutic options, clinical response remains heterogeneous, and genome-wide expression microarray has been be used to create gene signatures that can predict prognosis in ER + patients treated with adjuvant tamoxifen.[11] Patients with HER2 + - tumors and BLBC have higher rates of pathologic complete response to anthracycline-based neoadjuvant chemotherapy than those with luminal or normal-breast-like tumors, but they still have a worse overall and distant disease-free survival.[12,13] Although various long-term survival studies were not standardized to treatment modality, the poorer prognosis of HER + tumors and BLBC likely resulted from recurrence when pathologic complete response was not achieved after chemotherapy.[4,5,8,13]

Basal-Like Breast Cancer

The overall prevalence of BLBC is 15–25% in prospective and retrospective cohorts; however, premenopausal African-American women have a 39% prevalence of BLBC.[8] Breast tumors of patients carrying *BRCA1* mutations are generally BLBC.[5] BLBC is *triple-negative* for ER, progesterone receptor (PGR), and HER2 expression via immunohistochemistry (IHC), thus precluding medical therapy with tamoxifen, anastrozole, and the monoclonal HER2-antibody trastuzumab.[13,14] All triple-negative breast tumors cannot be empirically defined as BLBC because tumors of the normal-breast-like subtype are also negative for ER/PR/HER2 expression as judged by microarray.[3,4]

There is currently no standard for BLBC characterization by IHC, but the presence of one or more myoepithelial cytokeratins (CK) is highly specific.[15] Rakha et al. characterized 56% of triple-negative tumors as basal-like by CK5/6 + and/or CK14 + IHC and found this basal phenotype to be the best marker of poor prognosis in lymphnode-negative, triple-negative breast cancer patients.[16] Although epidermal growth factor receptor (EGFR, also known as ERBB1 or HER1) is not a myoepithelial marker per se, its frequent upregulation in BLBC has made combined EGFR and CK5/6 IHC widely accepted to characterize BLBC.[8,17] Specifically, compared with expression microarray, EGFR + and/or CK5/6 + IHC has been used to characterize ER−/HER2− breast tumors as BLBC with 100% specificity and 76% sensitivity.[17]

Current Clinical Trials Using Tumor Subtype Biomarkers

Future breast cancer research aimed at early diagnosis and therapeutic targets in BLBC is critical not only because of a high metastatic potential and mortality, but also because BLBC lacks the current drug targets ER and HER2. The small molecule lapatinib is a dual tyrosine kinase inhibitor of both EGFR and HER2. Lapatinib was approved in 2007 for use in combination with capecitabine (5-FU prodrug) to treat locally advanced or metastatic HER2 + breast cancer in patients previously treated with trastuzumab.[18] Lapatinib may overcome trastuzumab resistance either by interacting with the intracellular HER2-kinase domain without requiring an intact extracellular domain, as does trastuzumab, or by inhibiting EGFR, which may have become upregulated as a compensatory growth-promoting pathway.[18,19] Despite strong rationale, biomarker evaluation has not identified the subgroup of patients who fail to benefit from the addition of lapatinib to capecitabine.[18]

Further studies are required to determine lapatinib's utility as monotherapy or to treat HER− tumors.[19] Adjuvant Lapatinib and/or Trastuzumab Treatment Optimization (ALTTO) is an ongoing, international, randomized phase III trial for women with node-positive or high-risk (tumor size ≥1 cm) node-negative HER2 + breast cancer.[20] A randomized, double-blinded, active-control phase II trial with biomarker evaluation is underway to study the efficacy of neoadjuvant letrozole (aromatase inhibitor) versus letrozole plus lapatinib in hormone-sensitive, HER2− operable breast cancer.[21] In the endeavor to target the selected patient population that will benefit most, the well-established association of EGFR expression with BLBC indeed provides the rationale to include this particularly lethal breast cancer subtype in future lapatinib trials.[8,17] However, a correlation between EGFR expression and lapatinib response has yet to be uncovered.[19] Additional anti-EGFR drugs such as cetuximab

(chimeric monoclonal EGFR antibody) and erlotinib (EGFR tyrosine kinase inhibitor) are also in clinical trials studying triple-negative breast cancer.[14]

