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Molecular subtyping of breast cancer: opportunities for new therapeutic approaches

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Abstract. Evidence is accumulating that breast cancer is not one disease but many separate diseases. DNA microarray-based gene expression profiling has demonstrated subtypes with distinct phenotypic features and clinical responses. Prominent among the new subtypes is 'basal-like' breast cancer, one of the 'intrinsic' subtypes defined by negativity for the estrogen, progesterone, and HER2/neu receptors and positivity for cytokeratins-5/6. Focusing on basal-like breast cancer, we discuss how molecular technologies provide new chemotherapy targets, optimising treatment whilst sparing patients from unnecessary toxicity. Clinical trials are needed that incorporate long-term follow-up of patients with well-characterised tumour markers. Whilst the absence of an obvious dominant oncogene driving basal-like breast cancer and the lack of specific therapeutic agents are serious stumbling blocks, this review will highlight several promising therapeutic candidates currently under evaluation. Thus, new molecular technologies should provide a fundamental foundation for better understanding breast and other cancers which may be exploited to save lives. (Part of a Multi-auther Review)

Keywords. DNA microarrays, gene-based expression profiling, basal-like breast cancer.

Introduction

For many years, an important question in the clinical management of breast cancer has been how to avoid overtreatment (e.g. giving adjuvant chemotherapy to patients who do not need it) and undertreatment (e.g. failure to provide specific treatments for patients who do not respond optimally to standard therapies). Current predictive factors include hormone receptor status to predict response to tamoxifen, and HER2/neu (HER2) receptor status to identify patients who will benefit from Herceptin therapy (trastuzamab). There is a strong need to identify additional biomarkers in breast cancer that capture more of the underlying tumour heterogeneity [1–3]. Current estimates suggest that treatment failure occurs in approximately 30% of breast cancer patients [2]. For example, although the majority of breast cancer patients with axillary node-negative disease are cured by surgery alone, a significant proportion will relapse without more aggressive therapy.

The dilemma facing clinicians who treat breast cancer patients is clearly demonstrated in the results of recent clinical trials reported by Berry et al. [4]. Combining trial data from Cancer and Leukemia Group B (CALGB) and the US Breast Cancer Intergroup on breast cancer patients with node-positive disease, the authors reported a significantly greater benefit of adjuvant chemotherapy for patients with estrogen receptor alpha (ER)-negative compared to ER-positive disease. Some ER-positive patients appear to derive marginal improvement in survival from existing forms of chemotherapy, and better predictors are needed to identify ER-positive patients who could forgo chemotherapy or who might benefit from new

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therapeutic regimens. The authors state, 'Our analysis suggests that the biologic subtype of breast cancer is the most important predictor of the benefits of an improved therapeutic regimen', and emphasise the importance of identifying new biomarkers that capture tumour heterogeneity beyond hormone receptor status [4]. The response of ER-negative patients to chemotherapy is also not uniform [2], suggesting that both ER-positive and ER-negative breast cancer need to be further subdivided by tumour biology and response to therapy. A concerted effort to identify new biomarkers to inform breast cancer treatment reached a major breakthrough using molecular technologies [5]. Using DNA microarray-based gene expression profiling, Perou and colleagues identified at least five subtypes of breast cancer that are reproducible across patient populations and laboratories [6, 7]. The scientific community is just beginning to understand how these subtypes reflect tumour biology and response to therapy, and clinical trials have been initiated to test specific therapeutic regimens targeting molecular subtypes.

As ever, clinical decisions are based on the efficacy of a particular treatment for patients with a given subtype of tumour and the side effects which are likely to occur. There are currently a number of prospective clinical trials which are attempting to answer some of these questions. These include the MINDACT trial (Microarray In Node Negative Disease may Avoid ChemoTherapy). MINDACT will compare chemotherapy regimens to evaluate treatment efficacy with long-term toxicities as well as investigating the efficacy and safety of single-agent letrozole to sequential endocrine therapies. The primary objective of MINDACT is to confirm that patients with 'low-risk' molecular prognosis and high-risk clinical prognosis can be spared chemotherapy without affecting survival parameters. MINDACT will compare a 70-gene expression signature with a common clinical-pathological prognostic tool in selecting patients for adjuvant chemotherapy in node-negative breast cancer.

Another such study is TAILOR$_X$ (Trial Assigning IndividuaLized Options for Treatment (Rx) trial. TAILORx, will examine whether genes that are frequently associated with risk of recurrence for women with early-stage breast cancer can be used to assign patients to the most appropriate and effective treatment, in effect to design a methodology for personalising cancer treatment. TAILORx seeks to 'incorporate a molecular profiling into clinical decision making and thus spare women unnecessary treatment if chemotherapy is not likely to be of substantial benefit'. The study aims to enroll over 10 000 women at 900 sites in the United States and Canada. Women recently diagnosed with ER-α and/or progesterone (PR)-positive, HER2-negative breast cancer that has not yet spread to the lymph nodes are eligible for the study.

The purpose of this review is to provide an update on recent findings concerning molecular subtypes of breast cancer, with a special emphasis on basal-like breast cancer; to review clinical trials that utilise molecular subtype information; and to emphasise the need for future clinical trials and development of novel therapeutic approaches.

DNA microarray-based gene expression profiling

The advent of DNA microarray technology ushered in a new era in cancer research [8, 9]. The ability to monitor changes in expression of tens of thousands of genes simultaneously has yielded distinct, reproducible 'portraits' of many of the common cancer types [7]. The fact that expression patterns are similar across multiple samples from the same tumour, preserved in metastatic lesions, and are reproducible across laboratory platforms and patient populations provides strong evidence that molecular subtypes faithfully represent the underlying biology of human tumours. A variety of laboratory platforms have been employed whereby RNA from a tumour sample is converted to cDNA, labelled with distinguishable fluorescent dyes, and hybridised to a DNA microarray containing individual gene-specific probes (for review, see [8] and [10]). The field has undergone continued refinement of methods for sample collection and preparation, laboratory analysis, and approaches to statistical analysis [11]. Microarray results appear to be comparable across laboratories when common platforms and sets of procedures are used [12]. Currently, the greatest bottleneck to progress in this area is the availability of tumour samples from large cohorts of breast cancer patients with complete treatment information and long-term follow-up [12].

