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REVIEW

Combinatorial biomarker expression in breast cancer

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Abstract Current clinical management of breast cancer relies on the availability of robust clinicopathological variables and few well-defined biological markers. Recent microarray-based expression profiling studies have emphasised the importance of the molecular portraits of breast cancer and the possibility of classifying breast cancer into biologically and molecularly distinct groups. Subsequent large scale immunohistochemical studies have demonstrated that the added value of studying the molecular biomarker expression in combination rather than individually. Oestrogen (ER) and progesterone (PR) receptors and HER2 are currently used in routine pathological assessment of breast cancer. Additional biomarkers such as proliferation markers and 'basal' markers are likely to be included in the future. A better understanding of the prognostic and predictive value of combinatorial assessment of biomarker expression could lead to improved breast cancer management in routine clinical practice and would add to our knowledge concerning the variation in behaviour and response to therapy. Here, we review the evidence on the value of assessing biomarker expression in breast cancer individually and in combination and its relation to the recent molecular classification of breast cancer.

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Introduction

Breast cancer (BC) is a complex genetic disease characterised by the accumulation of multiple molecular alterations [1, 2]. Routine clinical management of BC relies on well-established clinicopathological factors. Although these factors show strong overall association with patients' prognosis and outcome, it has become clear that patients with similar features may show distinct outcomes and vary in their response to therapy [3]. For example, it has been shown that approximately one-third of patients with early stage BC develop recurrence [4], whilst a similar proportion of node positive patients remain free of distant metastases [5]. In an attempt to improve BC classification and to stratify patients into well-defined prognostic categories that can be used in management decision, these well-established prognostic factors have been combined to constitute prognostic indices, such as Nottingham Prognostic Index, which provides prognostic significance better than any of its components individually [6, 7]. In order to improve prediction of response to specific agents and to aid tumour classification and overcome the inherent subjectivity involved in histopathology, molecular biomarkers have been introduced. Currently, only hormone receptors (HR), including oestrogen (ER) and progesterone (PR) receptors, and human epidermal growth factor receptor 2 (HER2) are assessed and used in routine clinical practice at least in most centres [8]. Although several additional biomarkers are extensively studied, and some have shown prognostic (estimation of outcome after surgery alone) and predictive (estimation of

response to therapy) value in the research setting, only few biomarkers are likely to be included in routine clinical use, at least in some centres; these include basal markers and proliferation-related markers.

It has been estimated that all these traditional clinicopathological and molecular factors, which presently form the basis for determining adjuvant therapy, assign these patients into risk groups at an approximate absolute specificity level of only 10% to achieve an acceptable degree of sensitivity [9]. Therefore, there is an increasing need to improve patients risk stratification and targeting of treatment to those who will truly benefit, thereby avoiding iatrogenic morbidity in those who will not. Most importantly, whilst most predictive markers developed to date have acceptable negative predictive values (i.e. they identify the population of patients who will not benefit from a given therapy), their positive predictive values (i.e. their ability to identify the patients that will certainly benefit from a regimen) are clearly suboptimal. For instance, complete lack of ER expression does identify a group of BC patients that do not benefit from endocrine therapies (i.e. optimal negative predictive value), however, only a fraction of patients whose tumours express ER will benefit from endocrine therapy (i.e. suboptimal positive predictive value) [10]. Improved understanding of the molecular features of BC and the identification of the key genes that underpin the molecular heterogeneity of BC may lead to better prediction of tumour behaviour and treatment response.

Assessment of HR and HER2 in BC provides prognostic and predictive information on response to endocrine therapy and anti-HER2 targeted therapy, respectively. However, the expression of these biomarkers overlap and the prognostic and predictive value of these markers in combination need to be well-defined. Currently, it is recognised that a set of biological markers, rather than a single one, seem to be important to differentiate between a high or low chance for a response to systemic therapy [11]. In addition, recent microarray-based gene expression profiling studies (GEP) have demonstrated that the importance of assessment of key biomarkers in combination, which is expected to improve our understanding of the biology and behaviour of BC and help to tailor treatment [12]. GEP has also indicated that genomic fingerprints may refine prediction of the course of disease and response to adjuvant interventions. Currently, several commercially available prognostic BC tests based on the expression of multiple genes (using transcriptome) are available, including Oncotype DX (21 genes; Genomic Health, Redwood City, California, USA) [13], MammaPrint (70 genes; Agendia BV, Amsterdam, the Netherlands) [14], Theros H/I (2 genes; AvariaDX, Carlsbad, California, USA) [15], and Theros breast cancer index (a combination of Theros H/I and the molecular grade index, AvariaDx, Carlsbad, California, USA) which represent the first introduction of multigene assays into clinical application. Out of these technologies, only Oncotype DX has been included in the American Society of Clinical Oncology (ASCO) and National Cancer Centre Network (NCCN) guidelines for the management of BC patients [12].

