

# Is 'Basal-Like' Carcinoma of the Breast a Distinct Clinicopathological Entity? A Critical Review with Cautionary Notes

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## Key Words

Breast carcinoma · Basal-like carcinoma · Microarrays · Molecular classification of tumors · Immunohistochemistry

## Abstract

This review deals with studies that have used cDNA microarrays and immunohistochemistry to identify a subtype of breast carcinoma known as basal-like carcinoma. The key breast carcinoma studies are critically discussed to highlight methodological problems in cohort selection, definitions, interpretation of results and statistical analysis. The review concludes that basal-like carcinomas do not reflect a single, biologically uniform group of breast cancers, but show significant variations in their phenotypes, grades, immunoprofiles and clinical behavior, just as a wide range of subtypes and behaviors is observed among epithelial/luminal-derived breast carcinomas. Well-designed studies with comparison of low-grade nonbasal versus low-grade basal and high-grade nonbasal versus high-grade basal carcinomas are necessary before one can be convinced that this subtype represents a distinct clinicopathological entity.

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## Introduction

The traditional clinicopathological parameters such as tumor size, involvement of axillary lymph nodes (LNs), histological grade, nuclear grade, MIB-1 (Ki-67) index, expression of estrogen receptors (ERs) and progesterone receptors (PRs), overexpression (or amplification) of Her2/neu and mutations in the TP53 gene all have been successfully correlated to prognosis of patients with breast carcinoma [1–9]. With regard to the biology of breast cancer, however, the current prognostic factors provide limited information [9]. Moreover, the well-established prognostic and/or predictive factors have significant limitations in distinguishing breast cancer patients who may benefit from aggressive chemotherapy from those who do not need any adjuvant treatment [10]. Indeed, it has been shown that about 70% of patients with breast cancer receiving chemotherapy or antihormonal therapy would have survived without such treatments [10, 11].

The molecular-genetic heterogeneity and the large number of genes involved in controlling cell proliferation, apoptosis and differentiation clearly underline the importance of investigating multiple genetic changes in a variety of phenotypically different breast carcinomas. The introduction of complementary DNA (cDNA) and oligonucleotide microarrays in the mid-1990s [12, 13], the increasing application of these high-throughput tech-

niques and significant improvements in bioinformatic analyses have resulted in an era of genome-wide approaches to prognostication and outcome prediction in patients with breast cancer.

In 2000, Perou et al. [14] showed in their seminal paper that the phenotypic diversity of breast carcinomas is accompanied by a corresponding diversity in gene expression patterns (GEPs) that can be captured using cDNA microarrays. For the first time, they demonstrated that breast cancers could be classified into distinctive subtypes distinguished by characteristic differences in their GEP or their 'molecular portraits'. In their first study, they were able to identify 4 groups of breast cancers related to different molecular features of mammary epithelial cells composed of ER+/luminal-like, basal-like, Her2/neu+ and normal breast [14]. This study and the subsequent study in 2001 by Sorlie et al. [15] had a major impact on both molecular biologists and oncologists and also challenged the traditional histopathological classification system of breast carcinomas. An important implication of these 2 studies was that ER- breast carcinomas include at least 2 biologically distinct subtypes of tumors, namely basal-like and Her2/neu+ cancers, which may need to be treated as distinct entities [14, 15].

### **Essential Information Provided by Two Initial Publications in 2000 and 2001**

The first cDNA study performed by Perou et al. [14] analyzed variations of GEPs in grossly dissected normal and malignant human breast tissues from 42 individuals, consisting of 36 infiltrating ductal carcinomas (IDCs), 2 infiltrating lobular carcinomas (ILCs), 1 ductal carcinoma in situ (DCIS), 1 fibroadenoma and 3 normal breast samples. Fluorescently labeled cDNA was prepared from messenger RNA (mRNA) from each experimental sample. The authors prepared a pool of mRNAs isolated from 11 different cultured cell lines that served as a common 'reference' sample. The reference sample was labeled using a second distinguishable fluorescent nucleotide and provided an internal standard against which the gene expression of each experiment sample was compared. Using a hierarchical clustering method, the authors focused first on a set of 1,753 genes (about 20% of the 8,102 genes analyzed) in order to group genes on the basis of pattern similarity which their expression varied over all samples. Finally, the authors selected a subset of 496 genes ('intrinsic' gene subset) that consisted of genes with significantly greater variation in expression between different tumors

than between paired samples from the same tumor. Using the intrinsic gene subset, the cluster analysis revealed 4 distinctive groups, namely (1) luminal epithelial/ER+, (2) basal-like, (3) Her2+ and (4) normal breast-like carcinomas. This study concluded that application of cDNA microarrays and hierarchical clustering provide a distinctive molecular portrait of each breast cancer and that the breast tumors could be classified into subtypes based on differences in their molecular patterns [14].

In the subsequent important study of Sorlie et al. [15], which was published in 2001, the authors refined their previous classification by analyzing a larger number of breast carcinomas and explored the clinical value of the subtypes by searching for correlations between cDNA GEP and clinically established prognostic factors. The authors analyzed a total of 85 cDNA microarray experiments representing 78 breast carcinomas (including 71 IDCs, 5 ILCs and 2 DCIS), 3 fibroadenomas and 4 normal breast tissues. Using hierarchical clustering of the variations in gene expression, the authors were able to classify breast cancers into basal-like, Her2-overexpressing and normal breast-like groups. The previously identified luminal epithelial/ER+ group could be subdivided into at least 2 subgroups of luminal A and luminal B, each with a distinctive molecular genetic profile. In order to investigate whether the 5 distinctive groups represent clinically distinct subgroups of patients, univariate survival analyses comparing the subtypes with regard to disease-free survival (DFS) and overall survival (OS) were performed. This second study revealed highly significant differences in survival between the subtypes, with the Her2+ and basal-like subtypes associated with the shortest DFS and OS. The results of the second study indicated that the 'basal-like subtype may represent a different clinical' entity [15]. Furthermore, this study suggested that ER+ cancers are highly heterogeneous with respect to their GEPs and that luminal subtype can be divided into luminal A and luminal B (or even luminal C) associated with different clinical outcome. According to this study, the luminal subtype B (and C) carcinomas may represent a clinically distinct group with poorer prognosis compared to luminal A breast cancers [15].