GENE AMPLIFICATION AND MOLECULAR SUBTYPING

The structurally related transmembrane glycoproteins HER2 and EGFR are members of the epidermal growth factor receptor family of receptor tyrosine kinases, and both genes are targets for copy number amplification in breast cancer. Elevated gene copy number can occur by gene amplification and/or high polysomy (additional chromosomal copies). The HER2 + subtype essentially results from gene amplification within the *ERBB2* amplicon at 17q12-q22.[4,22,23] Amplification at the *HER2* locus has been characterized by fluorescent in situ hybridization (FISH) and CGH with 78.6% concordance.[23] Simultaneous expression of co-amplified genes contributes to tumor phenotype, and the *ERBB2* amplicon is a focus of HER2 therapeutics research.

Conversely, EGFR upregulation in BLBC is rarely due to gene amplification but often results from either high polysomy of chromosome 7 or transcriptional induction by the transcription factor YBX1 (Y box binding protein 1).[24,25] Both *EGFR* activating mutations and high gene copy number promote sensitivity to gefitinib (selective EGFR tyrosine kinase inhibitor), whereas altered drug uptake/efflux transporters and acquired alternative/downstream signaling are the common resistance mechanisms.[24,25] Combination drug therapy with multiple molecular targets may therefore provide the best approach to improve longterm treatment efficacy. In this regard, even though some BLBC cell lines are sensitive to gefitinib independent of activating *EGFR* mutations, combining gefitinib with suppression of YBX1 is necessary for drug sensitivity of anchorage-independent BLBC cells.[25]

ESR1 (estrogen receptor 1) gene amplification at the 6q25.1 amplicon has been demonstrated in 20.6% of breast cancers using FISH and a tissue microarray of more than 2,000 clinical breast samples.[26] *ESR1* amplification is highly specific (~95%) for ER expression in luminal tumors characterized by IHC; however, its low sensitivity (~33%) implies that other mechanisms of ER overexpression likely exist in luminal malignancies.[26] In situ carcinomas as well as certain benign proliferative lesions also exhibit *ESR1* amplification, suggesting that this mechanism is an early event in luminal breast cancer progression.[26] More important, survival is significantly longer in women receiving tamoxifen monotherapy for luminal breast cancer with *ESR1* amplification than in those without *ESR1* amplification.[26]

CANCER EPIGENETICS AND BREAST CANCER EPIGENOMICS

Epigenetics is defined as heritable changes in gene expression that are not due to changes in DNA coding sequence. Since the cancer phenotype is attributable to both genetic and epigenetic changes within the genome, several distinct aberrant epigenetic events have been observed during the process of tumorigenesis.[27] Prominent amongst these is a generalized loss of methylated cytosine residues within the genome. This global DNA *hypo*methylation is most often observed within repetitive sequences dispersed throughout the genome.[28] Global reduction in DNA methylation may be an important event in the adaptation of cancer

cells to changes in their microenvironment and likely contributes to the increased genomic instability, a phenotypic hallmark of tumor cells.[29,30]

The more studied cancer-associated epigenetic event is a process termed *epigenetic silencing*. This phenomenon is responsible for the transcriptional repression of numerous tumor suppressor genes (TSGs) and other growth regulatory genes in cancer cells. Among the epigenetic alterations linked to transcriptional silencing are specific posttranslational modifications to the histone subunits within nucleosomes. Specifically, deacetylation of histones H3 and H4, H3K9 methylation, and H3K27 trimethylation are all associated with chromatin in a transcriptionally repressed state.[31] These modifications, as well as those associated with transcriptionally active histone complexes (e.g., H3K4 trimethylation), have been termed the “histone code” and likely play a normal physiologic role in controlling gene expression.[32] In tumor cells, changes in the patterns of histone modifications can be observed as a genome-wide phenomenon, and accumulation of transcriptionally repressive marks occurs within the promoter region of epigenetically silenced genes.[33,34] More recently, the repositioning of nucleosomes within the promoter region of genes undergoing silencing is also implicated in the mechanics of epigenetic gene silencing.[35]

