

Review

Cancer Hallmarks, Biomarkers and Breast Cancer Molecular Subtypes

Xiaofeng Dai[✉], Liangjian Xiang, Ting Li, Zhonghu Bai

National Engineering Laboratory for Cereal Fermentation Technology, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu, P.R.China.

✉ Corresponding author: Xiaofeng Dai, PhD, National Engineering Laboratory for Cereal Fermentation Technology, School of Biotechnology, Jiangnan University, No. 1800, Lihu Avenue, Wuxi 214122, P.R.China. E-mail: Xiaofeng.dai@me.com, Tel.: +86-18611479958, Fax: +86-0510-85329306.

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Abstract

Breast cancer is a complex disease encompassing multiple tumor entities, each characterized by distinct morphology, behavior and clinical implications. Besides estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2, novel biomarkers have shown their prognostic and predictive values, complicating our understanding towards to the heterogeneity of such cancers. Ten cancer hallmarks have been proposed by Weinberg to characterize cancer and its carcinogenesis. By reviewing biomarkers and breast cancer molecular subtypes, we propose that the divergent outcome observed from patients stratified by hormone status are driven by different cancer hallmarks. 'Sustaining proliferative signaling' further differentiates cancers with positive hormone receptors. 'Activating invasion and metastasis' and 'evading immune destruction' drive the differentiation of triple negative breast cancers. 'Resisting cell death', 'genome instability and mutation' and 'deregulating cellular energetics' refine breast cancer classification with their predictive values. 'Evading growth suppressors', 'enabling replicative immortality', 'inducing angiogenesis' and 'tumor-promoting inflammation' have not been involved in breast cancer classification which need more focus in the future biomarker-related research. This review novels in its global view on breast cancer heterogeneity, which clarifies many confusions in this field and contributes to precision medicine.

Key words: cancer hallmarks, biomarker, breast cancer, subtype.

Introduction

Breast cancer is the most common neoplasm among women in the majority of the developed countries, accounting for one-third of newly diagnosed malignancies [1]. It is a highly heterogeneous disease, encompassing a number of biologically distinct entities with specific pathologic features and biological behaviors [1, 2]. Different breast tumor subtypes have different risk factors, clinical presentation, histopathological features, outcome, and response to systemic therapies [3-8]. Thus, stratification of breast cancer by clinically relevant subtypes is urgently required.

Immunohistochemistry (IHC) markers, together with clinicopathological variables such as tumor size, tumor grade, nodal involvement, histologic type, and

surgical margins, have been widely used for prognosis, prediction and treatment selection [9, 10]. Back to 1970s, breast cancer was divided into two subtypes according to the status of estrogen receptor (ER). With the advent of new technologies and incremental understanding of the complex tumorigenesis progress, new biomarkers and novel subtypes have been kept identified. This, on one hand, helps us in more accurate disease management, but, on the other hand, complicates our understanding towards breast cancer heterogeneity.

In 2000, Weinberg et al. have reported six hallmarks of cancer, i.e., 'sustaining proliferative signaling', 'evading growth suppressors', 'resisting cell death', 'enabling replicative immortality',

'inducing angiogenesis', and 'activating invasion and metastasis' [11]. The same authors have identified two emerging hallmarks, i.e., 'reprogramming of energy metabolism' and 'evading immune destruction', in 2011, and pointed out that all these hallmarks are enabled by two characteristics, i.e., 'genome instability and mutation' and 'tumor-promoting inflammation' [12]. As tumors are not conceivable as a single disease, breast cancer with different diagnostic features should differ in the hallmarks controlling their clinical differences. This review aims at identifying these dominant hallmarks driving breast cancer heterogeneity by focusing on identified biomarkers and the associated subtypes.

Hallmark 1: Sustaining proliferative signaling

Hormonal and growth receptors define basic breast tumor molecular subtypes

IHC markers including ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are classically used for breast tumor subtyping [13]. Experiments testing these markers have been routinely carried out in pathology laboratories, with staining and evaluation protocols well established worldwide [1]. These hormonal and growth receptors are known to mediate cell growth signaling. For instance, estrogen promotes the development of breast cancer and stimulates the growth *in vitro* of breast cancer cell lines having the corresponding receptors [14, 15]. Breast tumors are grouped into four basic subgroups according to these markers, i.e., [ER+|PR+]HER2- (tumors with either ER or PR positivity, and HER2 negativity), [ER+|PR+]HER2+ (tumors with either ER or PR positivity, and HER2 positivity),

ER-PR-HER2+ (tumors with ER and PR negativity, and HER2 positivity, also named HER2 positive), ER-PR-HER2- (tumors with ER, PR, HER2 negativity, also named triple negative). In this naming system, hormonal receptors (HR) are shown in the square brackets, '|' represents 'or', and '+/-' shows the receptor status [1]. In general, ER-PR- tumors (tumors with both ER and PR negativity) have relatively poorer prognosis than [ER+|PR+] cancers (tumors with either ER or PR positivity).

ER

ER is the most important and prevalent biomarker for breast cancer classification. It was first identified in the 1960s and used in breast cancer clinical management since mid-1970s as a primary indicator of endocrine responsiveness and a prognostic factor for early recurrence [16]. ER plays

crucial roles in breast carcinogenesis, whose inhibition forms the mainstay of breast cancer endocrine therapy. ER status has been shown to be the major determinant of breast cancer molecular portraits by recent gene expression profiling (GEP) studies [17-21]. It is comprised in the UK minimum data set for histopathology reporting of invasive breast cancer and routinely determined by a standardized technique [22].