### **Issues with Two Major cDNA Studies Published in 2000 and 2001**

Although, without any doubt, these 2 publications [14, 15] had a major impact on our current understanding and classification of breast carcinoma, one needs to reevalu-

ate them more cautiously. Indeed, there are a number of shortcomings that hamper the enthusiasm about these 2 papers. The problems are briefly discussed as follows.

#### *Use of Different Cancer Cell Lines as a Common Reference Sample*

As mentioned above, Perou et al. [14] used a pool of mRNAs isolated from 11 different cultured cell lines that provided an internal standard or reference sample against which the gene expression of breast cancer was compared. According to the Supplementary Information section of the first study [14], the reference sample was composed of equal mixtures of mRNA isolated from breast (MCF7, HS578T), ovarian cancer (OVCAR3), hepatoblastoma (HepG2), embryonal carcinoma (NTera2/D1), acute leukemia (NB4), T4 leukemia (MOLT4), multiple myeloma (RPMI8226), malignant melanoma (UACC62), liposarcoma (SW872) and colon carcinoma (COLO205) human cell lines. In contrast to the gene profiling study of van't Veer et al. [16], in which a pool of cDNA from each individual sporadic breast cancer patient was used as a reference, Perou and Sorlie and colleagues used a 'mixture of highly heterogeneous cell lines of epithelial, mesenchymal, and haematologic cancers of different organs as a control'. The crucial question is whether the reference sample used by Perou and Sorlie and colleagues is the appropriate one for genetic comparison, if one tries to better understand the biology of breast carcinoma. It is not clear why Perou and Sorlie and colleagues did not use a reference sample exclusively from frozen tissues of normal breast (tumor-free and normal breast tissue away from breast carcinoma) as an internal standard. Although human cell lines and primary cultures have been a popular choice due to their widespread availability as well as the ease of obtaining large amounts of RNA for microarray analysis, a reference sample exclusively consisting of normal breast tissue or, at least breast cancer cell lines, is a much more appropriate standard for comparison if one tries to demonstrate upregulation and downregulation of several thousands of genes in patients with breast carcinoma. As the field has evolved, investigators have realized the crucial issue of reference RNA samples used by different array platforms [17–19].

#### *Issue of Small Sample Size and Statistical Evaluation of Enormous Databases*

The first study examined 39 breast cancers consisting of 36 IDCs, 2 ILCs and 1 DCIS. cDNA analysis and hierarchical clustering of the results of this study revealed 6 (15%) breast cancers with 'basal-like' features (BLCs) [14].

In the subsequent study of Sorlie et al. [15], which also included correlation to clinical outcome, 78 breast carcinomas comprising of 71 IDCs, 5 ILCs and 2 DCIS were examined. This second study revealed 7 BLCs. It is important to point out that for survival analyses (DFS, OS), only a subgroup of 49 patients with locally advanced tumors and no distant metastases were investigated (only by univariate analysis). In fact, the total number of cases and the number of BLC cases in both studies were too small to allow meaningful and reliable statistical analyses. As correctly pointed out by Ioannidis [20], sample size plays a crucial role for analysis of results obtained by microarrays and molecular research: 'Microarrays need evidence and this cannot be obtained from a couple of small studies, no matter how high tech' [20]. Indeed, the same caveat applies not only in gene expression profiling, but also in proteomics and all discovery-oriented molecular research where enormous databases can be rapidly generated from just a small number of patients [20–22]. To achieve reliable and reproducible results, Ein-Dor et al. [23, 24] could recently prove that thousands of samples are needed to generate a robust gene list for predicting outcome in cancer.

#### *Hierarchical Clustering of Luminal-Like Subtype Using the Intrinsic Gene Subset*

A close look at the first study [see fig. 3 in 14] reveals that genes identified in luminal-like subtypes included prolactin receptor, myosin VI, hepatocyte nuclear factor 3, angiotensin receptor 1 and, most importantly, ER1 as well as estrogen-associated protein (LIV-1). It is, however, completely unclear why luminal-type cytokeratins such as CK8/18 and/or CK19 are not shown in this subtype of breast cancer [see fig. 3 in 14], although the term luminal-like would imply this. Indeed, it is a well-known fact that luminal-like carcinomas, which often express ER, show a strong immunoreaction for CK8/18 and or CK19 in the vast majority of cancer cells.

#### *Hierarchical Clustering of Basal-Like Subtype Using the Intrinsic Gene Subset*

The cluster analysis of the first study displayed a basal-like subtype of breast carcinoma. This subtype was characterized by 2 basal-type cytokeratins, namely CK5 and/or CK17 [but not CK14, as identified in many immunohistochemistry (IHC) studies]. Other genes of this subtype included laminin ( $\gamma$ 2), collagen type XVII, calponin1, caveolin 2 and heparin-binding growth factor 8. The first study also described a second basal epithelial cell-enriched gene cluster showing CK7 and CK13 (both

are not regarded as basal-type cytokeratins), epidermal growth factor receptor (EGFR), fatty acid-binding protein 7 (brain), P-cadherin placental, protein tyrosine phosphatase (receptor type, k), integrin ( $\beta$ 4) and tropoin. It is of note that this second gene cluster of basal-like subtype did not show basal-type cytokeratins such as CK5 and/or CK17.