Molecular subtypes of breast cancer

For patients with breast cancer, expression profiling has yielded new insights into tumour biology, prognosis, and response to therapy [8, 13, 14]. In the first such study to appear, Perou and colleagues [6] extracted mRNA from human breast tumours and compared expression patterns with human mammary epithelial cells grown in culture. The authors identified two principal gene clusters, including a set of genes involved in cell proliferation that showed high expression in the tumour samples and exhibited strong

correlation with Ki-67 staining using immunohistochemistry. The 'proliferation-associated cluster' was reproduced in a larger study of human breast tumours [7] using cDNA microarrays representing 8102 human genes. Using an unsupervised, hierarchical clustering method, the authors were able to identify an 'intrinsic' subset of 496 genes that identified four subgroups: ER-positive (luminal) tumours, two separate groups of ER-negative tumours (basal-like and HER2-positive), and a group with a pattern resembling normal breast. The proliferative cluster was closely associated with the basal-like subtype.

The terms 'luminal' and 'basal' refer to the principal types of epithelial cells found in the human mammary gland. In normal breast tissue, basal epithelial cells stain with antibodies to cytokeratin 5/6 and are in contact with the basement membrane, while luminal epithelial cells stain with antibodies against keratin 8/18 and form the surface lining layer of cuboidal secretory cells. The cell types of origin and lineage of basal and luminal cells are unknown. Basal cells probably represent a mixture of different cell types with high proliferative capacity, while luminal cells are more highly differentiated [15]. Whether one or both cell types include stem cells that are capable of self-renewal is also unknown. It is important to note, as pointed by Tischowitz and Foulkes [16], that current research on basal-like breast cancer does not implicate a distinct cell type of origin. As Gusterson et al. [17] point out, considerable confusion has arisen because the term 'basal' has historically referred to breast myoepithelium, while in the case of expression profiling, the term 'basal-like' refers to subpopulations of cells defined on the basis of cytokeratin staining and patterns of gene expression. According to these authors, gene-expression profiling and immunohistochemical characterisations of breast tumours are inherently descriptive, and do not directly address the lineage of the various molecular subtypes.

In a series of 78 breast cancer patients with clinical follow-up information, Perou and colleagues [18] confirmed the presence of the luminal, basal-like, HER2+, and normal breast clusters. Basal-like tumours exhibited a high frequency of somatic P53 mutations, and conferred a poor prognosis. Analysis of three independent datasets by the same group of investigators [19] yielded further evidence for the existence of the four subgroups. The investigators identified two subtypes of ER+ breast cancer, luminal A and luminal B, with the former conferring a more favourable prognosis. Once again, patients with the basal-like subtype fared poorly. In one of the three datasets, *BRCA1* mutation carriers were found to have a high frequency of the basal-like subtype. Subsequent analyses showed that the 'intrinsic' subtype classification system was preserved across a variety of microarray platforms and yielded clinically useful information in multiple datasets [20, 21].

The 'intrinsic' classification scheme showed significant agreement in predicting clinical outcome when compared with three other gene-expression-based classification schemes: the 70-gene prognostic classifier of [22], the recurrence score model [23], and the activated wound response model [24]. Furthermore, both the 'intrinsic' classification scheme and the 7-gene prognosis signature were preserved in metastatic lesions when compared with primary breast tumours [25]. These results provide strong evidence that gene-expression-based subtype classification systems are based upon stable biologic properties of breast tumours. As stated by Perou and colleagues [21], 'our findings show that while the individual brushstrokes (i.e. genes) may sometimes show discordance across data sets, the portraits created by the combined patterns of the individual brushstrokes is conserved and recognizable across data sets because of the similarities to the family portrait.' Additional studies also validated the existence of a proliferation-associated cluster of genes or 'proliferation signature' that separates grade 2 breast tumours into subgroups with high versus low risks of recurrence [26, 27].

Cross-validation studies such as those described above illustrate the value of public access to gene expression data [10,13]. As an additional example, Perou and colleagues [28] recently used gene-expression profiling to further subdivide ER-positive tumours into a poor prognosis group showing high expression of cell proliferation and anti-apoptosis genes, and a good prognosis group showing high expression of estrogen- and GATA-regulated genes. The outcome predictor was validated on three independent previously published public access datasets. Additional research is needed to address the issues raised by Berry et al. [4], namely whether expression-based predictors can be used to identify ER-positive patients who might forgo chemotherapy.

Applications to formalin-fixed tissue

Recent work by Perou and colleagues [29] demonstrated that a real-time quantitative reverse-transcriptase (qRT)-PCR assay comprising of 53 genes could reliably detect the 'intrinsic' subtypes. Methods have recently been developed for extracting RNA and performing qRT-PCR using formalin-fixed, paraffin-embedded tumour specimens [30]. Thus, the qRT-PCR assay would allow subtyping of previously banked tumour blocks where frozen tissue is not available, including patient cohorts and clinical trials

with extended periods of follow-up. An alternative and less expensive method for subtyping using fixed tissue would be to employ immunohistochemistry (IHC) surrogates for expression profiling. Nielsen et al. [30] identified a panel of four antibodies (ER, EGFR, HER2, and cytokeratin 5/6) to identify basal-like tumours, and Carey et al. [31] further refined the method by adding antibodies to EGFR and PR. The IHC panel was validated using a 930-case tissue microarray from the University of British Columbia. The definitions by Carey et al. [31] were as follows: luminal A (ER-positive and/or PR-positive, HER2-negative), luminal B (ER-positive and/or PR-positive, HER2-positive), basal-like (ER-negative, PR-negative, HER2-negative, cytokeratin 5/6-positive and/or EGFR-positive), HER2+/ER–, and unclassified (negative for all five markers).