ER positive tumors comprise up to 75% of all breast cancer patients, and constitute 65% and 80%, respectively, patients under and above 50 years [23]. ER positive tumors are largely well-differentiated, less aggressive, and associated with better outcome after surgery [24] than ER-negative ones [25]. Though ER alone provides limited prognostic value given the little difference on patients' long-term survival stratified by its status [26], it has been considered as the most powerful single predictive factor identified in breast cancer [20, 26-28]. In general, ER negative tumors are unlikely to respond to endocrine therapy, and approximately 50% ER positive patients are responsive to anti-estrogen or aromatase inhibitors [29]. A small proportion of ER negative tumors are documented to respond to hormonal therapy [30, 31]. Breast tumors differing in ER status are fundamentally different at the transcriptional level [17-19, 32], complexity of genetic aberrations [33-35], as well as the pathways and networks [17, 35, 36]. Besides ER, the importance of other biomarkers have been continuously reported in breast tumor subtyping with respect to their risk factors, clinical and biological behaviors [7, 29, 31].

PR

PR is induced by endocrine, whose activation suggests an active ER signaling [37-40]. PR positive tumors comprise 65% to 75% breast cancers and several studies have suggested its clinical implications in the classification of such tumors [41-46]. However, its classification role has been questioned by several researchers due to the lack of evidence supporting its predictive role over ER on endocrine therapeutic response [26, 29, 47]. PR positive tumors are hardly ER negative [47], i.e., 0.2% to 10% depending on the detection methods [16, 48-52]. Thus, strong PR positivity in an ER negative case may indicate a false discovery on ER negativity, which is commonly encountered in routine practice [50]. As demonstrated by Dowsett et al. [31], ER-PR+ patients benefit from endocrine therapy which would be excluded from such treatment if the decision was based on ER status alone. Approximately 40% ER positive tumors are PR negative [53]. ER+PR- tumors are less responsive to endocrine treatment than ER+PR+ tumors [53-56],

particularly for metastatic tumors under tamoxifen treatment [44, 45]. Lacking PR expression in ER positive tumors may suggest aberrant growth factor signaling that, in turn, contributes to tamoxifen resistance of such tumors [53-56]. PR is conventionally used together with ER in breast tumor subtyping, i.e., ER+PR+, ER+PR-, ER-PR+, ER-PR- are classified. The double positive group (ER+PR+) comprises 55% to 65% of breast tumors [24, 53, 57], among which 75% to 85% are responsive to endocrine treatment [31]. Compared with the other subgroups, patients of these tumors are associated with older age, lower grade, smaller tumor size and lower mortality rate. The double negative group (ER-PR-) comprises 18% to 25% of the tumors, among which around 85% are of grade 3. These tumors are associated with a higher recurrence rate, lower overall survival and do not respond to endocrine therapy [44, 45, 54, 57-59]. Tumors with concurrent negativity of ER and PR have, in general, good response to preoperative taxane/anthracycline-based regimen. Lots of evidences indicate that tumors of this class are highly heterogeneous [7, 60], which could be sub-divided into many groups based on the status of other markers such as HER2 [19, 20]. The single positive phenotype is consist of ER+PR- and ER-PR+ tumors, each accounting for 12% to 17% [24, 53, 57] and 0.2% to 10% breast cancer patients. Compared with double positive tumors, these cancers are more often of higher histological grade, larger tumor size, and are more likely to be aneuploidy and show higher expression of proliferation-related genes such as epidermal growth factor receptor (EGFR) and HER2 [53, 55]. Tumors with single hormone receptor positivity respond less well to endocrine treatment than those harboring double positive receptors [44, 54, 61], with only 40% responding to hormonal manipulation [31]. The single positive group is reported to show biological features somewhere in between the double positive and double negative groups [53, 62, 63].

Instead of using the binary representation, i.e., positive and negative, to define the receptor status in subtyping, the expression levels of ER and PR have been used to predict tumors' response to endocrine therapy [64]. Two categories of ER+PR+ tumors are reported, i.e., tumors over-expressing both ER and PR (ER > 50% and PR > 50%) and tumors expressing low levels of either or both receptors (10% < ER < 50% or PR < 50%), where the first category is highly sensitive to hormone treatment and the second is incompletely endocrine responsive [64]. The ER-/PR- group (ER < 10% and PR < 10%), on the other hand, is not shown to be beneficial from endocrine therapy [64]. A meta-analysis shows that the benefit of women from 5

years' tamoxifen treatment is proportional to the level of ER [29]. Further, Stendahl et al. recommend the use of a fractioned rather than dichotomized immunohistochemical evaluation of both ER and PR in the clinical practice [42].

Taken together, joint ER, PR assessment differentiates breast cancer variants better than using either one alone. Breast cancer subtypes classified by the two receptors can be ordered by ER+PR+, ER+PR-, ER-PR+, ER-PR-, with ER+PR+ being the most favorable and ER-PR- the most aggressive cancers regarding tumor size, grade, stage, patient outcome and response to hormonal therapies [24, 44, 45, 53-57, 65].

