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## REVIEW

# Molecular and Cellular Heterogeneity in Breast Cancer Challenges for Personalized Medicine

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Breast cancer is noted for disparate clinical behaviors and patient outcomes, despite common histopathological features at diagnosis. Molecular pathogenesis studies suggest that breast cancer is a collection of diseases with variable molecular underpinnings that modulate therapeutic responses, disease-free intervals, and long-term survival. Traditional therapeutic strategies for individual patients are guided by the expression status of the estrogen and progesterone receptors (ER and PR) and human epidermal growth factor receptor 2 (HER2). Although such methods for clinical classification have utility in selection of targeted therapies, short-term patient responses and long-term survival remain difficult to predict. Molecular signatures of breast cancer based on complex gene expression patterns have utility in prediction of long-term patient outcomes, but are not yet used for guiding therapy. Examination of the correspondence between these methods for breast cancer classification reveals a lack of agreement affecting a significant percentage of cases. To realize true personalized breast cancer therapy, a more complete analysis and evaluation of the molecular characteristics of the disease in the individual patient is required, together with an understanding of the contributions of specific genetic and epigenetic alterations (and their combinations) to management of the patient. Here, we discuss the molecular and cellular heterogeneity of breast cancer, the impact of this heterogeneity on practical breast cancer classification, and the challenges for personalized breast cancer treatment. (*Am J Pathol* 2013, 183: 1113–1124; <http://dx.doi.org/10.1016/j.ajpath.2013.08.002>)

## Breast Cancer Is a World Health Problem

Breast cancer is a major health problem in the United States and worldwide. The American Cancer Society estimates that 232,340 new cases of invasive breast cancer and 64,640 new cases of ductal carcinoma *in situ* (DCIS) will be diagnosed among women in the United States in 2013.<sup>1</sup> Worldwide, an estimated 1,383,500 new cases of breast cancer will be diagnosed in women in 2013.<sup>2</sup> Invasive breast cancer accounts for 29% of all cancer diagnoses among women in the United States and 23% of all cancer diagnoses among women worldwide. Breast cancer is now the second leading cause of cancer-related death in the United States, with approximately 40,030 deaths expected in 2013 for both men and women (or 39,620 deaths among

women, representing 14% of all cancer-related deaths among women) and remains the leading cause of cancer-related deaths among women worldwide, with 458,400 deaths expected in 2013 (representing 14% of all cancer-related deaths among women). A significant proportion of new breast cancer cases (691,300 new cases) and deaths (268,900 deaths) occur in economically developing countries.<sup>2</sup>

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This article is part of a review series on the molecular pathogenesis of breast cancer.

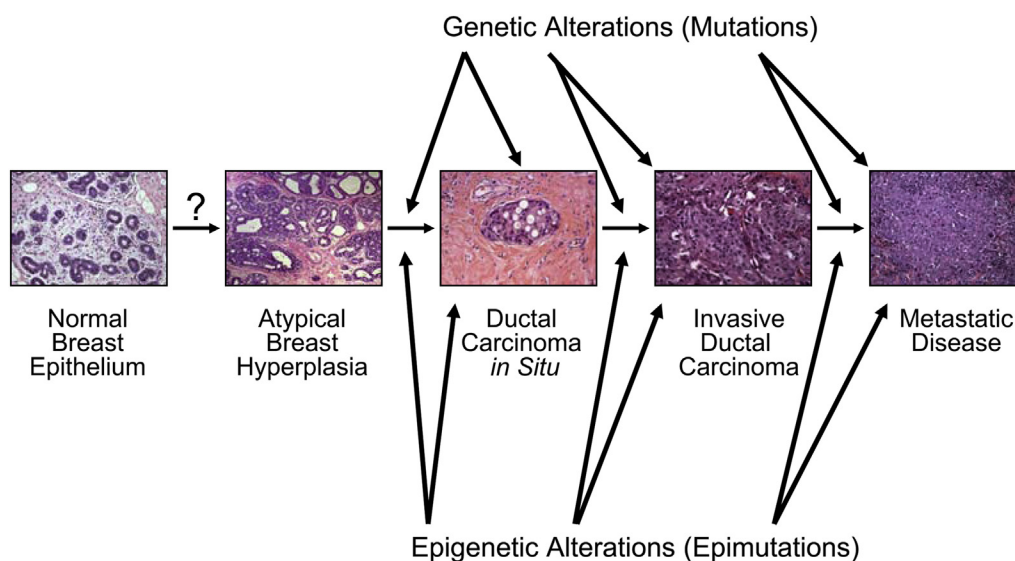
## Natural History of Breast Cancer

Clinical cancer develops over a long period of time, requires multiple molecular alterations, and involves evolution of cellular populations with increasingly aggressive phenotypic characteristics.<sup>3,4</sup> Although the time required for the process of carcinogenesis is not well established for any human cancer, estimates suggest that this multistep process unfolds over many years and possibly several decades. With the obvious exception of pediatric cancers, most cancers are diseases of old (or older) age. Sporadic breast cancers, in which there is no recognizable strong genetic component, generally emerge later in life (perhaps reflecting mostly postmenopausal breast cancers),<sup>5</sup> whereas hereditary breast cancers occur earlier in life (reflecting the contribution of genetic predisposition).<sup>6,7</sup> The relationship between time to emergence of a clinical disease and the hereditary or sporadic molecular underpinnings of the disease has been variously explained through the two-hit hypothesis of cancer and similar molecular concepts.

The leading hypothesis for the natural history of breast cancer development is stepwise progression from atypical ductal hyperplasia to DCIS, followed by evolution of this preinvasive lesion to invasive breast cancer<sup>8</sup> (Figure 1). DCIS is a commonly diagnosed breast lesion that accounts for 25% of breast neoplasms diagnosed in the United States.<sup>10,11</sup> DCIS is by definition noninvasive, but can vary from low-grade (and non-life-threatening) to high-grade lesions that may contain invasive elements. As such, DCIS, especially if high grade, is a risk factor for development of invasive breast cancer.<sup>12</sup> Consistent with this notion, the incidence of DCIS increases with age in parallel with the incidence of invasive breast cancer, and many

invasive breast cancers are associated with adjacent DCIS lesions. Although it is not entirely clear whether DCIS is a required precursor for development of invasive breast cancer, many invasive breast cancers are accompanied by DCIS at the time of diagnosis,<sup>13</sup> and there is consensus that DCIS will eventually progress to invasive disease in the absence of intervention. Genetic and epigenetic alterations (Figure 1) may accompany, or be required for, transitions between morphological stages and/or might occur among the altered cells that comprise the pathological lesions during development and progression of the disease.<sup>9</sup>