While, as mentioned above, cancer cells exhibit global genome hypomethylation, aberrant cytosine *hyper*methylation also occurs at discreet 5′-CG-3′ (CpG) dinucleotides within the genome. Generally, these CpG dinucleotides reside within clusters termed CpG islands and are often associated with the 5′ region of structural genes.[36] Moreover, it has been amply demonstrated that hypermethylation of CpG islands associated with TSGs is closely associated with their silencing.[37–39] Cytosine methylation, the only demonstrated post-replication modification in DNA, is catalyzed by a class of enzymes termed DNA methyltransferases (DNMTs).40 However, how specific genes are targeted for CpG hypermethylation and consequential silencing during the process of tumorigenesis is not well understood. Several studies indicate that transcriptionally repressive histone marks help to maintain DNA hypermethylation within the promoters of silenced genes, underscoring the complex and intricate nature of gene silencing.[41,42] Furthermore, epigenetic gene silencing has been documented in the absence of detectable CpG methylation, indicating that CpG methylation is not an a priori requirement for gene silencing.[43,44] In sum, it is now widely recognized that epigenetic silencing does not stem from a single aberrant event; rather, it arises from a complex set of biochemical modifications that transform chromatin from a transcriptionally active to an inactive state.[45,46]

Although it is now clear that epigenetic gene silencing is more complex than just CpG island hypermethylation, DNA methylation remains the most studied of the aberrant epigenetic events during tumorigenesis. In no small part this is attributable to a single technical fact: both DNA and methylated DNA can be amplified in vitro while polypeptides cannot. The methodologies used to study chromatin-associated proteins (i.e., chromatin immuno-precipitation, mass spectroscopy) require significant amounts of starting material, while the study of DNA can be accomplished from relatively small samples by the use of PCR. This allows the study of DNA methylation patterns not only in cell lines grown in the laboratory, but also in primary tumors and even in archived (i.e., formalin-fixed, paraffin-embedded)

pathology specimens. Thus, in this review, we focus on gene hypermethylation as a marker for epigenetic gene silencing in breast cancer.

Epigenomic Cancer Profiling

In a manner analogous to transcriptomic profiling, epigenetic profiling has potential to provide biomarkers useful in characterizing human malignancies and monitoring cancer progression based on a tumor-specific methylation signature. Toyota et al. observed that cancer-associated methylation of many genes was observed only within a subset of colorectal tumors, leading these investigators to propose that some tumors display a CpG island methylator phenotype (CIMP).[47] Furthermore, since the *MLH1* gene is one of the targets for silencing in CIMP + tumors, this phenotype is associated with microsatellite instability. More recently, epigenomic analysis of colorectal tumors indicates that sporadic colorectal cancer appears to arise from distinct but parallel pathways rather than a single linear progression model, two of which (serrated and villous adenoma) possess a CIMP + phenotype.[48–51] In addition to colorectal tumors, the CIMP phenotype has also been described in melanoma as well as gastric, oral, pancreatic, and hepatocellular cancer.[52–56] Specifically in melanoma, aberrant methylation of the *MINT17*, *MINT31*, *TFPI2*, *WIF1*, *RASSF1A*, and *SOCS1* genes was found to be associated with advanced stage cancer, and methylation of a panel of TSGs in esophageal adenocarcinoma is a predictor of poor prognosis when compared with tumors that do not display aberrant methylation of these genes.[56,57]

Epigenomic analysis has identified gene methylation profiles associated with the molecular subtypes of breast cancer via hormone receptor status, HER2 status, or both.[58–61] However to date, correlating epigenetic profiling with breast tumor type defined by histologic criteria has proven more challenging. Even though a hypermethylation signature can distinguish breast cancer from other non-breast tumor types, genome methylation profiling does not correlate with histologic type due to the relatively uni-modal distribution of methylation frequency between IDC, ILC, and mucinous carcinoma.[62–64] It is possible that profiling invasive breast lesions may not allow clear-cut tumor classification based on epigenomic criteria because disease-associated alterations in the epigenome occur early in the process of tumorigenesis, and significant variation in gene methylation can occur during breast cancer progression.[65,66] Nevertheless, numerous genes have been characterized as targets for silencing in breast cancer, and when considered either individually or in modest-sized gene panels, provide us with key insight into the mechanisms driving breast cancer progression and the role that aberrant epigenetic marks can play as additional prognostic/diagnostic biomarkers.