A consensus has not emerged on which IHC assays are most informative for identifying breast cancer subtypes. Livasy et al. [32] conducted a variety of IHC assays on breast tumours that had been classified using DNA microarray analysis. All basal-like tumours were ER-negative and HER2-negative. Most basal-like tumours showed immunoreactivity for vimentin, while few showed reactivity for myoepithelial markers. The latter finding provides evidence against a direct myoepithelial cell derivation for basal-like tumours. Basal-like tumours were high grade and showed high mitotic activity, necrosis, a pushing margin of invasion, and stromal lymphocytic response. Many basal-like tumours showed stained for both luminal (cytokeratin 8/18) and basal (cytokeratin 5/6) markers. EGFR expression was seen solely in the basal-like and HER2-positive/ER-negative subtypes. Abd El-Rehim et al. [33] used tissue microarrays (TMAs) of 1076 breast tumours to evaluate a panel of IHC markers related to epithelial cell lineage, differentiation, hormone and growth factors, and other cancer-specific markers. The authors identified five groups: two groups showing luminal epithelial cells phenotypes that were hormone receptor-positive; two groups characterised by high HER2 positivity and negative or weak hormone receptor status; and a final group showing strong basal epithelial characteristics, p53 positivity, negativity for hormone receptors, and weak to low luminal epithelial cytokeratin expression. Rakha et al. [34] examined 1944 breast tumours for a panel of basal myoepithelial IHC markers and using TMAs. Tumours were classified into two groups, those expressing basal markers (ck5/6 and/or ck14) and tumours showing myoepithelial phenotype (staining for SMA and/or p63). The basal phenotype was associated with high nuclear grade and larger size. The basal, but not myoepithelial, phenotype showed independent predictive value for clinical outcome.

Ries-Filho et al. [35] evaluated a series of metaplastic breast carcinomas, and showed that most tumours showed a basal-like immunoprofile, defined as ER-negative, HER2-negative, EGFR-positive, and/or ck5/6-positive. Fulford et al. [36] used the myoepithelial marker ck14 as a surrogate for the basal-like phenotype to evaluate a series of 453 breast tumours. Positive ck14 staining was associated with high mitotic index, tumour necrosis, and elevated nuclear/cytoplasmic ratio. Further confusion regarding the classification of basal-like breast cancers arises when such tumours are often referred to as ‘triple negative’, as defined by the absence of staining for the receptors ER, PR, and HER-2. Although this triple-negative phenotype is largely true for basal-like, there are a small number of these basal-like tumours that express one or more of these receptors. Conversely, a subset of triple-negative tumours do not express ck5/6 or EGFR. Thus, the triple-negative phenotype is an inadequate proxy for basal-like tumours. In the absence of clear definitions for basal-like tumours using IHC markers, the gold standard for clinical decision making in the future will likely be gene expression profiling. At present, IHC markers for breast cancer subtypes seem to be most useful as a research tool in situations where fresh tumour tissue is not available. An immunohistochemistry representation showing staining for keratins 8/18 and 5/6 in normal breast tissue, luminal breast tumour, and basal-like breast tumours is shown in Fig. 1 (taken from Perou et al. [12]).

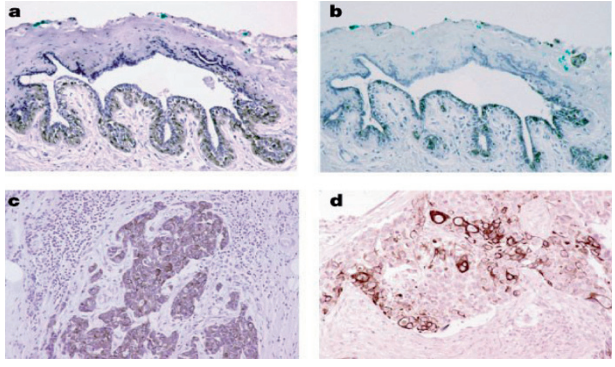

Figure 1. Immunohistochemistry showing staining for keratins 5/6 and 8/18 in normal breast tissue, luminal breast cancer, and basal-like breast cancer sections. (*a*) Normal mammary duct using antibodies against the basal keratins 5/6. (*b*) Normal mammary duct using antibodies against the luminal keratins 8/18 (adjacent tissues sections were used in *a* and *b*). (*c*) Luminal tumour: Tumour Stanford 16 using antibodies against keratins 8/18. (*d*) Basal-like tumour: Tumour New York 3 using antibodies against keratins 5/6. (Taken from Perou et al (2000) Nature 406, 747–752.)

Recent findings on basal-like breast cancer

Bergamaschi et al. [37] used array-based comparative genomic hybridisation (CGH) to evaluate a set of 84 breast tumours that had previously been analysed by microarray-based gene-expression profiling by Sorlie et al. [18]. Chromosomal gains and losses were most frequent in basal-like tumours, while DNA amplification was more common in luminal B tumours. These results suggest that different biologic mechanisms underlie the breast tumour subtypes.

The existence of a basal-like subtype of carcinoma *in situ* provides further evidence that this subtype is a distinct disease entity. Bryan et al. [38] evaluated 66 cases of high-grade ductal carcinoma *in situ* (DCIS) using antibodies to ER, PR, HER2, three basal cytokeratins (ck5/6, ck14, and ck17), EGFR, and c-kit. A small proportion (6%) of tumours were 'triple negative' (ER-negative, PR-negative, HER2-negative) and showed expression of basal cytokeratins and/or EGFR. No histologic features were observed that distinguished triple-negative from the remaining tumours; however, tumours were high-grade DCIS. Livasy et al. [39] evaluated a population-based series of 245 DCIS cases using a panel of four IHC markers. The following definitions were used: luminal A (ER-positive, HER2-negative), luminal B (ER-positive, HER2-positive), basal-like (ER-negative, HER2-negative, cytokeratin 5/6-positive, and/or EGFR-positive), HER2+/ER–, and unclassified (negative for all four markers). The prevalence of subtypes was luminal A (61%), luminal B (9%), basal-like (8%), HER2+/ER– (16%), and unclassified (6%). Basal-like DCIS was showed comedo necrosis, high-grade nuclei, p53 overexpression, and elevated cellular proliferation measured by Ki-67 index.