Since the late 1990s, invasive breast cancers have been characterized using gene expression analysis and classified on that basis into several molecular subtypes.<sup>14–18</sup> More recently, analyses of gene expression patterns in DCIS have identified similar molecular subtypes.<sup>19–24</sup> The correspondence between molecular subtypes of DCIS and invasive cancers suggests that the DCIS lesions are likely the direct precursors of invasive cancers. However, some recent molecular analyses of invasive breast cancers and associated preinvasive lesions suggest that common cellular ancestors with altered genomes (ploidy changes and mutations) may give rise to both.<sup>25</sup> Data suggest that the diversity of molecular subtypes observed in invasive breast cancers emerges from an evolution of low-grade to high-grade DCIS lesions.<sup>24</sup> Thus, early alterations in the breast epithelium leading to the development of preinvasive DCIS lesions may determine the severity of the invasive breast cancers that subsequently develop in many patients. Although risk factors for DCIS development appear to mirror those for invasive breast cancer, the molecular pathogenesis of DCIS is not well understood. Likewise, the nature of early molecular alterations preceding DCIS has not been characterized in detail,



**Figure 1** Natural history of breast cancer development. Breast cancer develops from normal breast epithelial cells that evolve through atypical hyperplasia (and eventually dysplasia), DCIS, and invasive breast cancer. Multiple molecular alterations occur during this process, involving genetic and epigenetic alterations in precursor and neoplastic cells. Genetic predisposition can contribute to this process, but early molecular alterations (preceding DCIS) have not been well characterized. Original magnification,  $\times 20$ . Modified from Rivenbark and Coleman,<sup>9</sup> with permission from Elsevier.

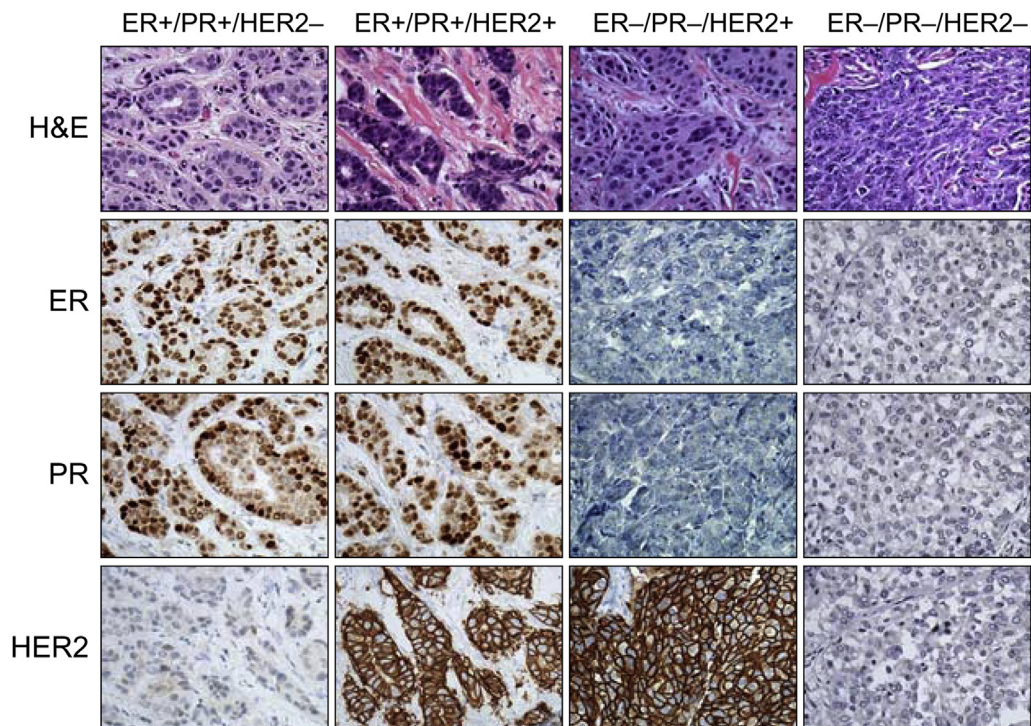
particularly in patients lacking strong genetic predisposition to breast cancer development.

## Breast Cancer Is Not Just One Disease

Breast cancer is a heterogeneous disease characterized by variant pathological features, disparate response to therapeutics, and substantial differences in long-term patient survival.<sup>26</sup> The heterogeneity observed among breast cancers reflects the now well-accepted notion that there is not just one disease with a few variant subtypes, but that breast cancer instead represents a collection of distinct neoplastic diseases of the breast and the cells composing the breast.<sup>26</sup> The distinct nature and character of these diseases can be realized through traditional pathological examination (ie, in terms of disease morphology),<sup>27,28</sup> but the actual extent of diversity among breast cancers can be appreciated only through molecular analyses.<sup>29</sup> Invasive ductal carcinoma is the most common morphological subtype, representing 80% of invasive breast cancers, and invasive lobular carcinoma is the next most common, representing approximately 10% of invasive breast cancers.<sup>27,28</sup> The less common subtypes include mucinous, cribriform, micropapillary, papillary, tubular, medullary, metaplastic, and inflammatory carcinomas.<sup>27,28</sup> These morphological subtypes of breast cancer can be further subdivided into classifications based on their molecular signatures (ie, expression of protein biomarkers or gene expression profiles).