Epigenetic Silencing in Breast Cancer Subtypes

Epigenetic silencing is one mechanism by which mammary epithelial cells repress ER expression, leading to the ER− molecular subtypes of breast cancer.[67] Although it is commonly asserted that *ESR1* promoter methylation is the principle mechanism of ER repression in ER− breast cancer, *ESR1* silencing is most consistently observed in several breast cancer cell lines.[68,69] Primary breast tumors represent a heterogeneous cell population, and while studies have shown that *ESR1* methylation is more common in ER−

than in ER + tumors, mapping the *ESR1* promoter CpG island by methylation-specific PCR (MSP) has not revealed clear and consistent results in tumors overall.[68,69] In contrast to ER + breast cancer cell lines, where the *ESR1* gene is unmethylated, many ER + breast tumors have shown evidence of *ESR1* methylation.[68] Furthermore, even though *ESR1* methylation has proven to be a significantly better predictor of clinical response to adjuvant tamoxifen than hormone receptor status scored by IHC, *PGR* methylation was actually the best predictor of ER status (inverse association) in a panel of 35 markers that included *ESR1*.[58] Lapidus et al. suggest that a heterogeneous *ESR1* gene methylation pattern may evolve during breast cancer progression and play a role in ER− recurrences or metastases in patients with ER + tumors.[68]

Breast tumors with *BRCA1* (breast cancer 1, early onset) methylation show a disproportionately higher frequency of *ESR1* promoter methylation.[69] BRCA1 is a nuclear phosphoprotein that contributes to genomic stability by promoting DNA double-strand break repair, recombination, and cell-cycle checkpoint control.[70] Unlike many other TSGs, *BRCA1* somatic mutations are extremely rare in sporadic breast cancer, but 9–13% of these tumors reveal aberrant *BRCA1* gene methylation, especially when loss of heterozygosity occurs at the *BRCA1* locus.[71–73] *BRCA1* methylation is associated with increased breast-cancer-specificmortality, but it was not clear in this study the relationship between *BRCA1* methylation, BLBC, and mortality.[74]

Although breast tumors of patients carrying *BRCA1* mutations are generally BLBC, there is no significant difference in *BRCA1* methylation between sporadic BLBC (14%) and sporadic non-BLBC controls matched for age and grade (11%).[5,75] *BRCA1* methylation also appears to be similar across molecular subtypes (14–17%).[76] *BRCA1* promoter methylation is associated with reduced expression by IHC and qPCR, which have been positively correlated.[75,76] Promoter methylation is thus one mechanism of *BRCA1* gene silencing in sporadic BLBC. Sporadic BLBC, however, more frequently contains unmethylated *BRCA1* coupled with high BRCA1 expression, consistent with a high mitotic rate and normal regulation of BRCA1 expression.[76] This fact points to an alternative mechanism for the phenotypic overlap between sporadic BLBC and hereditary BRCA1 tumors. Matros et al noted that many tumors with low BRCA1 expression are unmethylated, which may result from either a low proliferative rate or upstream inactivation of BRCA1 regulators in higher-grade proliferative tumors.[76] The significantly lower *BRCA1* messenger RNA (mRNA) expression in sporadic BLBC compared with age/grade-matched controls possibly stems from overexpression of ID4 (inhibitor of DNA binding 4), a negative regulator of the *BRCA1* promoter.[75]

Both *BRCA1* and *ESR1* gene methylation are also associated with medullary breast cancer, a histologic special type prevalent in *BRCA1* carriers.[69,72] Expression microarray and CGH have determined that medullary breast cancer is a distinct genomic subgroup of BLBC.[77,78] Furthermore, *SCGB3A1* (secretoglobin, family 3A, member 1) gene methylation is a potential surrogate marker of medullary breast tumors since it is methylated in most sporadic breast tumors and unmethylated in medullary carcinoma and BRCA1-deficient tumors.[79–81]