Population-based estimates of the prevalence of the 'intrinsic' molecular subtypes of invasive breast cancer are provided in two recent epidemiologic studies. Carey et al. [31] analysed a series of 496 incident population-based breast cancer patients from North Carolina. The prevalence of the basal-like subtype was 20% in the entire dataset, and basal-like breast cancer was more frequent among premenopausal African American women (39%) compared with postmenopausal African American women (14%) and pre- and postmenopausal white women (16%). Definitions for the subtypes were based on five IHC surrogates: ER, PR, HER2, CK5/6, and EGFR (as listed previously). The luminal A subtype was less frequent in premenopausal African American women (36%) compared with the other groups (51–59%). The HER2+/ER– subtype did not vary with race or menopausal status. Compared to luminal A, basal-like tumours had more P53 mutations (detected by single-strand conformation analysis and direct sequencing), higher mitotic index, more marked nuclear pleomorphism, and higher combined grade. Breast cancer-specific survival was shortest for patients with the HER2+/ER- and basal-like subtypes compared with luminal A. Yang et al. [40] used the same five IHC surrogates to identify molecular subtypes in a population-based series of 804 breast cancer cases from Poland. The overall prevalence of basal-like breast cancer was 12%. Basal-like tumours exhibited higher grade, and tended to be found among younger patients: the prevalence was 16% in premenopausal and 11% in postmenopausal patients.

Several other investigators reported a higher prevalence of ER-negative, PR-negative [41] or 'triple negative' (ER-negative, PR-negative, HER2-negative) [42] breast cancer in younger African American patients. Most recently, Bauer et al. [43] found that in a population-based series of 51074 incident breast cancer cases identified from the California Cancer Registry (United States), the prevalence of triple-negative breast cancer was 12%. Triple-negative disease was most common among patients under the age of 40 as well as African American and Hispanic women, and carried a poor prognosis. The prevalence of triple-negative breast cancer was 23% among patients under the age of 40, 16% for patients aged 40–49, and 11% for patients aged 50 and over. Triple-negative disease was found in 11% of non-Hispanic white, 25% of non-Hispanic African American, and 20% of Hispanic patients. It should be noted that triple-negative breast cancer was strongly associated with lower socioeconomic status and later stage at diagnosis. In addition, as noted previously, the triple-negative phenotype is not equivalent to basal-like breast cancer. The subtype definitions used by Carey et al. [31] and Yang et al. [40] serve as more specific surrogates for gene-expression profiling. When comparing the prevalence of molecular subtypes across patient populations, it is important to take into account the laboratory methods and definitions that are employed, as well as patient demographics and risk factor profiles that may affect the distribution of basal-like and other subtypes of breast cancer.

Calza et al. [44] conducted gene-expression profiling to identify the 'intrinsic' subtypes in 412 breast tumours from a population-based cohort in Sweden. Basal-like tumours were found in 14% of patients, who tended to be younger and have tumours that were P53 mutation-positive, higher grade, and genomically unstable (based on image cytometry). In patients who received tamoxifen as well as those who did not, the HER2-positive group showed the worst survival. Potemski et al. [45] used the IHC markers ER, HER2, ck5/6, and ck17 to identify basal-like tumours

in a hospital-based study of 195 breast cancer patients from Poland. The basal-like (ER-negative, HER2-positive, and ck5/6- and ck17-positive) and HER2-positive/ER-negative subtypes showed poor survival. Basal-like tumours were found in 24.6% of patients. In a multivariate model in which IHC markers and other tumour factors were considered singly, ck5/6 and ck17 were not independent predictors of survival. Significant predictors were nodal status, HER2 status, and cyclin E expression. In a series of 776 consecutive breast cancer patients from Korea, Kim et al. [46] classified breast tumour subtypes using TMAs and antibodies for ck5, ck14, ck8/18, EGFR, c-kit, ER, PR, p53, and HER2. Basal-like tumours showed a prevalence of 14.7%, and were more likely to be p53-positive and higher grade. Survival for patients with basal-like breast cancer was not significantly different than for luminal breast cancer. A high proportion of basal tumours showed metaplastic features.

A recent study by Carey et al. used immunohistochemical profiles to examine the relationship of neoadjuvant chemotherapy response to outcome among breast cancer subtypes [47]. Using ER positivity for luminal; ER negativity and HER2 positivity for HER2+; and ER negativity and HER2 negativity for basal-like, the authors found that HER2+ and basal-like tumours were actually more sensitive to neoadjuvant anthracycline-based chemotherapy than luminal cancers. The authors found that patients who had a pathological good response to chemotherapy had a good prognosis regardless of subtype. However, HER2+ and basal subtypes had a poorer prognosis, which was explained by the fact that they had a higher likelihood of relapse in patients who had not achieved a pathological complete response [47].

Epidemiology of basal-like breast cancer

The study of Carey et al. [31] provided the first population-based estimates of the prevalence of 'intrinsic' breast cancer in a population-based series of African American and white women. The study was limited by modest sample size and the use of IHC surrogates for subtypes based upon gene-expression profiling. As described previously, definitions of IHC surrogates for 'intrinsic' subtypes are still under development, and there is no agreed upon definition that is common to all studies. Reliable studies of the prevalence of breast cancer subtypes, particularly basal-like breast cancer, are needed to plan future clinical trials and to target recruitment efforts to groups of women most likely to benefit from specific therapies. In addition, few studies have examined risk factors for molecular subtypes of breast cancer and no large epidemiologic studies have examined risk factors for the 'intrinsic' breast cancer subtypes. Such studies could provide important information for identifying at risk individuals for behavioural or pharmacologic interventions targeted at reducing the risk of specific breast cancer subtypes.