The authors of these 2 studies also performed IHC on cases with gene profiling of CK5 and/or CK17 and reported positive staining for either CK5/6 or CK17 or both in all cases of basal-like subtype. The authors did not use a cutoff for positive immunoreaction in tumor cells of this subtype. Judging from 1 illustration [see fig. 2 in 14], the immunostaining for CK5/6 was heterogeneous. On the other hand, several IHC studies recognized a strong positivity for CK8/18 and/or CK19 (both being luminal-type cytokeratins) in BLCs. Indeed, it is extremely rare to observe immunostaining for CK5/6 or CK17 without positivity of breast cancer cells for CK8/18 or CK19. Moreover, the immunoreaction of tumor cells in BLCs for CK8/18 and/or CK19 is often much more intense and in much higher proportion than that of basal-type cytokeratins. This would imply that basal-like subtype should also show these luminal cytokeratins by means of cDNA microarrays, which is not the case. In other words, with respect to basal- and luminal-type cytokeratins, there is no clear correlation of GEP and IHC results. This discrepancy requires further explanation.

### Subsequent Molecular-Genetic Findings

There has subsequently been a number of publications that have used variations of the intrinsic gene set to confirm the existence of the molecular portrait, with particular attention to the BLC and its prognostic significance [25–30]. It is important to note that GEP studies of primary breast carcinomas performed by different laboratories have resulted in the identification of a number of distinct prognosis profiles or gene sets that share very little overlap in terms of gene identity [23, 31–33]. A recent study [33] examined 295 breast cancer samples and applied 5 different gene expression-based models. The results of GEP of the examined cases using intrinsic subtype [14], 70-gene profile [16], wound response [34], recurrence score [35] and the 2-gene ratio [36] were correlated with the probability of relapse-free survival and OS. An interesting finding of this current study [33] was that despite the absence of gene overlap, 4 of the 5 tested gene models (all but the 2-gene ratio approach)

showed significant agreement on the outcome predictions for individual patients. This study also revealed that a patient whose breast cancer is classified as basal-like based on the intrinsic gene set will most likely be classified as having a poor 70-gene profile, poor activated wound response and high recurrence score [33].

Using comparative genomic hybridization (CGH), a relatively recent publication [37] demonstrated that BLCs show losses at 16p, 17q, 19q and Xp. The BLCs displayed chromosome gains at 20q, 1q, 4q, 6q, 7q and 8q. This study found a striking similarity in the pattern of CGH alterations in BLCs and myoepithelial carcinomas (also known as malignant myoepithelioma or carcinoma with myoepithelial differentiation) [37]. Furthermore, the clinical outcome of patients with poorly differentiated non-BLC (IDC, NOS type) was compared with that of patients with BLC. A remarkable finding of this study was that the BLCs on their own do not convey a poor prognosis and that these tumors represent a heterogeneous group with only a subset showing a shorter OS [37].

### Immunohistochemical Features

While BLC of the breast was initially defined by gene expression profiling, most recently published works have used IHC to define BLC. BLCs commonly show a focal positive immunoreaction with antibodies against basal-type cytokeratins (or high-molecular-weight cytokeratins) such as CK5/6, CK14 and CK17. While most BLCs are negative for ER, PR and Her2 (triple negative), many of them express EGFR (Her1), immunohistochemically [38–43]. The vast majority of BLCs express both luminal-type cytokeratins (such as CK8/18 and CK19) and vimentin [38, 40, 43]. A positive reaction for c-kit (CD117) can also be identified in more than 50% of BLCs [38, 40, 43].

It is important to keep in mind that some of the myoepithelial markers in the breast such as smooth muscle actin (SMA), maspin, CD10, 14-3-3 sigma and CD29 can also be positive in tumor cells of BLCs, indicating that at least some of the BLCs show a myoepithelial differentiation, raising the possibility of myoepithelial origin of this subtype of breast carcinoma [42].

It is important to note that a wide variety of definitions have been used for immunohistochemical characterization of BLC. Using IHC, most previous studies have noted a heterogeneous and focal expression of basal-type cytokeratins in BLCs. The vast majority of these studies have shown a much more intense/diffuse expression of

luminal-type cytokeratins such as CK8/18 or CK19 in BLCs. Indeed, no minimal threshold for what constitutes a positive immunoreaction with antibodies against basal-type cytokeratin has been established or agreed upon. Consequently, there are several open questions that need to be addressed by an international consensus to define the precise immunohistochemical profile of BLCs:

(1) Considering the heterogeneous and often focal immunoreaction, what is the lowest threshold for accepting a lesion as positive? Is a positive reaction for keratins such as K5/6 or K17 in a single cell sufficient to consider a given breast cancer basal like? Or is 5, 10 or even more than 50% positivity a better requirement? Is there any difference between a lesion that is 10% positive and one that is diffusely (100%) positive?

(2) What type(s) of basal-type or high-molecular-weight cytokeratins should be used to confirm BLC? The original 2 studies by Perou and Sorlie and colleagues identified K5/6 and K17 in basal-like subtype by using cDNA microarrays and immunohistochemical validation. CK14 was not mentioned in these 2 studies. A number of recent IHC studies, however, have used CK14 as a marker for BLC. Can a positive immunoreaction for CK14 without positivity for K5/6 and/or K17 be used as a marker of BLC? Should we require a panel of 3 basal-type cytokeratins such as CK5/6, CK14 and CK17 to characterize BLC? Should we also require expression of p63, CD10 and other myoepithelial markers?