In addition to genomic stability, BRCA1 also contributes to epigenomic stability by functioning in maintenance of the inactive X chromosome (Xi), although its exact function is still controversial.[82] The heterochromatic Barr body forms during early female embryogenesis as the result of chromatin remodeling by X inactive-specific transcript, non-protein coding (XIST) RNA.[82,83] Both BRCA1-deficient tumors and BLBC frequently lack a Barr body and overexpress a subset of X-linked genes clustered in Xp22.[84] Thus, while a mitotic error leading to active X chromosome (Xa) isodisomy (XaXa) is most common, X-linked genes also escape silencing via epigenetic changes.[82,84,85] Female X-linked gene expression is quite variable, and further studies are needed to elucidate how epigenetic changes in Xi contribute to breast cancer progression and the gender specificity of certain tumors.[85]

Epigenetic Silencing in Breast Cancer Progression

Epigenetic silencing is considered to be a mechanism that can act as a *hit* in Knudson's two-hit hypothesis of tumorigenesis.[86] A number of TSGs possessing CpG islands that acquire dense methylation and subsequently undergo transcriptional silencing during breast carcinogenesis have been identified.[70] A subset of these genes is methylated in proliferative and in situ breast lesions, strongly suggesting a role for epigenetic silencing in the initiation and/or progression of breast cancer (Table 1).

RASSF1A (Ras association domain family 1 isoform A) is a TSG that contains a Ras association domain and potentially functions as a proapoptotic effector of Ras-dependent signaling.[87] Epigenetic silencing by promoter methylation is the most common mechanism of *RASSF1A* inactivation and occurs early in breast cancer progression.[65,87,88] Pyrosequencing has quantified increased *RASSF1A* methylation in epithelial hyperplasia with or without atypia, and methylation is significantly greater for in situ and invasive breast cancer compared with normal breast tissue.[88] Quantitative MSP has also scored dense *RASSF1A* methylation in epithelial hyperplasia as well as intraductal papilloma.[65] Ductal carcinoma in situ (DCIS) and microdissected invasive cells from the same specimens have shown similar extensive *RASSF1A* methylation by quantitative MSP.[65] These studies characterize *RASSF1A* hypermethylation as a biomarker of pathologic proliferation in breast epithelium and suggest that *RASSF1A* methylation occurs before invasive growth develops.[65,88]

SFN (stratifin), a TSG involved in cell-cycle control and regulated by TP53, is frequently epigenetically silenced in breast cancer.[89] Similar to *RASSF1A*, *SFN* methylation frequently occurs in microdissected atypical hyperplasia and DCIS.[65,90] *SFN* methylation, however, has also been scored in physiological breast proliferations and stromal breast tissue, emphasizing the importance of selective tissue sampling when using *SFN* methylation as a biomarker of breast cancer risk.[65,90]

RARB (retinoic acid receptor, beta) encodes a nuclear receptor that binds retinoic acid (biologically active vitamin A) and limits cell proliferation by regulating gene expression.[70] Bisulfite genomic sequencing (BGS) has revealed dense *RARB* promoter methylation in breast cancer cell line DNA.[91] Treatment of these cell lines with 5-aza-2′-deoxycytidine (5-azadC), a DNA demethylating agent, increases *RARB* expression consistent with epigenetic

silencing.[91,92] MSP has confirmed frequent *RARB* promoter methylation in human breast cancer at the same CpG site previously determined by BGS.[93] Furthermore, *RARB* methylation frequency positively correlates with increasing cytologic abnormality using the Masood cytology index, supporting a role for *RARB* methylation in breast cancer risk assessment.[93]

Several TSGs suppress cellular invasion and metastasis by promoting cell–cell adhesion and extracellular matrix integrity. Secreted cystatin E/M inhibits the lysosomal cysteine proteases that can degrade the extracellular matrix, and this tumor suppressor (referred to as *CST6*) is silenced by aberrant methylation in both DCIS and IDC.[94,95] *WIF1* (WNT inhibitory factor 1) is another secreted tumor suppressor that inhibits the Wnt signaling molecules functioning in cell–cell interactions. *WIF1* is frequently methylated in primary breast tumors, and reduced tumor expression has correlated with methylation when compared with patient-matched normal breast tissue.[96] *TGM2* (transglutaminase 2) catalyzes extracellular matrix cross-links and is commonly epigenetically silenced in primary breast tumors.[97] Diminished *TGM2* expression has also been observed in DCIS by IHC; thus, cells may acquire *TGM2* hypermethylation prior to becoming invasive.[97] More important, in vitro doxorubicin sensitivity is abrogated following 5-azadC treatment in breast cancer cells with *TGM2* hypermethylation, making this epigenetic event a potential biomarker for chemosensitivity.[97,98]