Several important clues to the etiology of breast cancer subtypes are provided by recent clinical series. However, these studies are relatively small and do not include unaffected population controls. Calza et al. [44] found that current users of hormone-replacement therapy were over-represented in the 'normal-like' or 'unclassified' breast tumour subtype. Symmans et al. [48] used RT-PCR to measure expression of GABApi, a marker highly expressed in the basal-like subtype, in tumours from Hispanic women diagnosed at the MD Anderson Cancer Center in Texas. GABApi expression was associated with younger age at diagnosis and shorter duration of breastfeeding among parous women. The latter results are intriguing given recent evidence that pregnancy confers specific gene-expression signatures on breast tissue and may effect the distribution and differentiation of potential breast cancer stem cells [49]. As suggested by Tischowitz and Foulkes [16], full-term pregnancy followed by failure to breastfeed or reduced duration of breastfeeding might result in retention of initiated progenitor cells that later develop into basal-like breast tumours. Interestingly, BRCA1 mutation carriers who breastfed for 1 year or more were less likely to develop breast cancer than mutation carriers who did not breastfeed; no effect of breastfeeding was seen for BRCA2 carriers [50].

To date, only one large population-based epidemiologic study examined risk factors for breast cancer based on molecular subtypes. Yang et al. [40] identified family history of breast cancer and younger age at menarche as risk factors for basal-like breast cancer. Additional studies are needed of the epidemiology of molecular subtypes of breast cancer, particularly among African American and Hispanic women [51]. Such studies could yield important information for designing behavioural and/or pharmacologic interventions to reduce future risk of specific subtypes of breast cancer.

BRCA1 and basal-like breast cancer

There are now numerous studies corroborating the finding of Sorlie et al. [19] that *BRCA1* carriers exhibit a high prevalence of basal-like breast cancer. Foulkes et al. [52] showed that ck5/6 expression was a feature of nearly all breast tumours from *BRCA1* carriers in a population-based study from Montreal, Canada. Tu-

mours that expressed ck5/6 were larger in size, more likely to p53-positive and ER-negative, and showed poor survival. In another study of 261 primary invasive breast cancers, p-cadherin, a marker of basal-like breast cancer, was found to be most strongly associated with high grade, ER and $p27^{kip1}$ negativity, ck5/6 and cyclin-E positivity, P53 mutation-positive and BRCA1 mutant breast cancers [53]. Laakso et al. [54] studied ck5/14 and ck8/18 in a cohort of 288 sporadic ductal breast tumours and found that ck5/14-positive tumours were more likely to be grade 3, steroid hormone receptor-negative and HER2-negative. In the same study using a separate set of 42 hereditary breast cancers, the authors reported that the majority of BRCA1 mutant tumours stained positive for ck5/14 (78%), but only one of 15 BRCA2 mutant tumours stained positive [54]. BRCA1 mutant tumours were also more likely to be ck8/18-negative [54]. Indeed, the phenotype of ER negativity combined with ck5/6 and ck14 positivity was found to be 36 times more common in BRCA1 mutant cancers than in BRCA1/2-negative controls [55].

Another common feature of BRCA1 germline tumours and basal tumours was their propensity to metastasise locally or to visceral sites, while non-basal-like or BRCA2 mutant tumours were more likely to spread to bone [56]. Enhanced lung metastasis may be a feature of both BRCA1 and basal tumours which contributes to poor prognosis [16]. Some authors have noted that this metastatic behaviour is accompanied by particular expression profiles, such as the co-expression of ck5/6 with the intermediate filament protein vimentin in ER–, grade 3 cancers with high invasion potential [57]. In a study by Perou and colleagues, vimentin was shown to be a highly selective marker for basal-like tumours and stained positive in 17/18 basal-like tumours with known microarray profiles [32]. Another marker postulated to be a basal-specific marker is p63. Ribeiro-Silva and colleagues investigated the expression of p63 in relation to ck5 expression, the luminal markers ck8/18, and BRCA1 expression in 102 formalin-fixed, paraffin-embedded ductal carcinomas [58]. They found strong co-expression of p63 with ck5, in particular coinciding with loss of BRCA1 expression and inverse staining for ck8/18, suggesting that p63 was a basal marker and may have a role in basal to luminal transition [58].

The observation that breast tumours from BRCA1 mutation carriers were predominantly basal-like led to the suggestion that BRCA1 expression levels may also be important in the regulation of the basal-like phenotype in sporadic breast cancers. A recent study by Turner et al. [59] showed that BRCA1 mRNA levels were two-fold lower in sporadic basal breast cancers than matched controls. In this study this effect was not thought to be due to BRCA1 promoter methylation since methylation-specific PCR of the BRCA1 promoter showed no significant differences compared to controls. Instead, expression of ID4, a known negative regulator of BRCA1 expression, was found to be nine times higher in basal-like tumours compared to control tumours [59]. However, heterogeneity in the basal subtype was also evident since BRCA1 promoter methylation was shown to occur five times more commonly in a small subset of basal cancers with a 'metaplastic' phenotype [59]. BRCA1 promoter methylation has also been reported to occur more frequently in other small subsets of breast tumours showing 'medullary' or 'mucinous' phenotypes which have been shown to be over-represented in BRCA1 families [60]. Consequently, sporadic breast cancers with decreased BRCA1 mRNA expression show similar triple-receptor-negative phenotypes to BRCA1 mutant tumours and may therefore also be classified as 'basal-like.'