## Immunohistochemical Classification of Breast Cancer

Routine histopathological subclassification of invasive ductal carcinomas is accomplished by immunostaining cancer tissues to detect expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth receptor 2 (HER2; alias c-ErbB-2 or, in rodents, Neu), as well as HER1 and various cytokeratins (eg, CK5/6).<sup>30,31</sup> The differential expression of these protein biomarkers provides a clinical classification for breast cancer (Figure 2) and dictates therapeutic approaches for treatment.<sup>32</sup> Approximately 70% of invasive breast cancers express ER,<sup>33</sup> and the majority of ER<sup>+</sup> cancers also express PR.<sup>34,35</sup> Although the presence of normal PR levels suggests an intact ER signal transduction pathway in the breast cancer cells,<sup>36</sup> discrepant ER and PR expression patterns (ER<sup>+</sup>/PR<sup>-</sup> and ER<sup>-</sup>/PR<sup>+</sup>) are sometimes observed.<sup>35</sup> In clinical practice, for many breast cancers the classification as ER<sup>+</sup>/PR<sup>-</sup> or ER<sup>-</sup>/PR<sup>+</sup> may be attributable to false-positive or false-negative results of immunohistochemical staining, although technical improvements have reduced errors significantly.<sup>32,37</sup> Collectively, the ER<sup>+</sup> malignant neoplasms are classified as luminal cancers. These cancers are further subclassified based on their HER2 status and proliferation rate, giving rise to the ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>+</sup> and ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>-</sup> subtypes (Figure 2). The ER<sup>-</sup> breast cancers are subclassified as HER2<sup>+</sup> and triple-negative based on HER2 overexpression or gene amplification status, basal cytokeratin expression, and EGFR (HER1) expression, giving rise to ER<sup>-</sup>/PR<sup>-</sup>/HER2<sup>+</sup>



**Figure 2** Clinical classification of invasive breast cancer based on expression of ER, PR, and HER2. Representative examples of invasive breast cancers that correspond to the general clinical classifications are shown. Cancer histology is depicted using H&E staining; expression of ER, PR, and HER2 is visualized using immunohistochemistry. Breast cancers are generally classified as positive or negative for hormone receptors ER and PR and for HER2, resulting in four major clinical groupings: ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>-</sup>, ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>+</sup>, ER<sup>-</sup>/PR<sup>-</sup>/HER2<sup>+</sup>, and the triple-negative ER<sup>-</sup>/PR<sup>-</sup>/HER2<sup>-</sup>. Original magnification,  $\times 40$ .

(HER2-enriched) and ER<sup>-</sup>/PR<sup>-</sup>/HER2<sup>-</sup> (triple-negative) subtypes (Figure 2).

## Molecular Classification of Breast Cancer

Early studies of transcription profiles using DNA microarrays identified several molecular subtypes of breast cancer.<sup>15</sup> Groupings of breast cancers were established using computational methods that evaluated similarities in the gene expression profiles generated for individual breast cancers among large cohorts of breast cancer samples. Clusters were identified based on common gene expression patterns driven by overexpressed genes. The first study of this type identified four major molecular subtypes of breast cancer: i) ER<sup>+</sup>/luminal, ii) HER2<sup>+</sup> (HER2-enriched), iii) basal-like, and iv) normal-like.<sup>15</sup> Subsequent transcription profiling studies of invasive breast cancer demonstrated that these molecular subtypes are distinct and reproducible between breast cancer cohorts and using different gene sets for cluster analysis.<sup>38</sup> The currently recognized molecular subtypes of breast cancer are i) luminal A (ER<sup>+</sup>), ii) luminal B (ER<sup>+</sup>/HER2-enriched), iii) HER2<sup>+</sup> (HER2-enriched), (iv) basal-like, v) claudin-low, and vi) normal-like.<sup>8,9,18–22</sup> Significantly, the molecular subtypes of breast cancer revealed by transcriptomic analysis are associated with different clinical outcomes.<sup>15,16,38–42</sup> Recent studies have shown that the molecular subtypes of breast cancer can be robustly identified based on other transcriptomic platforms, including quantitative real-time PCR (qPCR).<sup>43</sup> The reproducibility of molecular subtype classification of breast cancer based on transcriptomic analyses has been reviewed.<sup>44</sup> Although breast cancer classification methods show good reproducibility, suggesting that these are robust biological subtypes, breast cancers that are not classifiable are identified with regular frequency.<sup>44</sup>

### Luminal A and Luminal B Breast Cancers

ER<sup>+</sup> breast cancers occur most frequently and comprise two major molecular classifications: luminal A and luminal B. Luminal A breast cancers are the most common, with a frequency of 28% to 31%.<sup>44</sup> Luminal B breast cancers are characterized by ER positivity accompanied by amplification and/or overexpression of the HER2 gene. Luminal B breast cancers occur less frequently, typically representing approximately 20% of patients in any given data set.<sup>44</sup> The expression status of proliferation-associated genes is one major discriminator between luminal A and luminal B breast cancers.<sup>45,46</sup> In general, the two ER<sup>+</sup> breast cancer subtypes, luminal A and luminal B, are associated with a good prognosis and excellent long-term survival (approximately 80% to 85% 5-year survival), whereas the ER<sup>-</sup> subtypes (HER2<sup>+</sup> and basal-like) are difficult to treat and are associated with poor prognosis (approximately 50% to 60% 5-year survival).

The ability of patients with ER<sup>+</sup> breast cancers to survive their disease reflects the availability of effective targeted

therapy in the form of anti-estrogen treatment (eg, tamoxifen). However, among the ER<sup>+</sup> breast cancers, the luminal B neoplasms are associated with a significantly worse prognosis, compared with the luminal A subtype.<sup>38</sup> The differences in patient outcomes are due in part to variations in response of luminal A and luminal B breast cancers to anti-estrogen treatment.<sup>47</sup> Therapy targeted to HER2-overexpressing breast cancers (including the luminal B subtype) with trastuzumab (Herceptin; Genentech), either concurrent or sequential with adjuvant chemotherapy, has improved survival for these breast cancer subtypes.<sup>48</sup>

### HER2<sup>+</sup> Breast Cancers

HER2 is a member of the human epidermal growth factor receptor family, which also includes EGFR (alias HER1), HER3, and HER4. In breast cancer and some other cancers, HER2 behaves as an oncogene, exerting its oncogenic effects through overexpression, either via the normal gene or secondary to gene amplification.<sup>49</sup> Amplification in breast cancer of the HER2 gene, *ERBB2* (alias *CD340*, *HER-2*, *HER2*, *NEU*, and *NGL*), was first reported in 1987.<sup>50</sup> More recently, this subset of breast cancers was rediscovered through transcriptomic analyses that identified a cluster of breast cancers with strong expression of the *ERBB2* proto-oncogene.<sup>15</sup> HER2<sup>+</sup> breast cancers represent approximately 17% of all breast cancers, with a frequency of 12% to 21% across different data sets.<sup>44</sup> HER2 overexpression (HER2<sup>+</sup>) in breast cancer is associated with poor clinical outcomes,<sup>50</sup> but is also predictive of positive therapeutic responses to anti-HER2 drugs (eg, trastuzumab).<sup>49</sup> HER2<sup>+</sup> breast cancers are typically ER<sup>-</sup>, so treatment for these cancers does not include anti-estrogenic hormonal therapies. Rather, therapies for the HER2<sup>+</sup> breast cancers are based on combinations of targeted drugs (eg, trastuzumab) and cytotoxic chemotherapy.<sup>51</sup> Since the introduction of targeted therapy for HER2<sup>+</sup> breast cancers, the long-term outcomes for these patients have improved dramatically.<sup>52</sup>