Overlap exists in the methylation profiles of IDC and ILC, but there are also defining distinctions. *CDH1* encodes a cell adhesion glycoprotein, commonly referred to as E-cadherin (epithelial-cadherin), a member of the cadherin superfamily of genes. Both lobular carcinoma in situ (LCIS) and ILC characteristically lack E-cadherin expression by IHC, and promoter methylation is one mechanism known to silence *CDH1* in this histologic special type.[99] *SCGB3A1* encodes a putative cytokine and is a candidate TSG epigenetically silenced in primary breast tumors as well as DCIS and LCIS.[79] One or more genes among a five-gene panel including *SCGB3A1*, *RASSF1A*, and *RARB* was methylated in 100% of IDC and ILC, 95% of DCIS, and 69% of LCIS by MSP.[64] Methylation status for each gene was statistically similar between IDC and ILC except for *TWIST1* (twist homolog 1), which was methylated significantly less often in ILC than IDC.[64] Conversely, *DAPK1* (death-associated protein kinase 1) silencing by promoter methylation is significantly more frequent in ILC compared with IDC.[100]

Early Breast Cancer Diagnostics and Epigenomic Analysis

Noninvasive nipple aspiration (NAF), minimally invasive nipple ductal lavage (NDL), and fine-needle aspiration (FNA) are techniques that facilitate cytologic diagnosis for both palpable breast masses and suspicious nonpalpable lesions detected radiologically. Given the nature of aberrant epigenetic changes as early events in breast cancer progression, epigenetic analysis has the potential to improve the accuracy of cytology commonly used for breast cancer risk assessment, routine screening, diagnosis, and surveillance.[101]

To date, epigenetic analysis has been applied to cells collected by NAF, NDL, and FNA. An early study detected DNA methylation in ductal fluid from endoscopically visualized carcinomas, even when cellularity was inadequate for cytology.[102] Furthermore, the results

of this study seemed quite promising for using DNA methylation to detect cancer in NDLs taken from high-risk women with healthy mammograms, because methylation correlated to the cytology that led to a diagnosis of breast cancer in two women. However, more recent studies indicate the likelihood of obtaining sufficient material by either NAF or NDL limits the efficacy of these techniques as tools for risk assessment in women at high risk for breast cancer.[103,104] While aberrant DNA methylation was detected in NAF specimens from patients with DCIS or stage I cancer, Krassenstein et al. chose the markers used for each patient by first examining gene methylation in matched tumor DNA.[105] This study design allowed the investigators to conserve the limited amount of DNA isolated from aspirate fluid, but it has limited use in the diagnosis of cancer prior to surgery. To compensate for low sample cellularity, others have explored the use of more complex methods of DNA methylation analysis to assess cancer in NDL specimens.[106–108] Fackler and co-workers found that a panel of methylated gene markers proved a strong ancillary tool to cytology in the diagnosis of breast cancer.[106,107] And others concluded that aberrant DNA methylation corresponded to high sample cellularity rather than cellular atypia.[108] Thus, at present, the use of NAF and/or NDL as techniques to obtain material suitable for combined cytology and epigenetic analysis remains an attractive but equivocal diagnostic method.