Heterogeneity within the basal-like subtype of breast cancer has been proposed by a number of groups [16, 54, 61, 62]. It is possible that basal-like cancer itself contains a number of constituent subtypes, whilst having similar expression profiles may have different clinical responses. This has been prompted by the finding that many basal-like breast cancers co-express the basally restricted markers ck5/6 along with the luminal markers ck8/18 [32], whilst others, such as BRCA1 mutant tumours, do not [54]. In a recent review, Tischowitz and Foulkes [16] proposed a model in which BRCA1 may function as a breast stem cell regulator and BRCA1 mutant tumours represent the 'true basal breast cancers', showing ck5/6 and p63 positivity, and conversely ck8/18 negativity [16]. The authors propose that the loss of p63 expression is required for the transition from a basal to luminal phenotype.

The need for clinical trials for basal-like breast cancer

Traditional approaches to breast cancer diagnosis rely upon histological appearance coupled with pathological parameters such as tumour grading, tumour size, lymph node involvement, and presence of metastases. The growth of expression profiling technologies has substantially refined the classification of breast tumours, but this has not always been coordinated with response to therapy. Treatment information has not been comprehensively obtained in all studies and is a major confounding variable with respect to comparing potential markers across studies [13]. When examining correlations with survival, problems arise if treat-

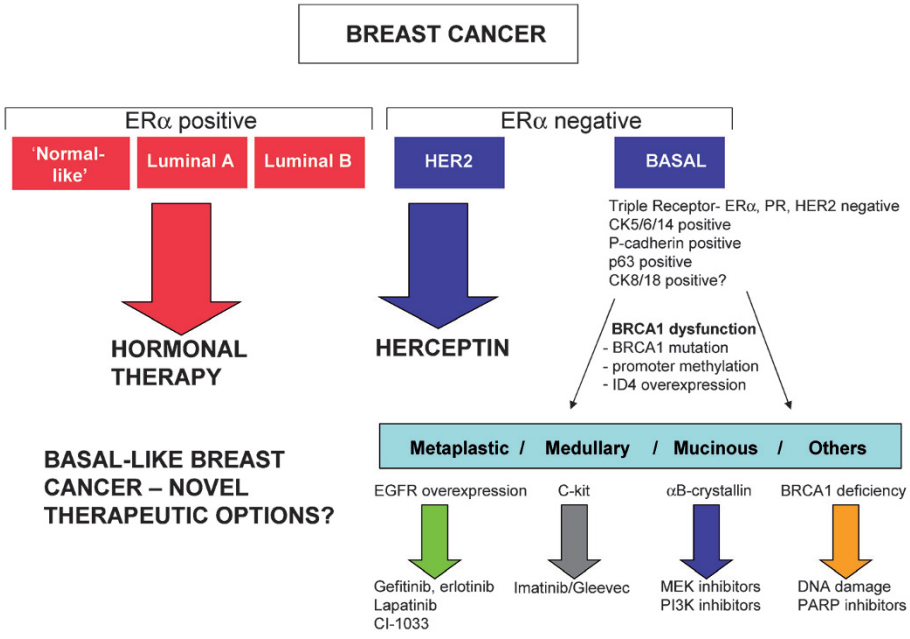

Figure 2. Potential novel therapeutic targets for basal-like breast cancer.

ment information is not included, since this mixes prognosis with predictive ability of the subtypes (and their corresponding responses to particular types of therapy). Other limitations which have hampered meaningful comparisons of studies include the fact that most are restricted to observational data, and do not always have well-defined clinical endpoints. The sample size of many studies also contributes to inbuilt errors in trial design, as small sample size will often be prone to overestimate environmental and lifestyle factors leading to the incidence of breast cancer subtypes. Many studies are hampered by the fact that they are retrospective in nature and therefore lack the flexibility to factor in treatments directed against specific molecular defects. This is particularly relevant with the advent of Herceptin (trastuzamab) to treat HER2+ and luminal B breast cancer subtypes, and it is important that prospective treatment information be obtained prior to the start of this type of study. Thus, the best evidence for the use of molecular subtypes as prognostic markers, and as predictive markers for specific therapeutic agents, would be prospective randomised trials where treatment information is taken into account [13]. In such a trial it is also important that enrolment protocols, follow-up protocols, and data analyses are standardised across contributing centres.

Therapeutic agents that may be effective for basal-like breast cancer

The absence of an obvious dominant oncogenic factor driving basal-like breast cancer (in the same manner that overexpression or amplification of HER2 drives HER2+ breast cancer) reflects the apparent heterogeneous nature of this type of breast cancer. The strong overlap between basal-like and BRCA1 mutant tumour types, however, does provide an insight into molecular defects which may be utilised for designing more specific therapies (Fig. 2). The strong association of both basal-like and BRCA1 mutant cancers with EGFR expression would suggest that EGFR tyrosine kinase inhibitors may offer a viable treatment option for these subtypes of breast cancer. Baselga and colleagues have described the efficacy of targeted therapies such as the receptor tyrosine kinase inhibitor (TKI) ZD1839 (gefitinib/IRESSA), which appears to have anti-tumour activity against certain solid tumour types, most notably NSCLC [63]. Single agent gefitinib and gefitinib combined with anastrazole have been shown to be well-tolerated and effective neoadjuvant treatments for reducing the size of breast tumours and estrogen receptor phosphorylation [64]. However, in a phase II study of advanced breast cancers by Baselga and colleagues, gefitinib was shown to have poor single-agent activity, with only 1 patient showing a partial response and another 3 showing stable disease from a cohort of 31 patients [65]. Another EGFR inhibitor, erlotinib/Terceva, has also been reported to have overall disappointing breast cancer response rates [66].