### Basal-Like and Claudin-Low Breast Cancers

Together, the basal-like and claudin-low molecular subtypes represent subsets of triple-negative breast cancers (as classified by immunohistochemistry), lacking expression of ER and PR (ER<sup>-</sup>/PR<sup>-</sup>) and also lacking amplification of *ERBB2* (HER2<sup>-</sup>).<sup>53</sup> The basal cell phenotype of breast cancer was first described in immunohistochemical studies,<sup>54</sup> and since then has reemerged through more recent transcriptomic analyses.<sup>15,16,38–42</sup> The basal-like subtype is typically HER2<sup>-</sup> and exhibits some characteristics of breast myoepithelial cells.<sup>55</sup> Basal-like breast cancers represent approximately 15% of all breast cancers.<sup>44</sup> The basal-like breast cancers have high rates of cell proliferation and extremely poor clinical outcomes.<sup>16,38</sup> These cancers are associated with distinct risk factors, including early-onset menarche, younger age at first full-term pregnancy, high parity combined with lack of breast feeding, and abdominal adiposity.<sup>56</sup> Basal-like breast cancers have been shown to be

over-represented in patients of certain age and ethnic groups, specifically young Black women.<sup>57</sup> However, this linkage is not related to genetics, and these cancers affect women of every age and ethnic group or continental origin.<sup>56</sup> Basal-like breast cancers respond to preoperative chemotherapy.<sup>58,59</sup> Despite the observation of pathological complete response in some patients with basal-like breast cancers, overall these patients have a very poor prognosis, perhaps related to a higher likelihood of relapse in those patients in whom pathological complete response was not achieved.<sup>59</sup>

Claudin-low breast cancers represent approximately 10% of all breast cancers.<sup>44</sup> These breast cancers are enriched for markers of epithelial-to-mesenchymal transition and stem cell-like and/or tumor-initiating cell features.<sup>60</sup> Similar to the basal-like cancers, claudin-low breast cancers respond to some chemotherapeutic agents, but patients have poor recurrence-free and overall survival outcomes. This observation may reflect the fact that these cancers exhibit mesenchymal properties and may not be sensitive to standard chemotherapy treatment.<sup>60</sup>

#### Normal-Like Breast Cancers

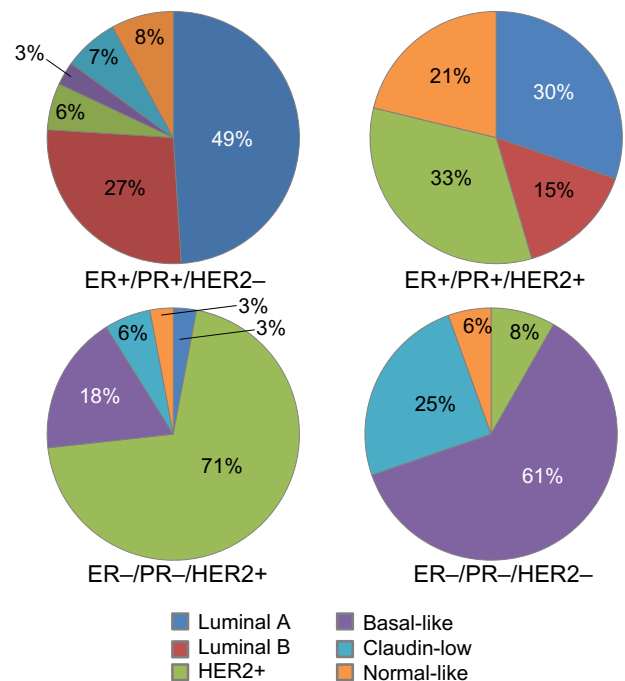
The normal-like breast cancers are so designated because they tend to cluster closely with normal breast epithelium in microarray studies.<sup>15</sup> It is not yet clear whether this is a distinct molecular subtype of breast cancer or simply a grouping of breast cancers that are not otherwise classifiable because of contaminating normal epithelium. Nevertheless, this subset of breast cancer is routinely reported in gene expression studies.

### Correspondence between Clinical and Molecular Classifications in Breast Cancer

Molecular classification of breast cancer based on complex patterns of gene expression provides a link between the molecular biology of breast cancer and the behavior of cancer cells in the corresponding subtypes.<sup>61</sup> However, molecular classification of breast cancer has not yet reached clinical implementation as a routine aspect of patient management.<sup>62</sup> Instead, immunohistochemical staining proxies for the molecular subtypes have been developed based on five basic biomarkers: ER, PR, HER2, cytokeratin 5/6, and HER1. The ER<sup>+</sup> breast cancers are subclassified as luminal A-like when they express an ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>-</sup> pattern and as luminal B-like when they express an ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>+</sup> pattern. Similarly, ER<sup>-</sup> breast cancers are subclassified as HER2<sup>+</sup> when they express an ER<sup>-</sup>/PR<sup>-</sup>/HER2<sup>+</sup> pattern and as triple-negative (ER<sup>-</sup>/PR<sup>-</sup>/HER2<sup>-</sup>) when none of these biomarkers are expressed. Basal-like breast cancers can be distinguished from other triple-negative breast cancers by expression of cytokeratin 5/6 (CK5/6<sup>+</sup>) and/or EGFR (HER1<sup>+</sup>). With this proxy method, many breast cancers are not classifiable because of mixed biomarker expression (eg, ER<sup>+</sup>/PR<sup>-</sup> or ER<sup>-</sup>/PR<sup>+</sup>) or lack of

expression of all five markers. Clinical classification has a major influence on treatment decisions for individual patients. Patients with ER<sup>+</sup> breast cancers are typically treated with anti-estrogenic drugs (eg, tamoxifen) in conjunction with chemotherapeutic drugs, and HER2<sup>+</sup> breast cancers are treated with anti-HER2 drugs (eg, trastuzumab) in conjunction with chemotherapy.