HER2

The clinical implications of HER2 amplification have been recognized since 1987 [66]. Numerous subsequent studies have revealed that HER2 gene amplification or protein over-expression is associated with poor prognosis and good clinical outcome receiving systemic chemotherapy treatment [67-69]. The protein over-expression and gene amplification of HER2 occur in 13% to 20% of invasive ductal breast cancer, more than half of which (around 55%) are ER-PR- [66, 70, 71]. The prognostic value of HER2 positivity is higher in node-positive than node-negative patients. Examining HER2 status has been established as a routine clinical practice before applying trastuzumab to advanced tumors or adjuvant treatment to potential HER2 positive early stage patients [72, 73]. Its predictive value on the outcome receiving anthracycline-based chemotherapy has been reported, with HER2 positivity being associated with favorable drug response [74-77]. It has also been suggested that HER2 positivity is predictive of better response to higher dose of anthracycline related regimens [78, 79], and to regimens containing taxane than those do not [80, 81]. Besides, HER2 positivity is associated with relative but not absolute resistance to endocrine therapies [82], which is consistent with the inverse relationship of HER2 and ER/PR at the expression level [71]. Note that such resistance does not apply to estrogen depletion therapies such as aromatase inhibitors [83, 84]. Despite the aforementioned treatments and strategies, HER2 is an important target of a variety of novel cancer therapies, including vaccines and drug lapatinib which is directed at the internal tyrosine kinase portion of HER2 protein.

A combination of various IHC markers including ER, PR and HER2, with or without additional markers such as basal and proliferation markers, has been used to define breast tumor subtypes, where the statuses of ER, PR and HER2

have been considered as the most important features. Using the dichotomized immunohistochemical evaluation of these three receptors, breast tumors could be classified into [ER+|PR+]HER2-, [ER+|PR+]HER2+, ER-PR-HER2+, and ER-PR-HER2-. [ER+|PR+]HER2- and [ER+|PR+]HER2+ are similar to luminal A and luminal B tumors defined by GEP nomenclature [85-89]. However, no unanimous consensus has been reached on such conversion between IHC and GEP classification. In Nielsen's study, all HER2+ cases ([ER+|PR+]HER2+, ER-PR-HER2+) are grouped as HER2 positive subclass [90], based on the evidence that HER2-amplified cases share similar genetic changes [91] and outcome [9, 18, 85] regardless of their hormonal statuses.

Preclinical and clinical data suggest that HER2 over-expression confers intrinsic resistance to hormonal treatment in ER+ or PR+ tumors, indicating that [ER+|PR+]HER2+ tumors may not benefit much from single-agent hormone therapy. Results from randomized clinical trials combining hormone treatment and targeted anti-HER2 therapy in [ER+|PR+]HER2+ postmenopausal patients indicate that this novel dual-targeting strategy could significantly improve patient outcome [92]. Konecny et al. find that HER2 expression is inversely correlated with that of ER, and suggest that the relative resistance of [ER+|PR+]HER2+ tumors to hormone therapy as compared with the [ER+|PR+]HER2-subtype is due to reduced ER or PR expression or high proliferation rates rather than HER2 positivity [82]. Other studies suggest that [ER+|PR+]HER2+ breast cancer might benefit more from anti-HER2 therapy plus chemotherapy [93]. It is reported that [ER+|PR+]HER2+ tumors have a good prognosis irrespective of the achievement of a pathological complete response (defined as the absence of any residual invasive cancer at the breast site and at the nearest axillary lymph node site [94]), whereas patients with ER-PR-HER2+ and ER-PR-HER2-tumors show the worst prognosis [95]. Hayes et al. have demonstrated that HER2+ tumors benefit from the addition of paclitaxel after adjuvant treatment with doxorubicin plus cyclophosphamide in node-positive breast cancer regardless of ER status, while ER+HER2- tumors gain little benefit from such treatment [81].

All current evidences indicate that [ER+|PR+]HER2- tumors have the best prognosis and response to hormone therapy. ER-PR-HER2+ and ER-PR-HER2- tumors are poorly differentiated, show aggressive behavior and poor outcome, and are least likely to respond to hormone therapy.

AR

Besides ER, PR and HER2, androgen receptor (AR) has also been used in breast cancer subtyping. AR is the prevalent sex steroid hormone receptor expressed in 90% ER positive and 55% ER negative tumors [96, 97]. It is a potential prognostic marker and therapeutic target in breast cancer. It seems to play a similar role as HER2. Lakes et al. have classified ER-PR- tumors into ER-PR-AR+ (molecular apocrine, abbreviated as MAC) and hormone receptor negative carcinomas (ER-PR-AR-) [32, 98], and a considerable overlap is observed between ER-PR-HER2+ and MAC tumors [98]. MAC accounts for 13.2% of all breast cancer cases and is often characterized by KI67+ [98]. Despite the higher risk of ER-PR- tumors regarding patient relapse and death, MAC tumors have a favorable outcome comparable with [ER+|PR+] tumors [32, 98]. Also, patients with MAC tumors have a favorable outcome on treatment containing taxane [32, 98].

Taken together, the classic breast tumor molecular subtypes are defined by hormonal and growth receptors according to the most prominent cancer hallmark, i.e., 'sustaining proliferative signaling'. With the decreasing response to proliferative signals, breast tumors exhibit increasing aggressiveness and decreasing number of available targeted therapy. Among the three hormonal receptors (ER, PR, AR) and the growth receptor (HER2), ER plays a determinant role on differentiating breast tumors regarding their proliferation ability (corresponding to the 'sustaining proliferative signaling'), while PR and AR exhibit a similar role with ER and HER2, respectively.

Proliferation markers deteriorate [ER+|PR+]HER2- tumors

More directly than hormonal receptors, proliferation markers have been used in breast tumor classification, especially among [ER+|PR+]HER2-tumors. It has been widely acknowledged that increased cell proliferation is a key determinant of clinical outcome among breast cancer patients [99, 100]. Chemotherapy agents including CMF (cyclophosphamide, methotrexate, 5-fluorouracil), taxanes and anthracycline-based treatment all affect cell division or DNA synthesis. Thus, concurrent assessment of proliferation and conventional IHC markers provides additional predictive value and more precise clinical implications than using IHC alone. Worth noting that proliferation markers are informative in further differentiating HR positive tumors and of limited value in ER-PR-HER2- or HER2 positive tumor classification [88].