(3) How should one classify a poorly differentiated breast carcinoma that displays positive immunoreaction for a variety of basal-type cytokeratins and also exhibits some positive reaction for ER or PR, but is negative for Her2? One has to keep in mind that although all 6 cases of BLC in the first study by Perou et al. [14] were negative for ER, the second study by Sorlie et al. [15] included 2 ER+ breast cancers among the basal-like subtype (2 of 7 cases). Should one classify a Her2+ but ER- and PR- breast carcinoma, which also expresses some positivity for basal-type cytokeratins, as BLC or do we need to reserve this subtype for triple-negative cases that express basal-type cytokeratins?

### Histopathological Features of BLC

The vast majority of publications uses the designation of basal-like phenotype for this subtype of invasive breast carcinoma. This subtype of carcinoma is, however, defined either by genotyping (GEP, using the intrinsic gene model) or IHC (using antibodies against basal-type cyto-

keratins). Therefore, the designation 'phenotype' is not quite appropriate, since it is actually the immunoprofile or immunotypic characteristics that define this subtype. In fact, the designation 'basal-like phenotype' would imply that carcinomatous cells histomorphologically look like basal cells. This is, definitely, not the case. In addition, it should be noted that epithelial and myoepithelial cells have long been recognized in the breast [1, 2]. It has also been known for quite some time that the normal epithelial cells variably express CK8, CK18, ER and PR, while the myoepithelial cells generally express CK5/6, CK14, CK17, p63, CD10, actin and calponin, but never express either ER or PR [1, 2]. Does replacing 'epithelial' with 'luminal' and 'myoepithelial' with 'basal' add anything? Nevertheless, there are some phenotypic features of carcinomas that are more likely to be associated with this recently recognized subtype of breast cancer. BLCs usually, but not always, show high nuclear atypicity, high mitotic activity including several atypical mitotic figures, high MIB-1 (Ki-67) index, solid aggregates of tumor cells often with pushing border of invasion and not infrequently a marked lymphocytic stromal reaction [41, 43]. Areas of central 'comedo' type or acellular ('geographic') necrosis within the invasive, solid aggregates can be present [41, 43–45]. The above-mentioned morphologic features are commonly observed in poorly differentiated IDC (grade 3, NOS type).

The vast majority of metaplastic (sarcomatoid) carcinomas of the breast show the characteristic immunohistochemical features of BLC [2, 42, 46–48]. Indeed, there is no sharp dividing line between metaplastic carcinoma and BLC. The metaplastic carcinomas with basal-like immunophenotype include adenosquamous carcinoma, spindle cell carcinoma (including carcinoma with myoepithelial differentiation), carcinoma with heterologous chondroid and/or osseous differentiation (including so-called matrix-producing carcinoma and carcinosarcoma) [1, 2, 42]. In addition, most medullary carcinomas and so-called atypical medullary carcinomas (IDC with medullary features) are triple negative and express basal-type cytokeratins [49].

It is important to note that although most examples of basal-like subtype are of high grade and show a very high mitotic activity, there are certain low-grade primary breast carcinomas which definitely show the immunoprofile of BLCs. These rare BLCs, which clinically have a very low aggressive behavior, include adenoid-cystic carcinoma, low-grade adenosquamous carcinoma, low-grade squamous carcinoma, low-grade spindle cell ('fibromatosis-like') carcinoma and secretory ('juvenile')

carcinoma [1, 2]. All these primary low-grade breast carcinomas express basal-type cytokeratins and often are negative for ER, PR and Her2 (triple negative) [2]. In sharp contrast to the high-grade BLCs, the low-grade variant of BLC lacks significant nuclear atypia and shows a very low MIB-1 (Ki-67) index or mitotic activity [2].

### A Distinct Clinicopathological Entity?

Since the first 2 publications of Perou and Sorlie and colleagues in 2001 and 2001 [14, 15], there has been a marked increase in publications concerning BLC. Although several publications have confirmed the distinctive nature of this cancer subtype, both genetically and clinicopathologically, there are some recent publications raising serious questions, particularly with regard to the poor prognosis of this subtype [50, 51]. The main reasons for advocating this newly recognized subtype as a distinct clinicopathological entity are discussed as follows.

#### *Prognosis*

GEP studies have repeatedly shown that the basal-like and Her2-overexpressing subtypes of breast carcinomas have significantly poorer DFS and OS than their luminal A/ER+ counterparts [15, 25, 28, 29]. However, many of these studies did not specifically describe histopathological features of the cohort of BLCs, whether they were mostly of high-grade nuclear atypia, contain a high number of mitotic figures, show a high MIB-1 (Ki67) index or were strongly positive for p53 immunostaining. In other words, if one takes high-grade BLCs in her/his study and compares the clinical outcome of this subtype with that of the luminal A/ER+, which often represents low- (grade 1) or intermediate-grade (grade 2) breast cancer, the prognosis (OS and DFS) will be, of course, much poorer in BLCs. On the other hand, none of the GEP studies seems to include special subtypes of breast carcinomas that are of low-grade nuclear atypia but characteristically are triple negative and express basal-type cytokeratins such as CK5/6, CK14 or CK17. A typical example of this low-grade variant of BLC is adenoid cystic carcinoma of the breast, which is well known to have an excellent prognosis [1, 2, 52]. Other examples of low-grade breast carcinomas with basal-like immunotype, that do not seem to be examined by previous GEP studies, are low-grade metaplastic carcinomas (low-grade adenosquamous carcinoma, low-grade squamous carcinoma, low-grade 'fibromatosis'-like or spindle cell carcinoma and low-grade 'matrix-producing' carcinoma). All these special types of

breast carcinomas express several basal-type cytokeratins, are mostly triple negative and associated with a very low proliferative activity (low MIB-1 index, low mitotic activity) [1, 2]. It is well known that all these low-grade carcinomas have a good (or excellent) prognosis, yet they are not included in the category of BLCs in GEP studies. Therefore, the selection of BLCs in the previous GEP studies with clinicopathological correlation is not representative of all variants.