Although an immunohistochemical staining proxy can be used to stratify and classify breast cancers in a clinical setting, the correspondence between clinical (ie, immunohistochemical) and molecular (ie, gene expression) classification is not very good. This situation is illustrated in Figure 3 for 381 breast cancers for which both immunohistochemical staining data and molecular classification were available.<sup>63</sup> The ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>-</sup> subset contained 100/111 (90%) of the luminal A breast cancers, but this represents only 49% of the cancers that were classified as ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>-</sup> (Figure 3). Thus, more than half of patients with ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>-</sup> breast cancers have disease that will behave clinically (with respect to therapy response, disease-free outcomes, and long-term survival) in a manner that is more similar to the molecular subtypes with poor prognosis (luminal B, HER2<sup>+</sup>, basal-like, and/or claudin-low).

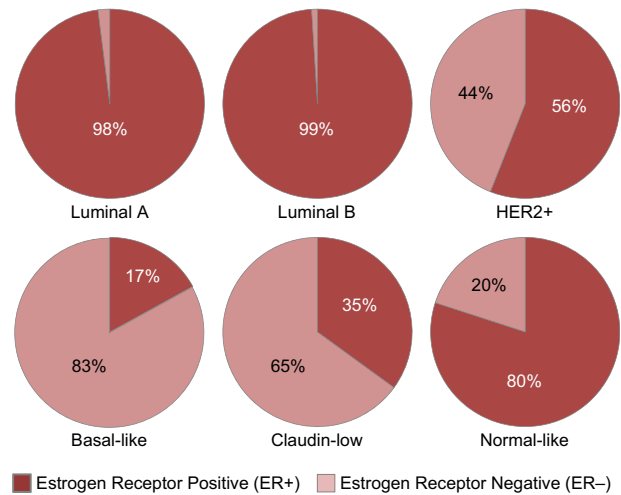


**Figure 3** Correspondence between immunohistochemical and molecular classification of breast cancer. This analysis reflects data from a cohort of breast cancers for which complete immunohistochemical staining results for ER, PR, and HER2 were known (no missing values) and for which molecular classification had been performed based on gene expression patterns.<sup>63</sup> The 381 breast cancers in this cohort were 205 ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>-</sup>, 33 ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>+</sup>, 34 ER<sup>-</sup>/PR<sup>-</sup>/HER2<sup>+</sup>, and 109 ER<sup>-</sup>/PR<sup>-</sup>/HER2<sup>-</sup> based on immunohistochemical staining and 111 luminal A, 60 luminal B, 57 HER2<sup>+</sup>, 80 basal-like, 43 claudin-low, and 30 normal-like based on gene expression analysis.

Among luminal B breast cancers, 55/60 (92%) were classified as ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>-</sup> (representing 27% of this classification) and only 5/60 (8%) were classified as ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>+</sup> (representing 15% of this classification) (Figure 3). This is in conflict with what might be expected, that most luminal B breast cancers would be clinically classified as HER2<sup>+</sup>. The ER<sup>-</sup>/PR<sup>-</sup>/HER2<sup>+</sup> subset was enriched for HER2<sup>+</sup> breast cancers (71% of this subset), but this accounted for only 24/57 (42%) of all HER2<sup>+</sup> breast cancers. The remaining 58% of HER2<sup>+</sup> breast cancers were assigned other clinical classifications, with 23% (13/57) in the ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>-</sup> subset and 16% (9/57) in the ER<sup>-</sup>/PR<sup>-</sup>/HER2<sup>-</sup> subset. Thus, 39% of patients with HER2<sup>+</sup> breast cancers would be misclassified clinically into HER2<sup>-</sup> subsets and would be unlikely to receive anti-HER2 therapy.

As might be predicted, the majority of basal-like (67/80, or 84%) and claudin-low (27/43, or 63%) breast cancers fell within the ER<sup>-</sup>/PR<sup>-</sup>/HER2<sup>-</sup> subset (together accounting for 86% of this clinical classification), but some of these aggressive cancers fell into other clinical subsets (Figure 3). These very aggressive and difficult-to-treat breast cancers may contribute to adverse patient outcomes (with respect to relapse and survival) among clinical subsets of breast cancer (eg, ER<sup>+</sup>/PR<sup>+</sup>/HER2<sup>-</sup>) in which an excellent long-term prognosis is predicted. This analysis strongly suggests, consistent with the observations of others,<sup>32</sup> that molecular classification of breast cancer cannot be reliably inferred based on an immunohistochemical staining surrogate.

The most commonly used targeted therapies in breast cancer treatment are anti-estrogenic (eg, tamoxifen) or anti-HER2 (eg, trastuzumab).<sup>64</sup> Clinical classification of these biomarkers therefore affects the therapeutic strategy applied to every patient. The frequency of ER positivity and ER negativity (based on immunohistochemical staining) varies for each of the molecular subtypes of breast cancer (Figure 4).<sup>63</sup> The luminal A and B subtypes are considered to be ER<sup>+</sup> breast cancers, and indeed these are rarely ER<sup>-</sup>. Likewise, normal-like breast cancers are more often ER<sup>+</sup>. In contrast, HER2<sup>+</sup>, basal-like, and claudin-low breast cancers are considered ER<sup>-</sup> breast cancers, but these breast cancer subtypes are clinically classified as ER<sup>+</sup> with some frequency (Figure 4). The HER2<sup>+</sup> breast cancers are most heterogeneous for ER expression, exhibiting positivity in approximately 56% of cases, whereas the basal-like and claudin-low breast cancers are ER<sup>+</sup> in 17% and 35% of cases, respectively (Figure 4). The frequency of HER2 positivity and negativity (based on immunohistochemical staining) also varies for each of the molecular subtypes of breast cancer (Figure 5).<sup>63</sup> Luminal B and HER2<sup>+</sup> breast cancers are considered HER2<sup>+</sup>, but the majority of luminal B breast cancers (90%) are HER2<sup>-</sup> by immunohistochemical staining, and although the HER2<sup>+</sup> molecular subtype is enriched for HER2<sup>+</sup> breast cancers (based on immunostaining), 39% are classified as HER2<sup>-</sup> (Figure 5). The basal-like and claudin-low breast cancers are typically HER2<sup>-</sup> (Figure 5). The normal-like breast cancers are most often