KI67

The most widely used proliferation marker in breast cancer is KI67, which is predominantly present in cycling cells [101]. KI67 has been used to predict the neoadjuvant response [102-106] or outcome from adjuvant chemotherapy (endocrine therapy for ER positive tumors) for breast cancer. It has also been used in combination with other markers in breast cancer to provide prognostic and predictive values [9, 105, 107]. Chang et al. have used KI67 in addition to ER, PR and HER2 to classify breast tumors, where [ER+|PR+] tumors are divided into three prognostically distinct subclasses based on the expression of KI67 and HER2 [9]. In their study, [ER+|PR+]HER2- tumors are classified into [ER+|PR+]HER2-KI67- and [ER+|PR+]HER2-KI67+ tumors, respectively, with [ER+|PR+]HER2-KI67+ being associated with poorer outcome regardless of systemic therapy [9]. This accords with the hallmark of cancer on 'sustaining proliferative signaling', with the more propensity on cancer cell proliferation the poorer patient clinical outcome. This classification subdivides the intrinsic luminal B tumors (as will be described in the next section) into two groups, i.e., [ER+|PR+]HER2-KI67+ and [ER+|PR+]HER2+KI67+, demonstrating the importance of the combined use of KI67 with ER, PR, HER2 in differentiating tumors with positive hormone receptors. Further, the joint use of these four markers has been shown to provide as much information as some expensive molecular assays in breast cancer subtyping [92].

TOP2A

Topoisomerase II alpha (TOP2A) catalyzes the breakage and reunion of double-stranded DNA, and thus leads to relaxation of DNA supercoils [108]. It plays crucial roles in a number of fundamental nuclear processes including DNA replication, transcription, chromosome structure, condensation and segregation [108], thus heavily affects cell proliferation. TOP2A expression is found correlated with that of KI67 [109, 110]. TOP2A aberration is frequently found in HER2-amplified breast cancer, accounting for approximately 30%-90% of such tumors. It has been suggested as a potential biomarker with aberrations being associated with increased responsiveness to anthracycline-based chemotherapy [108, 111-113].

Cell cycle genes

Aside from KI67, other proliferation markers have also demonstrated their importance in differentiating [ER+|PR+] tumors. Cell cycle genes are known to be associated with proliferation, whose over-expression is prognostic of poor clinical

outcome. Loi et al. have identified a 97-gene signature, mostly comprising genes involved in cell cycle regulation and proliferation [114]. Using these genes they classified [ER+|PR+] tumors into two groups significantly differ in prognosis despite whether or not tamoxifen is given [115]. Perou et al. have reported that a cluster of genes, whose expression considerably vary among subtypes, are correlated with cellular proliferation rates [18, 87].

Taken together, over-expression of proliferation markers tend to accelerate the hallmark of 'sustaining proliferative signaling' among [ER+|PR+]HER2-tumors, leading to worse clinical outcome.

Hallmark 2: activating invasion and metastasis

Basal markers deteriorate ER-PR-HER2-tumors

ER-PR-HER2- tumors are undeniably one of the most relevant subtypes among breast tumors given the lack of targeted therapies and their aggressive clinical behavior. These patients can be clustered into at least two distinct molecular classes, i.e., the basal phenotype and non-basal ER-PR-HER2- tumors [85, 89, 90, 116-119], which differ in their behavior, outcome and therapeutic response. An expanding number of basal IHC markers have been used to define the basal tumors, among which cytokeratins (CK) 5/6, 14, 17, 8/18, EGFR are the most widely accepted [13, 85, 89, 90, 118-123]. Various combinations of these basal markers have been used to identify the basal subtype. The most pragmatic and widely accepted definition of the basal subtype is ER-PR-HER2- tumors with positive expression of CK5/6 and EGFR [85, 123]. Rakha et al. have proposed the use of CK5/6, CK14, CK17 and EGFR in characterizing basal tumors from ER-PR-HER2-cancers [118]. Matos et al., have reported the combined assess of P-cadherin, TP63 and CK5 in distinguishing the basal subtype from ER-PR-HER2-tumors via immunoprofiling [89]. The basal subtype has also been identified from double negative (ER-HER2-) tumors using basal markers. Nielsen et al. have used CK5/6 and EGFR to identify basal tumors from hormone receptor negative cancers regardless of PR status [90]. In addition to CK5/6 and EGFR, Livasy et al. have added CK8/18 and vimentin (VIM) in their panel to characterize such tumors within the ER-HER2- group [124]. Cytokeratins alone have been used to identify the basal subtype. For instance, CK5/6 and CK14 have been jointly assessed to identify the basal subtype [120], and CK14 alone is reported to define a proportion of breast tumors carrying morphological features strongly associated

with basal carcinoma [13]. The basal markers used for identifying the basal subgroup from ER-PR-HER2-breast tumors in various publications are summarized in Table 1.

Despite the various inclusions of basal markers in triple negative breast tumor classification, markers of this class are associated with the cancer hallmark of 'invasion and metastasis'. Recent studies have shown that cytokeratins, P-cadherin and vimentin are closely linked with tumorigenesis and metastasis. Cytokeratins are proteins of keratin-containing intermediate filaments in the intracytoplasmic cytoskeleton of epithelial tissue. Vimentin is the major intermediate filament protein of mesenchymal cells [125]. It regulates the interaction between cytoskeletal proteins (including cytokeratins) and cell adhesion molecules (such as P-cadherin), and thereby participates in cell adhesion, migration, invasion and cell signal transduction in tumor cells [125]. TP63 has been reported to play a tumor suppressive role in cancer metastasis [126].