The situation is more complex and confusing if one focuses on a number of studies that solely used IHC for their definition and characterization of BLCs. Due to the lack of uniform IHC definition and probably some biased selection of the cases, these IHC studies revealed contradictory results with regard to the prognosis of BLCs as being an independent poor prognostic factor.

In this section, 8 recent IHC studies are analyzed in order to discuss relevant issues concerning contradictory results in BLCs. First, the focus is on 3 large studies from the Nottingham group [53–55] in which the basal-like subtype was defined as IHC positivity for CK5/6 and/or CK14 and which have shown BLCs to be associated with poorer OS and/or DFS than nonbasal breast carcinomas. In the following discussion, these 3 publications are referred to as Nottingham study 1, 2 and 3.

Using tissue microarray (TMA) technology, the first study from this group (Nottingham study 1), by Abd El-Rehim et al. [53], examined a high number of 1,944 cases of invasive carcinoma with antibodies against basal (CK5/6, CK14) and luminal cytokeratins (CK8, CK18 and CK19) to determine the frequency of expression of each cytokeratin subtype and compare their relationships with well-established clinicopathological prognostic factors. Any positive immunoreaction (even single tumor cells) for CK5/6 and/or CK14 was regarded positive. This study found that basal marker expression was significantly related to poor prognosis, ER negativity and younger patient age [53]. Multivariate analysis revealed that CK5/6 (but not CK14) was an independent indicator for DFS. OS of BLCs, however, was not significantly different from that of non-BLCs. The multivariate analysis of the results of this study included grade, nodal status, tumor size, Nottingham prognostic index, status of ER, vascular invasion and patient age. It is of note that 2 well-established and important pathological parameters, namely MIB-1 (Ki67) index and immunoreactivity for p53 were not included in the multivariate analysis.

The second TMA study (Nottingham study 2), by Rakha et al. [54], also examined 1,944 cases and evaluated morphological and immunophenotypical characteristics

of breast carcinomas with basal and myoepithelial immunotype (differentiation). The results of this study were correlated with outcome data. The authors were able to identify 2 groups of breast cancers: (1) tumors with basal immunotype that expressed one or both CK5/6 and/or CK14 and (2) carcinomas with myoepithelial immunotype expressing SMA and/or p63. Positivity was defined as the detection of 10% or more of tumor cells positive for CK5/6, CK14, SMA and p63 staining. Multivariate analysis in this study showed that tumors with basal, but not myoepithelial, immunotype has an independent value in predicting outcome, associated with reduced DFS and OS [54]. On the other hand, carcinomas with the combined basal and myoepithelial immunotype showed the shortest DFS and OS [54].

What is missing in the second Nottingham study is a comparison of outcome (DFS and OS) between grade 3 IDCs (NOS type) of nonbasal type with grade 3 BLCs. In other words, this study is unclear about whether nonbasal, but triple-negative, grade 3 carcinomas of NOS type have a different clinical behavior than grade 3 BLCs. It is also of note that the second study used a cutoff of 10% for all basal and myoepithelial markers, whereas in the first Nottingham study any immunoreaction for CK5/6 and/or CK14 was considered positive.

Another issue with the second study [54] is the separation of tumors with basal versus myoepithelial (immuno)phenotype (differentiation) just based on very few immunohistochemical markers. It is well known that normal and neoplastic myoepithelial cells can show positive immunoreaction for some myoepithelial markers, but can also be completely negative for several other established myoepithelial markers [1, 2]. Like salivary glands, depending on functional activity and state of differentiation of myoepithelial cells, the immunoreaction may vary significantly among several established myoepithelial markers such as SMA, smooth muscle myosin, calponin, p63, CD10, maspin, 14-3-3 sigma and CD29 [2]. In the second study, Rakha et al. [54] used IHC for only 2 myoepithelial markers (SMA, p63). Clearly, the negative immunoreaction for these 2 markers, by no means excludes the possibility of myoepithelial differentiation in a given breast cancer. Furthermore, p63 is a marker for both myoepithelial and basal cells (for example, basal cells of skin and prostate) and therefore is not an appropriate marker if one tries to distinguish between carcinomas with myoepithelial cell differentiation and carcinomas with basal-like differentiation.

The third paper (Nottingham study 3), which was published in 2006 [55], focused on a large number of 1,872

invasive breast carcinomas with a long-term follow-up to investigate the clinical significance of BLCs as defined by IHC for CK5/6 and/or CK14 (cutoff 10%). This study confirmed the previous findings that BLCs as a whole were associated with shorter OS and DFS in both LN- and LN+ subgroups.

An important additional finding of the third study [55] was that when tumors were stratified by histological grade, basal phenotype was not of significant prognostic value in grades 1 or 2, indicating that expression of basal-type cytokeratins on its own has no prognostic value. It is of note that the third study, like the 2 previous studies, is unclear on whether grade 3 and triple-negative, nonbasal-like cancers behaved differently from grade 3 BLCs or not.

An IHC study by van de Rijn et al. [56] has found expression of CK17 and CK5 as an independent factor (poor clinical outcome) in breast carcinomas. Using TMA technology, the authors examined 600 breast cancers immunohistochemically. Tumors with even focal and weak immunoreaction for CK17 and/or CK5 were considered basal-like. The authors of this study found that in the group of breast cancer patients with known LN metastases (229 cases), the expression of CK17 and/or CK5/6 had no predictive value. However, in the group of patients without LN metastases (245 patients), CK17 and/or CK5/6 expression was associated with significantly shorter survival. Multivariate analysis on all patients (with and without LN metastases) revealed that the prognostic association of basal cytokeratin expression with poor outcome was not independent from tumor size, LN status and histological grade [56]. Only in patients without LN metastases was the expression of basal cytokeratins identified as an independent prognostic factor [56].