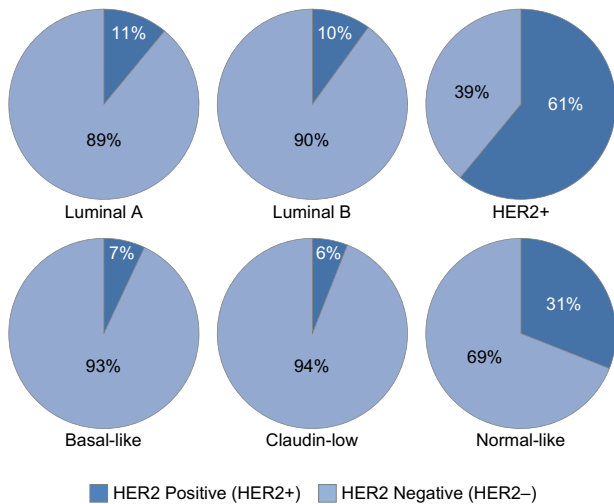


**Figure 4** Expression of ER among molecular subtypes of breast cancer. This analysis reflects data from a cohort of breast cancers for which ER status based on immunohistochemical staining was known and molecular classification had been performed based on gene expression patterns.<sup>63</sup> The 804 breast cancers in this cohort were 548 ER<sup>+</sup> and 256 ER<sup>-</sup> breast cancers based on immunohistochemical staining and 195 luminal A, 162 luminal B, 144 HER2<sup>+</sup>, 138 basal-like, 89 claudin-low, and 76 normal-like based on gene expression analysis.

HER2<sup>-</sup> (69%), but a substantial fraction (31%) are classified as HER2<sup>+</sup>.

## Application of Molecular Signatures to Predict Outcomes in Breast Cancer

Prediction of outcome in breast cancer is very difficult. Numerous clinicopathologic parameters can be used to predict outcomes for individual breast cancer patients, but many of these have limited predictive power.<sup>65</sup> Since the introduction of gene expression analysis using microarrays, and comparable massively parallel technologies, molecular assays have been developed for use in predicting breast cancer outcomes. Several such molecular assays are currently used in the clinical assessment of breast cancer, including MammaPrint (Agendia), *oncoPrint* DX (Genomic Health), Ipsogen MapQuant Dx (Qiagen Marseille), PAM50 (PAM50 Breast Cancer Intrinsic Classifier; ARUP Laboratories), and Breast Cancer Index (bioTheragnostics).<sup>32,66</sup> Each of these assays attempts to provide reliable measures of outcome risk based on complex gene expression signatures. For example, MammaPrint is a 70-gene prognosis profile, approved by the Food and Drug Administration, that is offered as a prognostic test for breast cancer patients who are ER<sup>+</sup> or ER<sup>-</sup>, lymph node-negative (stage I–II), and under the age of 61.<sup>67</sup> This assay stratifies patients at low or high risk for metastasis based on a gene expression score.<sup>67,68</sup> Patients at high risk for metastasis are recommended for aggressive chemotherapy, with more conservative therapy for those with a lower score.



**Figure 5** Expression of HER2 among molecular subtypes of breast cancer. This analysis reflects data from a cohort of breast cancers for which HER2 status based on immunohistochemical staining was known and molecular classification had been performed based on gene expression patterns.<sup>63</sup> The 498 breast cancers in this cohort were 101 HER2<sup>+</sup> and 397 HER2<sup>-</sup> breast cancers based on immunohistochemical staining and 128 luminal A, 90 luminal B, 92 HER2<sup>+</sup>, 96 basal-like, 53 claudin-low, and 39 normal-like based on gene expression analysis.

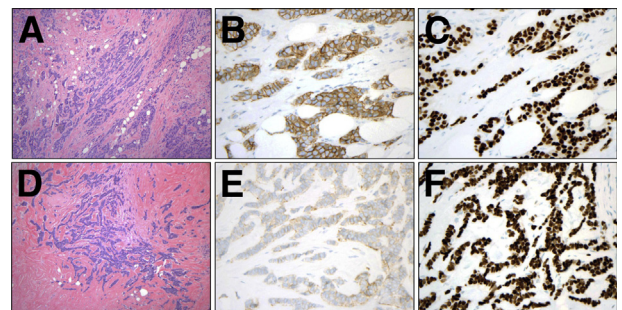
In similar fashion, the *oncoType* DX is a 21-gene prognostic and predictor assay based on a continuous variable algorithm that is used to predict the likelihood of relapse among patients with ER<sup>+</sup>, lymph node-negative early-stage breast cancer.<sup>69–71</sup> The predictive gene expression signature is identified through supervised analysis of microarray data.<sup>69</sup> The recurrence score generated using *oncoType* DX reflects the potential for recurrence among patients with early-stage breast cancer for whom the clinicopathological assessment suggests good prognosis, stratifying the patients who require aggressive therapy (ie, those likely to experience recurrence despite other, favorable predictive factors) versus those for whom conservative treatment will suffice. PAM50 is a 50-gene predictive assay that is based on qPCR for assessment of gene expression levels.<sup>43</sup> This assay stratifies breast cancers according to intrinsic molecular subtype predictive of outcome.<sup>16,38</sup> Ongoing clinical trials are expected to provide insight into the clinical utility of these various molecular assays in the management of breast cancer patients.<sup>66</sup>

### Cellular Heterogeneity among Breast Cancers

The majority of breast cancers are morphologically classified as invasive ductal carcinoma. Although these cancers are not perfectly uniform with respect to cellular characteristics and growth patterns, they are remarkably consistent with respect to histopathological features. Despite the common morphological appearance of these cancers, their clinical behaviors are extremely disparate. Clinical stratification of this group of breast cancers has been achieved

primarily in terms of hormone receptor (ER/PR) status and HER2 status (Figure 2). The clinical outcome of breast cancers has been linked to ER status, with patients having ER<sup>+</sup> breast cancers faring better than those having ER<sup>-</sup> breast cancers. In the case of ER<sup>+</sup>/luminal A breast cancers, the excellent patient outcomes are likely to reflect the molecular character of these cancers (ie, lack of aggressive features associated with cell proliferation),<sup>15</sup> as well as the general responsiveness of these cancers to anti-estrogenic therapies.<sup>72</sup> Likewise, HER2 status has also been linked to patient outcomes. ER<sup>+</sup>/HER2<sup>-</sup> breast cancers are associated with good prognosis, and HER2<sup>+</sup> breast cancers are typically responsive to anti-HER2 drugs. Thus, despite the aggressiveness and poor outcomes associated with HER2<sup>+</sup> breast cancer, the availability of targeted therapies has fundamentally altered outcomes for these patients.<sup>51</sup>