In contrast to NAF and NDL, FNA will produce sufficient cellularity for molecular analysis and breast cancer risk stratification. Indeed, random periareolar FNA (RPFNA) cytology has been effectively combined with nonquantitative methylation analysis of *RASSF1A* and/or *RARB* to enhance breast cancer risk assessment.[93,109] Furthermore, studies conducted on benign FNA samples taken ipsilateral or contralateral to a diagnosed cancer show that *RASSF1A* methylation correlates with increased breast cancer risk and atypical cytology.[110] *BRCA1* methylation, however, does not enhance risk assessment and does not predict mammary atypia in RPFNA samples from “high-risk” women.[111] Similarly, *ESR1* methylation predicts neither mammary atypia in RPFNA nor persistent atypia after 12 months of tamoxifen chemoprevention, despite its utility predicting clinical response of invasive breast cancer to adjuvant tamoxifen.[58,112]

Additionally, gene methylation may have value for improving the diagnostic capability of FNA, particularly when cytology is atypical or indiscriminant. Although an early study failed to show significant differences between benign and malignant lesions based on qualitative methylation analysis of a gene panel commonly methylated in cancer (*CDH1*, *GSTP1*, *BRCA1*, *RARB*), this result could be attributable to the specific genes selected for study and/or the nonquantitative nature of the assay used.[113] In support of the former, Pu et al. used the same non-quantitative methylation analysis but a different panel of genes (*RARB*, *RASSF1A*, *CCND2*) to score methylation in 17 archival FNAs of indeterminate diagnosis, and this assay differentiated malignancy with 100% specificity and 67% sensitivity in the limited number of samples studied.[114] In support of the latter possibility, FNA samples were analyzed using approaches that *quantify* DNA methylation, and statistical analysis revealed a high diagnostic specificity when three out of four genes (*RASSF1A*, *SCGB3A1*, *APC*, and *CCND2*) displayed methylation in a validation set of 45 FNA washings with indiscriminant cytomorphology.[115]

Patient blood samples have also been explored as a source of epigenetic biomarker detection. Aberrant methylation of at least one gene in a three-gene panel (*RASSF1A*, *APC*, *DAPK1*) was positive in the serum of 76% of preoperative patients with in situ or invasive breast cancer.[116] Furthermore, *RASSF1A* methylation in DNA isolated from serum is an independent indicator of poor prognosis in patients with primary or metastatic breast cancer.[117] In addition, serum *ESR1* and *SFN* methylation have shown promising results when used together to differentiate between breast cancer patients and healthy individuals.[118] Most recently, plasma from women with stage I–IV breast cancer were analyzed using a four-gene panel (*APC*, *GSTP1*, *RASSF1A*, and *RARB*), and quantitative methylation of at least one gene in this panel had moderate predictive capability for breast cancer detection.[119]

CONCLUSION

Breast tumors have diverse phenotypes affecting their interaction with the endocrine system, and translating tumor subtype microarray results from the bench to bedside is an important advance in personalizing therapeutic strategies. BLBC is a particularly aggressive subtype that currently lacks effective treatment options and differentially affects a health disparity group. Consequently, further molecular characterization of breast cancer is required to impact the future of anticancer breast therapies, and clinical trials with biomarker evaluation are underway for both HER2 + and HER2− breast cancers. Both gene copy number amplification and epigenetic gene silencing are prominent molecular mechanisms that can propel a malignant phenotype and the development of a specific breast tumor subtype. Because TSGs often undergo promoter methylation prior to observable histopathologic changes, epigenetic analysis in conjunction with minimally invasive techniques has high potential to improve risk assessment and diagnostic accuracy. Although further work is required to define and refine the accuracy of gene panels used in epigenetic analysis, determining the epigenetic signature of a suspicious breast lesion may become a routine part of clinical workup in the foreseeable future.

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TABLE 1

Tumor suppressor genes hypermethylated during breast cancer initiation/progression

	BRCA1	CDH1	CST6	DAPK1	ESR1	RARB	RASSF1A	SCGB3A1	SFN	TGM2	TWIST1	WIF1
Proliferative breast lesions							+		+			
Atypia						+	+		+			
DCIS			+			+	+	+	+	+		
LCIS		+			+	+	+	+				
Primary breast tumors	+	+	+	+	+	+	+	+	+	+	+	+
Ductal carcinoma			+	−		+	+	+			+	
Lobular carcinoma		+		+		+	+	+			−	
Medullary carcinoma	+				+			−				
References	69,71–76	99	95	100	58,67–69	64,93	64,65,87,88	64,79–81	65,89,90	97	64	96

DCIS ductal carcinoma in situ, *LCIS* lobular carcinoma in situ

+Methylated, −unmethylated