The basal tumors, accounting for 10% to 25% of breast cancers [127, 128], have a worse prognosis than the other ER-PR-HER2- breast tumors. These two subtypes have distinct molecular and biological characteristics and differ in their response to neoadjuvant chemotherapy [129, 130]. Thus, accurate identification of basal tumors from the ER-PR-HER2-subtype is required for precise therapeutic strategy making. It has been suggested that tumors expressing more than one basal keratin are more likely to have a dysfunctional BRCA1 pathway [131]. Consistent with this, several other studies have also suggested the

predictive value of basal keratins on BRCA1 mutation [132, 133]. Preclinical models of tumors with dysfunctional BRCA1 have been shown to be exclusively sensitive to cross-linking agents and inhibitors of the poly (ADP-ribose) polymerase [134], indicating the efficient therapeutic treatment of tumors of this class.

Though basal markers are primarily expressed among ER-PR-HER2- tumors and, particularly, the basal subtype, a small percentage of tumors with hormonal receptor positivity also exhibit basal marker expression (accounting 1% to 18% [ER+|PR+] tumors). This has led to the question that whether these patients belong to the luminal or basal tumors, and how the corresponding treatment should be given. It has been observed that [ER+|PR+] tumors with basal marker expression exhibit a poorer prognosis than the conventional luminal tumors [16]. This may suggest a potential link between luminal B and basal breast tumors [19, 20]. Aside from this, basal markers are also reported to be present in a small proportion of HER2 positive tumors, which are less responsive to Herceptin treatment than the conventional HER2 positive tumors [135].

The poor prognosis of triple negative tumors is associated with the 'activating invasion and metastasis' hallmark. The ambiguities exhibited in tumor classification when basal markers are included suggest that breast tumors, regardless of which subtype they belong to, once harboring the 'activating invasion and metastasis' hallmark, exhibit either poorer prognosis (luminal tumors with basal markers) or drug resistance (HER2 positive tumors with basal markers).

Table 1. Different immunohistochemical marker combinations used to define the basal phenotype in various publications.

Journal article	Immunohistochemical marker										
	ER	PR	HER2	CK5/6	CK14	CK17	CK8/18	EGFR	Vimentin	P-cadherin	TP-63
Carey et al., 2006[85]	-	-	-	+/- ^a				+/- ^a			
Cheang et al., 2008[123]	-	-	-	+/- ^a				+/- ^a			
Rakha et al., 2009[184]	-	-	-	+/- ^a	+/- ^a	+/- ^a		+/- ^a			
Matos et al., 2005[89]				+					+	+	
Nielsen et al., 2004[90]	-		-	+/- ^a				+/- ^a			
Livasy et al., 2006 [124]	-		-	+			+	+	+		
Rakha et al., 2007[120]				+/- ^a	+/- ^a						
Fulford et al., 2006[13]					+						

^aPositivity for at least one of the highlighted markers.

EMT, Stem cell markers deteriorate ER-PR-HER2-tumors

Epithelial to mesenchymal transition (EMT) is a reversible biological process that involves the transition from motile, multipolar or spindle-shaped mesenchymal cells to planar arrays of polarized cells called epithelia. EMT is a necessary process for metastasis. Markers of EMT primarily include VIM, SNAI1, SNAI2, TWIST1, TWIST2, ZEB1, ZEB2, CDH1, CLDN3 (claudin 3), CLDN4 (claudin 4), CLDN7 (claudin 7) [136, 137]. Molecules conventionally considered as stem cell markers include CD44, CD24, EpCAM, CD10, CD49, CD29,

MUC1, THY1 and ALDH1A1 [138]. Aided by these markers, two groups of breast tumors, i.e., claudin-low and metaplastic breast cancer (MBC), could be further differentiated from ER-PR-HER2-tumors. Both subtypes share many similarities regarding tumor characteristics, genomic aberrations as well as drug response and clinical outcome. They are characterized by low expression of GATA3-regulated and cell-cell adhesion genes, enriched for EMT markers, and displaying stem cell characteristics [139, 140]. It is claimed that claudin-low and MBCs may arise from a more primitive cell than the precursor to luminal or basal tumors, and define a novel chemo-resistant ER-PR-HER2- disease exhibiting a signature similar to that of breast tumor-initiating cells [139]. Though both breast tumor subtypes share a significant similarity regarding the EMT and stem cell markers, MBC breast cancer are distinct from claudin-low tumors by harboring PIK3CA, AKT or KRAS mutations [139]. For example, PIK3CA mutations are detected in 47.4% MBCs while no such mutation is found among claudin-low tumors [139]. Also important is that these subtypes are not mutually exclusive, with a small proportion overlapping [139].

Claudin-low tumors are ER-PR-HER2- tumors characterized by low gene expression of tight junction proteins claudins 3, 4 and 7 as well as the calcium-dependent cell-cell adhesion glycoprotein CDH1 [141]. These tumors account for around 7% to 14% of breast tumors [141]. Molecular characterization of the claudin-low subtype reveals that they are enriched for EMT, stem cell-like and tumor initiating cell (TIC) genomic signatures, lack of common epithelial cell features and show low expression of luminal and proliferation-associated genes [141]. Though claudin-low cancers show some chemotherapy sensitivity, patients harboring these tumors suffer from poor overall survival [141].