Classification of breast cancer based on these immunohistochemical biomarkers is extremely important to the routine management of the individual patient. In some cases, however, classification is complicated.<sup>73</sup> At the extreme, breast cancers can have a mixed immunohistochemical phenotype, making classification of the cancer impossible. This is illustrated in Figure 6 by routine immunohistochemical staining of ER and HER2 in two different aspects of a single breast cancer occurring in an individual patient. This breast cancer contained elements of tissue that were ER<sup>+</sup>/HER2<sup>+</sup>, but also distinct elements of tissue that were ER<sup>+</sup>/HER2<sup>-</sup>, thus exemplifying variable overexpression of HER2 in a single breast cancer. Additional examples of cellular heterogeneity within breast cancers have been documented, including variable expression of ER, as well as cancers exhibiting regions of tissue that are triple negative.<sup>74</sup> These mixed-phenotype cancers present a challenge to clinicians, because use of targeted anti-estrogenic drugs or anti-HER2 drugs in breast cancers that contain ER<sup>-</sup> or HER2<sup>-</sup> regions cannot treat the entire



**Figure 6** Heterogeneity of HER2 expression in a single invasive breast cancer. Heterogeneity for HER2 positivity but uniform positivity for ER expression is observed in a single cancer in a single patient. **A:** H&E staining of an ER<sup>+</sup>/HER2<sup>+</sup> region of an invasive breast cancer. **B** and **C:** This region of the cancer demonstrates strong (3<sup>+</sup> positivity) HER2 expression (HER2<sup>+</sup>) by immunostaining (**B**) and uniform strong ER expression (**C**). **D:** H&E staining of an ER<sup>+</sup>/HER2<sup>-</sup> region of the same invasive breast cancer. **E** and **F:** This region of the cancer demonstrates weak (1<sup>+</sup> positivity) HER2 expression (HER2<sup>-</sup>) by immunostaining (**E**) and uniform strong ER expression (**F**). Original magnification:  $\times 10$  (**A** and **D**);  $\times 40$  (**B**, **C**, **E**, and **F**).

disease (although it may effectively target a portion of the disease). Using a single or a few protein biomarkers can reveal marked variability in a given breast cancer. Of greater significance may be the underlying molecular heterogeneity, which is much more difficult to observe. With clonal evolution of a developing breast cancer, it is likely that multiple subpopulations of cells with specific molecular alterations will emerge and persist in the clinical cancer.

The presence of cellular heterogeneity within a breast cancer likely reflects the natural history of the lesion and the successive outgrowth of subpopulations of altered cells during carcinogenesis.<sup>13</sup> An enduring paradigm of cancer biology is that cancer is a complex multicellular disease that originates from a single cell. The clonal evolution of breast cancer, beginning with a normal epithelial cell and ending with a clinical metastatic cancer, occurs through many steps, with numerous molecular alterations (both genetic and epigenetic) and multiple emergent cellular populations with altered phenotypic characteristics. During this protracted process of breast carcinogenesis, successive populations of altered cells emerge; some persist and others decline. However, the appearance of a new dominant clonal population is not necessarily accompanied by the disappearance of other subpopulations of cells. Clonal outgrowth of successive populations of nascent cancer cells results in a complex cellular landscape in the tissue, reflecting the molecular and cellular heterogeneity of the subpopulations of cells contained therein.<sup>26</sup>

Throughout carcinogenesis, tumorigenesis, and progression, individual cells acquire molecular alterations, whether genetic mutations or epimutations (ie, epigenetic alterations), that confer new cellular phenotypes and behaviors. Thus, activation of a given growth pathway or inactivation of a tumor suppressor pathway (or some combination of these) confers growth advantages in affected cells, compared with neighboring cells without the same molecular alteration. The end result is a heterogeneous mixture of altered cell populations with adjacent normal cells, only some of which will acquire all of the necessary changes to produce a cancerous mass lesion. The cellular heterogeneity observed in breast cancer has important implications for patient management. Subpopulations of breast cancer cells can represent cancer stem cells (or tumor-initiating cells) and/or treatment-resistant cells.<sup>26</sup> The presence of cancer stem cells may account for regrowth of a breast cancer in which pathological complete response was achieved through initial clinical interventions. Furthermore, resistant cell populations (cancer stem cells or other) may give rise to cancer recurrence, despite good therapeutic response by the bulk cancer.

## Molecular Heterogeneity among Breast Cancers

The development of breast cancer occurs in response to an accumulation of genetic and epigenetic abnormalities that drive uncontrolled growth of breast epithelial cells (Figure 1).

The primary manifestations of the genetic and epigenetic abnormalities occurring in breast cancer are reflected in the cellular behaviors observed in cancer cells (autonomy of growth control, resistance to growth suppression and apoptosis, and ability to invade local and metastasize to distant sites) and in the underlying gene expression patterns. However, the genetic and epigenetic alterations occurring in breast cancer cannot be fully understood and represented in gene expression patterns alone.

In 2012, the Cancer Genome Atlas Network published results related to analyses of gene expression patterns, gene mutations, DNA copy number, DNA methylation, and miRNA expression patterns among a large cohort of approximately 800 breast cancers.<sup>29</sup> This study demonstrated clearly that breast cancer is a heterogeneous disease with multiple distinct molecular subtypes and that there is great diversity among the recognized major molecular subtypes.<sup>29</sup> It follows that the molecular processes governing the pathogenesis of breast cancers of a given molecular subtype can vary, involving different mechanisms for gene activation or inactivation and different genes representing positive and negative mediators of neoplastic development and progression, and that, therefore, no singular molecular mechanism of breast cancer pathogenesis exists. Further investigation will be required to elucidate the various pathways that can lead to breast cancer development and the key molecular events that contribute to tumorigenesis and progression (driver versus passenger genetic and epigenetic events).