It is of note that van de Rijn et al. [56] did not state in their study what they exactly meant with poor clinical outcome. It is unclear whether they meant reduced OS, reduced DFS or both. Furthermore, it is not clear whether patients with low-grade carcinomas with some positivity for CK17 and/or CK5/6 showed different clinical outcome than those with grade 1 cancers without expressing basal cytokeratins.

In contrast to the Nottingham studies [53–55] that found worse prognosis in BLCs in both LN+ and LN- patients, van de Rijn et al. [56] found a poor prognosis in BLCs only in LN- patients. Moreover, in contrast to the study by van de Rijn et al. [56], Malzahn et al. [57] reported a statistically significant association of basal/myoepithelial keratin expression with poor prognosis only in patients with LN+ patients but not in LN- breast cancer patients.

With regard to the prognosis of BLCs, there are 4 more publications that need to be addressed. In contrast to the above-mentioned studies, all these studies have found that BLC on its own does not convey a poor prognosis.

A study by Jones et al. [37] investigated 43 grade 3 IDCs positive for basal CK14 as well as 43 grade- and age-matched CK14- controls by means of IHC and CGH. CK14 was the only basal cell marker examined in this study. In the cohort of grade 3 carcinomas, CK14 expression was not associated significantly with prognosis. Only a subset of grade 3 IDC with basal immunotype showing certain CGH patterns revealed significantly shorter OS than other grade 3 tumors, indicating that even high-grade BLCs represent a heterogeneous group of breast cancer [37]. In addition, the authors found striking similarities of genetic alterations between BLCs and previously reported myoepithelial carcinomas (carcinomas with myoepithelial differentiation) [58].

In a following study by Fulford et al. [59] from the same group (Breakthrough Breast Cancer Research), 443 grade 3 IDCs were examined by IHC for CK14 and the results were correlated with the established clinicopathological parameters. An important finding of this study which drastically contrasts many other studies was that in patients without metastatic disease, DFS in CK14+ cases was significantly better than in CK14- carcinomas. The OS in CK14+ and CK14- patients was similar at 5 years, but long-term survival was much better in CK14+ patients [59]. Moreover, Fulford et al. [59] reported that both OS and DFS were significantly better for diffuse than for focal CK14 immunostaining. While focally positive tumors had OS and DFS similar to the nonbasal carcinomas, the prognosis for diffuse immunostaining was markedly better [59]. It is of note that CK14 was the only basal cell marker used in the study by Fulford et al. [59] and patients with special-type carcinomas, such as metaplastic carcinoma and medullary carcinoma, were not included in this study.

A recent IHC study performed by Kim et al. [50] analyzed 776 patients with invasive breast carcinoma. Positivity for CK5, CK14 and CK8/18 was defined as detection of at least 1% of malignant tumor cells showing strong cytoplasmic and membranous staining. EGFR (Her1), c-kit (CD117), ER, Her2 and p53 were also evaluated immunohistochemically. Clinicopathological characteristics of breast cancers included age, histological grade, nuclear grade, tumor size, status of nodal metastasis, tumor type, locoregional recurrence and distant metastasis. Histologically, most BLCs were IDCs, NOS type (98 cases, 86%), with high nuclear and/or histologi-

cal grades, and most metaplastic carcinomas (6 of 8 cases) were of the basal-like subtype. All BLCs indentified in this study were of histological grades 2 (22 cases, 19%) or 3 (92 cases, 81%). Both histological and nuclear grades of BLCs were significantly higher than those of other subtypes. On multivariate analysis adjusting other prognostic factors, Her2-overexpressing subtype was the worst subgroup of breast cancers with poorer prognosis than other subtypes, showing poorer prognosis than BLCs. In contrast to earlier findings, no statistically significant survival differences were evident between BLCs and other subgroups except for Her2-overexpressing subtype [50]. It is of note that the cohort of 776 breast cancer patients in this study was exclusively from South Korea, which should be taken into consideration for the analysis of contradictory results obtained in Western countries.

Finally, another recent IHC study by Potemski et al. [51] is of particular interest. The authors examined 195 breast carcinomas and defined BLC as tumors positive for CK5/6 and/or CK17 but negative for ER, PR and Her2. Twenty-five percent of tumors were classified as BLC. Positive immunostaining for CK5/6 and/or CK17 (no cutoff) was associated with worse cancer-specific survival in all examined breast cancer cases and in the node-negative group, but not in the node-positive group. To determine the real prognostic value of basal-like cytokeratins, cancer-specific survival in a group of ER- patients was investigated depending on CK5/6 and/or CK17 expression. Importantly, no influence of basal-type cytokeratins on survival was identified [51]. In multivariate analysis, independent prognostic factors affecting survival in the whole group included nodal involvement, Her2 status and cyclin E expression. Potemski et al. [51] concluded that the poor prognosis associated with BLC is not related to positive immunostaining for CK5/6 and/or CK17, but is determined by ER absence and cyclin E expression.

In summary, with regard to the poor prognosis of BLCs and expression of basal-type cytokeratins in breast cancers as an independent prognostic factor, it is fair to state that the results of current studies are contradictory. The observed discrepancies on the prognostic significance could be due to the lack of uniform IHC definition of BLCs, analytic methods, patient populations and treatment modalities. For future studies it is crucial to investigate and compare age- and grade-matched low-grade basal versus nonbasal as well as high-grade basal versus nonbasal carcinomas. Important independent prognostic factors such as MIB-1 index and immunoreactivity for p53 need to be included in multivariate analysis of both



GEP and IHC studies. Indeed, these 2 prognostic parameters are missing in the vast majority of current studies of BLCs.