An interesting fact is that, so far, no clear association has been observed between EGFR levels and response to EGFR-targeted agents in breast cancer. However, a caveat for gefitinib therapy in breast cancer could be found in tamoxifen-resistant ER+ breast cancers (ER+ TAMR). In this instance, tamoxifen resistance is often accompanied by the upregulation of erbB signalling components, including EGFR and HER2. Within this context, gefitinib showed efficacy against ER+ TAMR breast cancers (66%), but much lower efficacy in ER-negative tumours (11%), despite ER-negative tumours having a greater overall expression of components of the erbB pathways [67]. It was noted that patients with ER+ TAMR tumours showing clinical benefit (CB) had lower EGFR than ER+ TAMR non-responders. The majority of these ER+ TAMR patients showing CB in response to gefitinib also showed decreases in pEGFR expression during treatment, but this was not universal in this study [67]. From these data it must be concluded that the prevalence of EGFR overexpression in ER-negative tumours may not in itself be beneficial for gefitinib-based therapies.

In general, the use of EGFR inhibitors in breast cancer trials to date has been disappointing, but factors such as heavy chemotherapy pretreatment in metastatic patients and lack of information about tumour subclassification have also contributed to their overall targeting inefficacy. Preclinical evidence suggests that a number of pathways may be involved in acquired gefitinib resistance, such as IGFR, AKT, and PKCδ activation [61, 68]. It also remains to be clinically proven if dual erbB TKIs such as lapatinib (EGFR and HER2) or indeed pan-erbB inhibitors such as CI-1033 will be more therapeutically effective against breast cancer in general, or basal-like breast cancer in particular.

A basal-like marker offering a potential therapeutic option is the c-kit tyrosine kinase receptor (CD117). This marker has been reported to be associated with a particular subset of basal-like cancers called adenomic cystic carcinoma of the breast, which has been shown to be estrogen-, progesterone-, and HER2-negative [69]. However, this subtype of basal-like carcinoma was also associated with favourable outcome, again highlighting the heterogeneous nature of basal tumours. The value of c-kit as a predictive (or prognostic marker) was also questioned in another study where the expression of EGFR in basal cancers (54%) predicted for poor outcome independent of nodal status or tumour size, whilst c-kit did not influence prognosis [70]. C-kit has previously been exploited as a therapy for gastrointestinal stromal tumours (GIST) by virtue of the fact that it shows a high degree of sensitivity and clinical response to imatinib/Gleevec, the tyrosine kinase inhibitor specifically designed to target bcr/abl kinase in chronic myelogenous leukaemia (CML). The efficacy of targeting c-kit in basal breast cancer or its role as a predictive marker of response is still very much a matter of conjecture and will depend on the prevalence of c-kit in basal tumours as well as the role of c-kit in disease progression.

Several additional molecular defects associated with basal-like breast cancer offer opportunities for new therapeutic approaches. Loss of the phosphatase and tensin homologue (PTEN) tumour suppressor has been reported as a common event in ER- and HER2-negative breast cancers [71, 72]. This suggests that loss of control of the PI3K/AKT pathway could be driving the aggressive basal-like phenotype and may be amenable to PI3K/AKT inhibitors. In addition, 45% of basal-like breast cancers also express the small heat-shock protein αB-crystallin, an independent predictor of poor survival [73]. Overexpression of αB-crystallin lead to an enhanced tumorigenic phenotype including EGF- and anchorage-independent growth, increased cell migration and invasion, and constitutive MEK activation [73]. The PI3K-AKT pathway also appears to be activated following αB-crystallin overexpression [73]. αB-crystallin is a stress protein which has been shown to inhibit apoptosis through disrupting the activation of cell death by inhibiting the activation of caspase 3. Interestingly, MEK inhibitors abrogated the αB-crystallin-transformed phenotype, suggesting that constitutive MEK activation may be driving many of the proliferation genes downstream of αB-crystallin and could represent a promising pathway to target therapeutically [73]. Other putative targets which have been reported to be overexpressed in basal-like breast cancers include genes involved in epithelial to mesenchymal transition and/or invasion such as transforming growth factorβ2 (TGFβ2), matrix metallopeptidase 14 (MMP14), and transmembrane-4 L six family member 1 (TM4SF1) [72].

Perhaps the most reliable predictive marker in basal-like breast cancer could be the expression levels of BRCA1. BRCA1 has been postulated to be an important determinant for both DNA-damaging and microtubule-damaging chemotherapy in both preclinical and clinical studies (for review see [74]). The presence of mutant BRCA1 in tumours predicts much better for response to DNA damaging chemotherapy such as anthracycline-based chemotherapy than either BRCA2-mutant or sporadic breast cancers [75]. Conversely, preclinical models show that the presence of wild-type BRCA1 confers sensitivity to microtubule-damaging agents such as paclitaxel and the vinca alkaloids [76]. The abrogation of BRCA1 function, however, may not be restricted simply to

BRCA1 mutation. Downregulation of BRCA1 expression (as in basal-like tumours) may confer chemotherapeutic responses reminiscent of BRCA1 mutant tumours. This appears to be the case in preclinical studies where downregulation of BRCA1 by siRNA (short interfering RNA) or antisense constructs resulted in enhanced responses to DNA damaging agents [76]. This hypothesis would appear to be very relevant to basal breast cancer since it was shown in a retrospective study of 55 non small cell lung cancers that measurement of BRCA1 mRNA levels could predict clinical outcome following neoadjuvant cisplatin/gemcitabine chemotherapy [77].

The finding that BRCA1 mRNA levels are depleted in sporadic basal-like breast cancers may offer another therapeutic alternative utilising its role as an integrator of multiple DNA damage responses. A deficiency in BRCA1 and BRCA2 function has been exploited in mouse embryonic stem (MES) cell models to target an intrinsic DNA repair defect in both BRCA1 and BRCA2 mutant cells [78]. In these models, MES cells with targeted deletions of BRCA1 and BRCA2 and were found to be highly sensitive to PARP1 inhibitors by virtue of the fact that they were unable to repair double-strand breaks which had resulted from PARP1 inhibition. This was subsequently shown by siRNA knockdown experiments not to be exclusive to functional BRCA1 and BRCA2 but to multiple genes involved in the homologous recombination repair pathway of double-strand DNA breaks [79]. The fact that BRCA1 siRNA knockdown experiments could also render such cells sensitive to PARP inhibition raises the possibility that basal-like breast cancer may also respond in a similar manner. Whilst the heterogeneity of basal breast cancer may also be a problem with this therapeutic approach, BRCA1 mRNA levels could theoretically serve as predictive markers of response to PARP inhibition.