MBC are aggressive ER-PR-HER2- tumors characterized by the co-existence of carcinoma with non-epithelial cellular elements [142], which accounts for 1% of breast cancer [143]. Tumors of this subtype are aggressive, chemoresistant, and associated with poor outcome [143]. MBC can be further classified into homogeneous spindle cell/sarcomatoid carcinoma, heterogeneous carcinosarcoma/carcinoma with sarcomatous differentiation (osseous, chondroid and rhabdoid), and pure epithelial malignant tumors with metaplasia such as adenosquamous and pure squamous cell carcinomas [144]. Many pathologic and clinical parameters of MBCs are distinct from the other breast tumors. The incidence of nodal involvement among MBC ranges from 6% to 26%, which is less frequent than typical breast tumors

[145-147]. These tumors tend to be present in an advanced stage, have a propensity for local recurrence and metastasize [143]. The recurrence rate for node-negative MBC ranges from 45% to 62% within 2 to 5 years from the initial diagnosis, which is much higher than the 17% to 20% recurrence rate for invasive ductal carcinoma of comparable tumor size [148]. As contrary to the basal tumors where neoadjuvant chemotherapy is typically associated with high pathological complete response rate, MBCs rarely benefit from such a treatment [149]. The stem cell features and frequent genomic aberrations activating the phosphatidylinositol 3-kinase (PI3K)/AKT signaling may suggest the source of MBC chemoresistance [139]. Accordingly, the PI3K/AKT pathway has been suggested as a therapeutic target in MBC [139]. Further, EMT and stem cell-like features are likely to contribute to the poor outcomes of these tumors and suggest novel therapeutic strategies [139].

Taken together, basal, EMT or stem cell markers, which represent the properties of the 'activating invasion and metastasis' hallmark, are more likely to be enriched in triple negative tumors. The more markers as such are enriched, the more aggressive the tumors are.

Hallmark 3: Evading immune destruction

Immune response genes rescue ER-PR-HER2-tumors

The interferon-rich subtype is recently identified from ER-PR-HER2- tumors, which is characterized by the over-expression of interferon-regulated genes [93, 150]. These tumors account for approximately 10% of breast tumor cases [93]. Among the interferon-regulated genes differentiating tumors of this subtype from the other ER-PR-HER2- cancers, STAT1 and SP110 are of the most importance, where STAT1 is the transcription factor mediating interferon-regulated gene expression [93], and SP110 is reported to have the prognostic value [150]. The relapse free survival of interferon-rich breast tumors is somewhere between the basal cancers (comparable with ER-PR-HER2+) and luminal A tumors, and is comparable with luminal B tumors [93], suggesting that the easier tumors fire the immune system the better outcome the patients show, and the more effective the appropriate therapeutic strategy might achieve.

Markers representing these three cancer hallmarks contribute to the current breast tumor classification. All conventional subtypes are summarized in Table 2.

Hallmark 4: Resisting cell death

BCL2 shows dual roles on tumor outcome prognosis and prediction

The protein BCL2 is a suppressor of apoptosis, which has been verified in a variety of *in vitro* and *in vivo* experiments [151-153]. Its expression is shown to be inversely correlated with that of TP53, and its function could be substituted by TP53 mutation [154]. The prognostic value of BCL2 has been investigated by many studies [155, 156]. It is found that moderate to strong BCL2 expression (abbreviated as BCL2+ tumors) is intensely associated with several favorable prognostic features, such as low mitotic count, low S-phase fraction size, low cathepsin D expression, high histological grade of differentiation, as well as lacking p53 expression and tumor necrosis [155]. Also, patients with BCL2+ tumors have a more favorable short-term but similar long-term breast cancer specific death as compared with those carrying BCL2- tumors [155]. Early studies report that BCL2 expression is associated with low-grade slowly proliferating ER+ breast tumors [157, 158], and the improved survival associated with such tumors is attributed to its correlation with ER status [159-161]. Several recent projects have suggested that BCL2 is a clinically valid and powerful prognostic marker for all types of early-stage breast cancer, independent of ER, HER2 and adjuvant therapy received [156, 162]; and its strong correlation with hormonal receptor might contribute to the superior survival observed for BCL2+ breast patients [156]. The predictive value of BCL2 is reported for ER-PR-HER2- breast tumors, with ER-PR-HER2-BCL2- patients found beneficial from anthracycline-based regimen [163]. These indicate that 'resisting cell death' is not a determinant

factor for breast tumors to be aggressive but is important for triple negative tumors to develop anthracycline resistance.

Hallmark 5: Genome instability and mutation

TP53 dysfunction increases tumor drug resistance

The tumor suppressor TP53 plays a critical role in many cellular signaling controlling cell proliferation, survival, apoptosis and, most importantly, genomic integrity [154, 164]. It acts as a gatekeeper of the genome when cells experience stress conditions such as DNA damage, hypoxia and oncogene activation. Thus, TP53 deficiency may lead to uncontrolled proliferation of damaged cells as the genomic stability is hampered which leads to a faster mutation speed. Approximately 25% to 30% tumors have a mutation on TP53 [165-167], which has been reported as an important prognostic marker in breast cancer independent of tumor size, node status and hormone receptor content [164]. An interaction between TP53 and PR is revealed, where TP53-PR-tumors are found associated with the worst prognosis among all breast cancers [164]. It has been shown that p53 mutation adversely affects breast cancer response to tamoxifen [164]. Increasing evidences have suggested that TP53 dysfunction is responsible for the development of anti-oestrogen resistance among ER+ tumors [168, 169], and ER-TP53- tumors may suffer from chemotherapy treatment failure [165, 170, 171]. These evidences altogether suggest that 'genome instability and mutation' contributes to tumor drug resistance regardless of which subtype it belongs to.