It is highly likely that breast carcinomas with high nuclear atypia, high MIB-1 index (or high mitotic activity) and positivity for p53 have a very poor prognosis regardless of expression of basal cytokeratins such as CK5/6, CK14 or CK17. The fact that triple-negative, special-type carcinomas of the breast such as adenoid cystic carcinoma, secretory (juvenile) carcinoma and low-grade metaplastic carcinoma with low-grade nuclear atypia and low mitotic activity express basal cell markers (such as CK5/6, CK14 or CK17, p63) and yet are associated with excellent prognosis clearly indicates that expression of basal (myoepithelial) cell markers by its own does not affect the prognosis in patients with breast cancer.

#### *The Basal-Like Subtype and BRCA1 and BRCA2 Mutations*

Several recent studies have characterized the morphological and immunohistochemical features of breast carcinomas arising in patients harboring germline mutations in the BRCA1 and BRCA2 genes [60–62]. Breast carcinomas in patients with BRCA1 germline mutations are mostly of higher grade, have higher mitotic index, are usually triple negative and show mutations in Tp53 gene compared with aged-matched sporadic breast cancers [60]. A number of studies have demonstrated a strong association between BLCs and BRCA1 germline mutations [60, 61], as this subtype is present in 44–88% of BRCA1-associated breast cancers. However, there is little information on the association of sporadic breast carcinomas with basal-like features (which represent the vast majority of BLCs) and BRCA1 mutations. A recent study by Lakhani et al. [60] investigated 183 breast cancers in BRCA1 mutation carriers, 63 BRCA2 mutation carriers and 109 controls (breast cancers unselected for mutation status). The authors used IHC for 5 basal markers (CK5/6, CK14, CK17, EGFR and osteopontin) and ER in order to develop predictive tests for identification of high-risk patients. In multivariate analysis, CK14, CK5/6 and ER were significant predictors of BRCA carrier status [60]. In contrast, the frequency of all basal markers in BRCA2 cancers was not significantly different from controls. Based on the results of this study, the authors suggested that such information can be used to predict more accurately the probability of carrying a BRCA1 mutation. Accordingly, a screening test based on selecting women who are ER– and CK5/6+, would have a sensitivity in BRCA1 car-

riers of 56% and a specificity of 97%, with a positive predictive value of 28% and a negative predictive value of 99% [60].

Concerning BRCA2, there is no significant association between the BLCs and BRCA2 gene mutations at present. Interestingly, a recent study [62] demonstrated that BRCA2-associated breast carcinomas are predominantly high-grade NOS-type ductal carcinoma of non-basal subtype often showing positivity for ER.

#### *Patient Age and Race*

The average age of patients with BLCs ranged from 47 to 55 years in 3 large IHC studies [29, 50, 63] and was 54 years in 1 GEP study [28]. On the contrary, 2 large population-based studies [28, 29] have found significant differences between the intrinsic subtypes regarding patient's age, with the basal-like subtype having the lowest average age among the classifiable cases in both studies. As apposed to this finding, there were no significant differences between the intrinsic molecular subtypes regarding patient age among the 804 patients that were enrolled in the Polish Breast Cancer Study [63].

One study showed that almost 40% of breast carcinomas in premenopausal African Americans were basal-like, as compared to 14% in postmenopausal African Americans and 16% in non-African Americans (pre- or postmenopausal) [29].

#### *Pattern of Distant Metastasis*

Three recent studies have found an increased rate of brain metastasis for BLCs and BRCA1-related carcinomas [44, 45, 59]. Furthermore, 1 study [59] found BLC to be less likely associated with liver and bone metastases compared with poorly differentiated, nonbasal IDCs (NOS type). One study reported that the likelihood of lung and pleural metastases in basal and nonbasal breast carcinomas was not different [59].

#### *Therapeutic Response*

A few recent studies examined whether the different molecular subtypes of breast carcinoma responded differently to (neo)adjuvant chemotherapy. A study performed by Rouzier et al. [65] examined fine needle aspiration of 82 breast carcinomas obtained before starting preoperative (neoadjuvant) paclitaxel followed by 5-fluorouracil, doxorubicin and cyclophosphamide chemotherapy. Gene expression profiling was done with Affymetrix microarrays and the previously reported intrinsic gene set was used for hierarchical clustering and molecular classification. The authors of this study found that

basal-like and Her2+ subtypes were associated with the highest rates of pathological complete response at 45% each, whereas the luminal subtype cancers had a pathological complete response rate of 6%. However, it is important to point out that the 'molecular class in this study was not independent of conventional clinicopathologic predictors of response such as estrogen receptor status and nuclear grade' [65].

In another study, Sorlie et al. [66] analyzed cDNA expression data from 81 breast carcinomas from 2 patient series, one treated with doxorubicin alone and the other treated with 5-fluorouracil and mitomycin. The authors observed a low frequency of progressive disease within the luminal A subtype from both series and a high frequency of progressive disease among patients with luminal B subtype treated with doxorubicin [66]. However, aside from these 2 observations, no other consistent association between response to chemotherapy and tumor subtype was observed in this study. Using supervised analysis, Sorlie et al. [66] 'could not uncover a gene profile that could reliably (more than 70% accuracy and specificity) predict response to either treatment regimen'.