Existing clinical trials for basal-like breast cancer

The clinicopathological significance of basal-like breast cancer was investigated by a large retrospective study of breast cancers (776 cases) using tissue microarrays in a Korean population [46]. They found that in comparison to western countries which showed basal cancers to have the poorest clinical outcome, Korean breast cancer cases showed HER2 expression to be the most important prognostic factor. Another study was performed by Rouzier and colleagues in a cohort of 82 breast tumours aimed to determine if different molecular subtypes of breast cancer (luminal, basal, normal-like, and HER2+) responded differently to preoperative chemotherapy consisting of paclitaxel followed by 5-fluorouracil, doxorubicin, and cyclophosphamide. The results were striking in that 45% of basal and 45% of HER2+ tumours showed a pathological complete response (CR), whilst only 6% of luminal tumours and none of the normal-like tumours showed a CR. They concluded that basal-like and HER2+ subtypes of breast cancer (which usually are associated with the worst prognostic outcome) are more sensitive to paclitaxel- and doxorubicin-containing preoperative chemotherapy than the luminal and normal-like cancers. These chemotherapy responses are reminiscent of studies including BRCA1 mutant tumours which also have very poor prognosis if left untreated but seem to derive greater benefit from DNA-damaging adjuvant chemotherapy than those with sporadic breast cancer [80].

The need for new therapeutic agents

The exemplar of molecularly targeted therapy in breast cancer is perhaps best typified by the success of Herceptin (trastuzamab) in targeting HER2+ tumours. The strong association of basal-like breast cancers with EGFR expression would initially indicate a similar approach targeting EGFR in this aggressive cancer subtype. However, as detailed previously, results from clinical studies to date have shown that targeting EGFR expression in breast cancer, particularly in ER-negative breast cancer, has met with very limited success. Given the poor clinical outcome associated with the basal subtype there is clearly a need to identify specific therapeutic targets. In this context the overriding question is: What is likely to work and what will not work? The question will only be answered in time following a more comprehensive review of the basic biology of basal-like cancers. It is now becoming increasingly evident from expression profiling that this seemingly uniform subtype is a mixture of poorly defined subtypes that may have their own intrinsic biology with profound implications for clinical outcome. The role of BRCA1 within the basal-like subtype umbrella would appear to be a key component of pathogenesis. The BRCA1 pathway may be amenable to therapeutic exploitation by specific drugs such as the PARP inhibitors. It is probably unrealistic to assume that this heterogeneous group of basal-like breast cancers will be respond to a common therapeutic strategy as in the Herceptin example, but more detailed preclinical examination of their biological defects should yield valuable information for prospective clinical studies. One method for identifying potential therapeutic targets for basal-like breast cancer is resequencing of genes that are overexpressed in specific breast cancer

subtypes. For example, Usary et al. [81] discovered somatic mutations in *GATA3*, a gene showing high expression in luminal A and luminal B (ER-positive) breast tumours. Comprehensive evaluation of the spectrum of mutations occurring in breast tumours, as reported recently by Sjoblom et al. [82], could provide important information, especially if tumours are subdivided according to molecular subtype. A complete catalogue of somatic alterations in breast cancers subdivided according to the intrinsic subtypes could identify new therapeutic targets and help to design new treatment regimens. An additional approach for developing therapeutic targets would be to identify chemotherapy-induced gene expression signatures that differ according to breast cancer subtype. Troester et al. [83] used cell lines as well as patient tumour samples taken before and after chemotherapy to show that luminal and basal-like tumours exhibit specific gene expression patterns that may influence response to chemotherapy. It is likely that any new agents for treatment of specific subtypes will need to be evaluated in combination with existing agents, with the goal being eliminating exposure to agents that have side effects that are not beneficial to specific subgroups of patients [4].

Conclusions and perspectives

DNA microarray-based gene expression profiling identifies reproducible and distinct subtypes of breast cancer. The 'intrinsic' subtypes of luminal A, luminal B, HER2+, and basal-like have been observed across multiple patient populations, although immunohistochemical methods for identifying these subtypes using formalin-fixed tissue have not been universally agreed upon. Evidence to date suggests that these subtypes differ according to prognosis and patient survival. Further work is needed to determine the utility of these subtypes in predicting therapeutic response. A variety of candidate therapeutic agents have emerged as a result of increased knowledge regarding gene expression, particularly for basal-like breast cancer. Large, prospective clinical trials are needed that enroll patients with specific breast cancer subtypes and that incorporate information on treatment as well as long-term patient survival. For basal-like breast cancer, patient age and other demographic characteristics will need to be taken into account to ensure that future clinical trials enrol the patients who most need access to novel therapeutic agents.

Preliminary epidemiologic data suggest that gene-expression profiling may also identify breast cancers with different underlying aetiologies. Information on risk factors for specific subtypes could yield important information for designing behavioural and/or pharmacologic interventions to reduce risk of disease. If breastfeeding or other modifiable activities are shown to reduce specific forms of breast cancer, this would represent a tremendous advance in our understanding of the pathogenesis of breast cancer. It would also provide important information for women who are determined to be at risk for hormone-receptor negative breast cancer and who thus may not benefit from tamoxifen and related chemopreventive agents.

In the future, although gene-expression profiling and/or immunohistochemical surrogates could help to tailor therapeutic approaches to individual patients, it is likely that these new technologies will continue to be used in combination with existing biomarkers and prognostic indicators. Thus, prospective clinical trials will need to incorporate side-by-side comparisons of new and traditional methods of tumour classification. Only by using the best of the old as well as the best of the new will we ensure that patients receive maximal therapeutic benefit with the least possible risk of adverse side effects.

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