Table 2. Summary of the breast tumor molecular subtypes.

Subtype	Alias	Biomarker status	Grade	Outcome	Additional features	Prevalence ^Δ
Luminal	Luminal A*	[ER+ PR+]HER2-KI67-	1 2	Good	Luminal cytokeratin+, FOXA1+, ADH1B high; cell-cell adhesion genes high	23.7% ^[9]
	Luminal B*	[ER+ PR+]HER2-KI67+ [ER+ PR+]HER2+KI67+	2 3	Intermediate Poor	Luminal cytokeratin+; TP53-; FGFR1 and ZIC3 amp; ADH1B low; cell-cell adhesion genes high	38.8% ^[9] 14% ^[9]
HER2 positive	HER2 over-expression*	ER-PR-HER2+	2 3	Poor	TP53-; GRB7 high; cell-cell adhesion genes high	11.2% ^[9]
Triple negative	Basal*	ER-PR-HER2-, basal marker+	3	Poor	BRCA1-, TP53-; CDKN2A high; RB1 low; FGFR2 amp; cell-cell adhesion genes high	10-25% ^[1] 12.3% ^[9] ^{28]}
	Claudin-low	ER-PR-HER2-, EMT marker+, Stem-cell marker+, claudin-	3	Poor	GATA3-regulated genes, cell-cell adhesion genes low; CDH1 low; Claudins low	7-14% ^[14] 1
	Metaplastic breast cancer (MBC)	ER-PR-HER2-, EMT marker+, Stem-cell marker+	3	Poor	GATA3-regulated genes, cell-cell adhesion genes low; PIK3CA-, AKT- or KRAS-	1% ^[143]
	Interferon-rich	ER-PR-HER2-, interferon regulated genes+	3	Intermediate	STAT1, SP110 high	~10% ^[93]
Molecular apocrine cancer (MAC)	Molecular apocrine cancer (MAC)	ER-PR-AR+	2 3	Poor	KI67+	13.2% ^[98]

*Subtypes with detailed expression patterns and clinical implications discussed in the text, which take the majority of the breast tumor cases and are most commonly referred to.

^Δ The percentages could not be added up, as triple negative tumor subtypes are not mutually exclusive and the percentages are taken from different publications.

^Δ The prevalences shown here are for all breast tumor cases, which are taken from one particular publication (as indicated in the square brackets) and can vary by different studies.

Hallmark 6: Deregulating cellular energetics

VRD provides novel view on breast tumor classification

Higher level of circulating vitamin D metabolites is shown to be associated with decreased breast cancer risk [172-175], and the statuses of vitamin D receptor (VRD), AR and ER are known to be correlated with tumor differentiation state [176]. Santagata et al. [176] have proposed a novel breast tumor classification method jointly assessing the statuses of these three receptors, according to which breast carcinomas could be quantitatively classified into 4 categories, i.e., HR3 (ER+AR+VDR+), HR2 (ER+AR+, AR+VDR+, ER+VDR+), HR1 (ER+, VDR+, AR+), and HR0 (ER-AR-VDR-). This classification differs from the conventional ER-PR-HER2 based grouping. For example, in the samples collected by Santagata [176]: among ER+ tumors, 75.1% are HR3, 23.4% are HR2, 1.5% are HR1; for HER2+ tumors, 29.4% are HR3, 43.5% are HR2, 22% are HR1 and 5.1% are HR0; and of TNP tumors, 36.5% are HR0, 44.6% are HR1 and 18.6% are HR2. Tumors classified using these markers have different clinical outcomes, with HR3 tumors being associated with the best survival and HR1 and HR0 tumors the most aggressive [176]. An intriguing implication of this novel classification is targeting VDR and AR in conjunction with ER for patients receiving hormone therapy, which is potentially a more efficient therapeutic strategy [176, 177].

Discussion

Among ER+ tumors, the traditional classification of breast tumors into four categories using ER, PR, and HER2 has been frequently challenged by samples with exceptional clinical associations. The role of KI67 in characterizing these subtypes has been gaining attention, suggesting the prominent role of the 'sustaining proliferative signaling' hallmark played in [ER+ | PR+] tumors.

Many ER-PR-HER2+ tumors overlap with ER-PR-AR+ (MAC), which are both associated with poor prognosis and tumors of grade 2 or 3. These seemingly imply a similar role played by HER2 and AR which, however, differ in that HER2 is a growth factor receptor and AR is a hormonal receptor involved in the control of male characteristics. Note that MAC tumors are also characteristic of KI67+ (Table 2), and KI67 is frequently recognized together with hormonal receptor positive tumors (above paragraph) and indicative of proliferation. Thus, the progressive nature of hormonal receptor (ER, PR, or

AR) positive subtypes may be governed by 'sustaining proliferative signaling'.