With regard to the clinical outcome and response to chemotherapy of patients with BLCs, a recent retrospective study by Banerjee et al. [67] analyzed 49 patients with BLC (as defined by CK5, CK14 and CK17) and 49 controls matched for age, nodal status and histological grade. Histological features, status of ER and PR as well as Her2 and clinical outcome (DFS, OS) after adjuvant chemotherapy (anthracycline) were compared between the 2 groups. This study showed that patients with BLC had a significantly higher recurrence rate and were associated with significantly shorter DFS and OS. Furthermore, in the group of patients who received anthracycline-based chemotherapy, both DFS and OS were found to be significantly shorter in the patients with BLC [67]. The authors of this study concluded that BLC is a distinct clinical and pathological entity, with a more aggressive clinical course. The authors also concluded that standard adjuvant chemotherapy seems to be less effective in BLC and new therapeutic modalities are indicated [67]. A critical review of the above-mentioned publication reveals that it suffers from some methodological problems with significant impact on its conclusions. According to the Material and Methods section of this study, all tumors were of grade 3 using the modified Bloom-Richardson-Scarff grading system (Nottingham or Elston-Ellis grading system). Medullary carcinomas and high-grade metaplastic carcinomas were excluded from this

study. Tumors were considered to be positive for ER and PR when nuclear reactivity was observed in more than 10% of tumor cells at any intensity (caveat: this cutoff is no longer used in most breast cancer centers). For CK5, CK14 and CK17, any cytoplasmic expression in neoplastic cells or tissue was considered positive. Most importantly, 'carcinomas with expression of at least one basal cytokeratins were considered to be the basal-like, regardless of the expression of ER, PR, or Her2'. In other words, even triple-positive tumors with very focal immunoreaction for basal cytokeratins were considered BLC. One needs to precisely define characteristic immunohistochemical features of BLC if one claims that poorly differentiated BLC as a distinct pathological and clinical entity has a much more aggressive clinical course compared to high-grade nonbasal and triple-negative carcinoma. It is well known that the vast majority of BLCs are triple negative and this property in conjunction with expression of basal-type cytokeratins distinguishes this subtype from luminal and Her2-overexpressing subtypes. It is likely that Banerjee et al. [67] included some cases of breast carcinomas with ER and/or Her2 positivity as BLC in their study. Therefore, the comparison of the 2 groups of high-grade carcinomas in this study is probably inaccurate and even misleading. According to the definition of BLC, as described in the Material and Methods section of the study by Banerjee et al. [67], a case with grade 3 IDC that shows positivity for CK5/6 in only very few tumor cells, while displaying strong positivity for ER and Her2, would be classified as BLC. On the other hand, a high-grade carcinoma which is completely negative for ER, PR and Her2, but reveals immunoreaction for CK5/6 or CK17 in more than 50% of tumor cells, would also be considered BLC. Obviously, these 2 tumors represent biologically completely different groups and, therefore, cannot be included in the same category of BLC as indicated by the above-mentioned study. Interestingly, a publication of the same group 2 years earlier found that IDC with basal-like immunotype on its own does not convey a poor prognosis [37].

In contrast to the study by Banerjee et al. [67], another recent study [65] showed that basal-like and Her2+ subtypes were more sensitive to anthracycline-based neoadjuvant chemotherapy than luminal A breast carcinomas [68].

With regard to chemotherapy, there is some evidence that patients with LN+ high-grade BLC may benefit significantly more from high-dose adjuvant chemotherapy (high-dose chemotherapy accompanied by autologous peripheral blood progenitor cell transplantation) than

conventional chemotherapy [69, 70]. In the West German Study Group AM-01 Trial, in which 236 node-positive breast cancer patients were randomized into conventional dose-dense and high-dose adjuvant chemotherapeutic arms, patients with basal-like and Her2+ carcinomas that received the high-dose therapy had event-free survival and OS comparable with the luminal A/ER+ group [69]. Patients with BLC that were treated with the conventional chemotherapy had event-free survival and OS that were significantly worse than those whose breast carcinomas were luminal A/ER+ [69]. Currently, no information is available with respect to high-dose chemotherapy (accompanied by autologous peripheral blood progenitor cell transplantation) of node-negative patients with basal-like and Her2+ breast carcinomas.

Finally, it has been suggested, and it is certainly of hope, that BLC may represent a group of breast carcinoma that could benefit from EGFR-targeted therapeutic strategies using either monoclonal antibodies against extracellular domain of the receptor or small inhibitory molecules binding to its intracellular domain (tyrosine kinase inhibitors) [71, 72]. Indeed, EGFR (Her1) is overexpressed by IHC in more than 50% of cases with BLC (including a variety of metaplastic carcinomas and sarcomatoid carcinomas with myoepithelial differentiation) [43, 71–73]. Future studies are needed to show whether determination of EGFR (Her1) by IHC versus in situ hybridization (FISH or CISH) could be used as a reliable method for selection of patients that may benefit from EGFR-targeted therapies.

## Conclusions with Cautionary Notes

The aim of this review was to critically analyze several major publications to find out whether the basal-like subtype represents a distinct clinicopathological entity. A critical survey of the current publications reveals that it is too early to consider this subtype as a distinct entity and the scientific and medical communities need to interpret these studies more cautiously. A thorough review of the literature reveals several limitations and methodological problems of the current studies on this subject [14, 15, 20–23, 74–76]. Due to the major problems/limitations and because of divergent or even contradictory results of the current studies, as discussed in this review, one has to seriously question the common claim of the distinctive nature of this breast cancer subtype.

It is important to emphasize that BLCs do not reflect a single, biologically uniform group of breast carcinomas. Indeed, there is a range of myoepithelial or basal-derived carcinomas with variation in their phenotype, immunoprofile, grades and clinical behavior, just as a wide range of subtypes and behaviors is observed among epithelial/luminal-derived breast carcinomas. It is highly likely that a poorly differentiated, triple-negative BLC shows similar aggressive clinical behavior as that of a poorly differentiated, triple-negative but nonbasal breast carcinoma. In other words, the expression of basal-type cytokeratins in a given breast cancer, per se, does not have an impact on the clinical course in patients with BLC. As a subtype of breast carcinoma, however, it is of hope that at least some patients with BLC could benefit from EGFR-targeted therapies and/or certain types of chemotherapy.

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