Using multiple platforms for gene expression analysis, including microarrays and next-generation, high-throughput sequencing, the Cancer Genome Atlas Network study reproduced the well-recognized ER<sup>+</sup> and ER<sup>-</sup> molecular subtypes of breast cancer.<sup>29</sup> In addition, certain molecular subtypes of breast cancer were found to be associated with specific genetic alterations. For instance, HER2<sup>+</sup> and basal-like breast cancers exhibit a high rate of somatic mutation in the *TP53* tumor suppressor gene (72% to 80%), whereas other molecular subtypes exhibit *TP53* gene mutations much less frequently (12% to 29%).<sup>29</sup> Luminal A, luminal B, and HER2<sup>+</sup> subtypes exhibited significant rates of mutation in the *PIK3CA* gene (45%, 29%, and 39%, respectively), whereas basal-like breast cancers are rarely associated with mutation of this gene (9%).<sup>29</sup> It is notable that very few genes were found to be mutated at greater than 10% frequency within or across the molecular subtypes of breast cancer, but numerous genes (including at least 177 cancer-associated genes) were mutated in smaller numbers of cancer (>20,000 nonsilent somatic mutations among 510 breast cancers).<sup>29</sup> Copy number variations reflecting gene deletions and amplifications were found to affect numerous genes and gene regions, including amplifications in *PIK3CA* and *ERBB2* chromosomal regions and deletions in *TP53* and *MAP2K4* chromosomal regions, among others.<sup>29</sup>

Cancer-associated alterations in DNA methylation include global hypomethylation and gene-specific hypermethylation.<sup>75-77</sup> Recent evidence suggests that these



epigenetic mechanisms play a major role in breast carcinogenesis.<sup>78–85</sup> Genes that have been determined to be directly silenced by DNA methylation in breast cancer include cell-cycle control genes (*CDKN2A*), steroid receptor genes [*ESR1* (alias *Era*, *NR3A1*), *PGR* (alias *PR*, *NR3C3*), and *RARB* (alias *HAP*, *NR1B2*, *RRB2*)], tumor suppressor genes (*BRCA1*), genes associated with cancer metastasis (*CDH1*, *TIMP3*), and many others.<sup>86–90</sup> Loss of expression of ER is frequently associated with hypermethylation of the *ESR1* gene. In addition to hypermethylation of specific genes, hypomethylation affecting large chromosomal regions can be associated with aberrant or inappropriate expression of genes that contribute to cancer development and progression. Furthermore, genome-wide demethylation contributes to chromosomal instability by destabilizing pericentromeric regions of certain chromosomes.<sup>91–93</sup> Thus, epigenetic mechanisms operating in breast cancer may contribute to altered expression of specific genes, altered expression of genes located in common chromosomal regions, and/or genetic instability resulting in copy number alterations.

## The Future of Personalized Breast Cancer Therapy

Investigations into the molecular pathogenesis and biology of breast cancer have increased our understanding of the disparate clinical behaviors observed among invasive breast cancers. Although it is clear that traditional histopathological evaluation of breast cancer has value and can be used to classify these cancers based on fundamental phenotypic properties, such as ER and HER2 expression status, the actual complexity of the disease cannot be fully evaluated without consideration of the breast cancer genome, transcriptome, and proteome. It is clear from the available literature that there is great diversity among breast cancers when these measures are considered individually or in combination.<sup>29</sup> The available genomic, transcriptomic, and proteomic data could be interpreted to suggest that every breast cancer is a distinct entity. The recent findings of the Cancer Genome Atlas Network suggest that the molecular signature of each breast cancer is unique whether compared with its closest neighbors (based on clustering) or compared with other breast cancers of the same molecular classification or compared across all cases.<sup>29</sup> The degree of molecular diversity observed increases with greater numbers of genes evaluated. This same observation can be made for gene mutations, copy number variations, pathway activation, and proteomics data.<sup>29</sup>

The molecular alterations observed in any given breast cancer represent either driver events that are necessary for cancer development or progression or passenger events that are secondary to other changes and/or that are not necessary for disease development or progression. Given the available data, it is tempting to speculate that there are many drivers

and/or driver pathways (rather than only a few major pathways) leading to breast cancer, and that each driver pathway accounts for only a small percentage of cancers. We assume that the driver events and affected driver genes convey some advantage to the emergent neoplasm, either through activation of a positive mediator of neoplastic development (eg, proto-oncogenes) or inactivation of a negative mediator of neoplastic development (eg, tumor suppressor genes). In contrast, the passenger events may represent collateral damage or secondary consequences of the driver events. However, this is not to suggest that the passenger events and genes do not contribute to the clinical behavior of the primary breast cancer and/or progression of the disease. It is likely that disparate responses to a given drug regimen among an otherwise similar cohort of breast cancers reflect underlying molecular alterations that render the treatment ineffective or promote resistance. Additional studies will be required to elucidate the complex relationships between the molecular biology of a given breast cancer and how that cancer responds to a specific therapeutic strategy (ie, drug combination, dose, and schedule).

If we accept the premise that every breast cancer is unique and reflects distinct qualitative and quantitative molecular traits, then knowledge of the entirety of molecular traits carried in any given breast cancer and patient is required for true personalized therapy to be realized. The need to evaluate more completely the various omics associated with a given patient and their breast cancer presents a number of challenges. The methods used would need to be practical (rapid and inexpensive), but provide in-depth molecular information. Next-generation, high-throughput sequencing technologies offer a technological basis for the rapid and practical assessment of the comprehensive breast cancer molecular signature.<sup>94</sup> Next-generation sequencing applied to cancer-derived RNA and DNA provides data that can be mined for gene expression patterns, copy number variations, and gene mutation status.<sup>95</sup> This technology will surely provide valuable research tools as investigators address the contribution of individual molecular lesions to the biology of breast cancers, and may lead to identification of new drug targets or new biomarkers for disease detection or prognostication. In addition, detailed molecular data from patients who have been subjected to specific therapeutic strategies (drugs or drug combinations) may enable correlation analysis to identify molecular lesions that could guide treatment (by conferring sensitivity) or might confound treatment (by conferring resistance).

In the future, data from next-generation sequencing or comparable technologies of breast cancers from individual patients may find utility in treatment decisions and choosing appropriate therapeutic strategies.<sup>96</sup> However, for any molecular technology used in this manner, the presence of cellular and molecular heterogeneity among breast cancers necessarily presents a significant barrier. Given the biological significance of cancer stem cells or other small populations of cells within a breast cancer that might influence

disease recurrence or drug resistance, failure of molecular methods to detect genomic, transcriptomic, or proteomic alterations within these cells necessarily results in prognostic failure. For current basic science, clinical, and translational researchers, the challenge is to evaluate large numbers of breast cancers (with known treatment and clinical response measures) to link specific qualitative or quantitative molecular traits with positive or negative responses to a variety of targeted and nontargeted drugs. With advancement of knowledge of comprehensive breast cancer molecular signatures, personalized medicine can be incrementally implemented for subsets of patients through prospective trials that increase the evidence base related to the pharmacogenomics of breast cancer.

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