ER-PR-HER2- tumors are notorious for their poor diagnosis, lack of efficient therapeutic treatments and high heterogeneity. It has been suggested that ER status is shifted from positive to negative in up to 70% of tumors showing acquired resistance and as the disease progressed from primary to metastatic state [178-183]. This, on one hand, shows the more aggressive nature of ER- tumors than ER+ ones in general and, on the other hand, suggests a switch on cancer hallmarks during carcinogenesis as well as its connection with ER status. Basal markers, EMT markers, claudins, immune response genes have been revealed to identify basal [85, 89, 90, 116-119], MBC [139], claudin-low [139] and interferon-rich cancers [93, 150] from tumors of this kind. CK are the most widely used basal markers, which contribute to cell-cell adhesion, and claudins are a family of proteins that are the most important components of the tight junctions. These suggest that 'activating invasion and metastasis' drives the differences among basal tumors, MBC, and claudin-low cancers, despite the different markers used for their identification. Indeed, as shown by Table 2, these ER-PR-HER2- tumors are all associated with poor clinical outcome and of grade 3 cancers. Also, recall that MBC and claudin-low tumors share many similarities regarding their, e.g., genomic aberrations and drug response, in addition to clinical outcome. The interferon-rich subtype, as indicated by its name, may differentiate itself from the other ER-PR-HER2- tumors by 'evading immune destruction'.

Novel biomarkers keep emerging, with more and more cancer hallmarks unveiled critical in deciphering breast cancer heterogeneity. For example, BCL2 is related to 'resisting cell death', TP53 represents the hallmark of 'genome instability and mutation', and VRD is associated with the level of circulating vitamin D metabolites and thus the 'deregulating cellular energetics' hallmark.

Concluding Remarks

As a tumor consisting of a collection of different diseases, various biomarkers have been identified to categorize them into different subtypes. Despite the novel subtypes being kept identified, the dominant cancer hallmarks driving such heterogeneity stay invariant. In summary, the extent to which cells having 'sustaining proliferative signaling' is of particular importance in breast tumor classification, especially among HR positive tumors such as [ER+ | PR+]HER2-, [ER+ | PR+]HER2+, and MAC.

In tumors lacking hormone receptors, i.e., ER-PR-HER2-, other cancer hallmarks take the role

and are often associated with more aggressive properties. Among these, 'activating invasion and metastasis' is of the most importance, based on which basal, claudin-low and MBC are differentiated from ER-PR-HER2- tumors. Also important for tumors of this kind is the emerging hallmark 'evading immune destruction', according to which a novel subtype, interferon-rich cancer, is identified. These ER-PR-HER2- subtypes, though distinct, are not mutually exclusive.

Other hallmarks, such as 'resisting cell death', 'genome instability and mutation' and 'deregulating cellular energetics' help to refine tumor classification and contribute, in particular, on predictive value.

With the arrival of the times of precision medicine, precise molecular characterization of the heterogeneity of complex diseases such as breast cancer has become of particular importance. Though the basic receptors (ER, PR, HER2) classifying breast tumors stay the same, novel biomarkers and approaches in subtyping of such tumors have been kept reported. This, in turn, has led us to an overwhelming realm of breast tumor subtypes that are not mutually exclusive, complicating our understandings towards breast cancer classification. This, on one hand, is due to the inconsistent criteria used for breast tumor identification and, on the other hand, suggests that we may have lost the global view on unveiling breast tumor heterogeneity. Here, by reviewing the current biomarkers and their associations with cancer hallmarks, we claim that 'the divergent outcome observed from cancer patients are driven by cancer hallmarks but not biomarkers; thus, biomarkers may vary among studies but cancer hallmarks driving such differences should stay invariant'.

Among the 10 cancer hallmarks, 6 have been covered by the current studies on breast tumor classification. This suggests that further efforts in this area should be inclined to the rest four hallmarks, i.e., 'evading growth suppressors', 'enabling replicative immortality', 'inducing angiogenesis' and 'tumor-promoting inflammation', assuming that all cancer hallmarks contribute to breast tumor heterogeneity.

Abbreviations

IHC: immunohistochemistry; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; HR: hormonal receptors; AR: androgen receptor; [ER+ |PR+]HER2-: tumors with either ER or PR positivity, and HER2 negativity; [ER+ |PR+]HER2+: tumors with either ER or PR positivity, and HER2 positivity; ER-PR-HER2+: tumors with ER and PR negativity, and HER2

positivity, also named HER2 positive; ER-PR-HER2-: tumors with ER, PR, HER2 negativity, also named triple negative; ER-PR- tumors: tumors with both ER and PR negativity; [ER+ |PR+] cancers: tumors with either ER or PR positivity; MAC: ER-PR-AR+, also named molecular apocrine; GEP: gene expression profiling; EMT: epithelial to mesenchymal transition; CMF: cyclophosphamide, methotrexate, 5-fluorouracil; TIC: tumor initiating cell; TOP2A: Topoisomerase II alpha; CK: cytokeratins; EGFR: epidermal growth factor receptor; VRD: vitamin D receptor; VIM: vimentin; MBC: metaplastic breast cancers; PI3K: phosphatidylinositol 3-kinase

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Competing Interests

The authors have declared that no competing interest exists.

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Author biography

Dr. Xiaofeng Dai, Ph.D., is an associate professor at the School of Biotechnology, JiangNan University, China. She got two master degrees, one in Biochemistry & Molecular Biology and the other in Bioinformatics. She received her first doctoral degree in Computational System Biology at Tampere University of Technology, Finland, and the second in Quantitative Methods in Economics at Aalto University, Finland. She conducted her postdoctoral researches at the Institute for Molecular Medicine Finland and the department of Obstetrics and Gynecology in Helsinki University, where she started her career in breast cancer. Dr. Dai got the "Chinese Government Award for Outstanding Self-financed Students Abroad" in 2009, "Best Paper Award" from "the international conference BIOTECHNO", Bucharest, 2009, and received a research financial support of 800000¥ from "National Natural Science Foundation of China" in 2014. Dr. Dai researches focus on breast cancer heterogeneity and subtyping, basal breast cancer stem cell signaling network construction and drug target exploration, as well as bioinformatics studies in biology.