Here, we present an overview of the significance of assessment of expression of biomarkers used in routine BC management and the added value of their combinatorial expression.

Hormone receptors

Oestrogen receptor

The oestrogen receptor (ER) was first identified in the 1960s and subsequent studies have provided the evidence that ER is important in the carcinogenic process, and its inhibition, through endocrine targeting, either directly using oestrogen agonists (Selective ER Modulators) or indirectly by blocking the conversion of androgens to oestrogen (e.g. aromatase inhibitors), forms the mainstay of BC endocrine therapy [9, 16–18]. Therefore, ER status has been used since the mid-1970s in the clinical management of BC both as an indicator of endocrine responsiveness and as a prognostic factor for early recurrence. It has also been reported that ER expression in BC is stable and phenotypic drift from primary to metastatic breast carcinoma is reported to be an exceedingly rare phenomenon [19]. In addition, recent GEP of BC has also indicated that ER is a major determinant of the molecular portraits of BC [20–23]. ER status currently forms part of the UK minimum data set for histopathology reporting of invasive BC and it is routinely determined using a standardised technique [8].

Oestrogen (ER)-positive tumours (ER+) comprise the majority of breast cancers, accounting for up to 75% of all cases. Up to 65% of tumours developing in women aged <50 years are ER+, whereas this figure increases to 80% in women >50 years [24]. Although ER+ tumours are generally well-differentiated, show other less aggressive primary tumour characteristics and are associated with better clinical outcome largely independent of other clinicopathological variables after surgery [25, 26], long term survival studies have reported that ER status loses its predictive significance and that the long term outcome of ER+ and ER− tumours is not different [10]. In fact, ER status provides limited prognostic information; currently, the major clinical value of determining ER status is to assess the likelihood that a patient will respond to endocrine therapy, and are unlikely to gain additional benefit from adjuvant chemotherapy [27, 28]. Most reports have concluded that ER is probably the most powerful single predictive factor identified in BC [18, 23, 29, 30].

Although ER expression is an accurate negative predictor of response to hormonal treatment, i.e. ER− tumours are unlikely to respond to hormone therapy, it provides limited positive predictive information, given that only approximately 50% of patients with ER+ tumours respond to hormone treatment [18]. It is also documented that a small proportion of ER− cancers respond to hormonal therapy [31, 32]. These observations, in addition to the fact that ER+ tumours comprise a large proportion of BC, demonstrate that ER-positivity per se defines a heterogeneous group of tumours with respect to their risk factors, clinical behaviour and biology [12, 18, 33]. Unsupervised analysis of the transcriptome of BC has revealed that at the transcriptional level, ER+ and ER− tumours are fundamentally different [20, 22, 34]. Furthermore, the type, pattern and complexity of genetic aberrations appear to be different in ER+ and ER− disease [35–37], and it has been observed that the molecular pathways and networks driven by copy number aberrations appear to some extent to be determined by the ER status of a tumour [36, 38].

Progesterone receptor

Progesterone receptor (PR) is an oestrogen-regulated gene and its expression is therefore thought to indicate a functioning ER pathway [39–41].

Progesterone receptor (PR)+ tumours comprise 55–65% of BC. Multiple studies have provided evidence for the prognostic and predictive importance of PR assessment in BC [33, 42–47]. PR+ cancers have been shown to have a better prognosis that PR− tumours, and there are some data to suggest that PR status can help to predict respond to hormone treatment, both in patients with metastatic disease [45] and in the adjuvant setting [32, 47–50]. However, it is also important to mention that some authors questioned the value of assessing PR status in BC [18, 51], and in the latest (2009) guidelines published by the National Institute for Health and Clinical Excellence (NICE) in the UK for early and locally advanced BC, it is recommended not to routinely assess PR status in patients with invasive BC (www.nice.org.uk/CG80). The argument was based mainly on the lack of evidence to support PR being of additional predictive over ER status with respect to response to endocrine therapy [10]. It has also been stated that PR positivity hardly exists amongst ER− tumours [51]. However, false ER negativity has been reported in routine practice [52] and strong PR positivity in an apparent ER− case may be an indicator of a false negative ER result.

Combinatorial expression of ER and PR

It is recognised that ER expression is used as the main determinant of response to hormone therapy in BC. Approximately 40% of ER+ tumours are PR− [33]. Lack of PR expression in ER+ tumours may be a surrogate marker of aberrant growth factor signalling that could contribute to tamoxifen resistance and that ER+/PR− tumours are generally less responsive than ER+/PR+ tumours [33, 48, 53, 54], particularly for Tamoxifen in the metastatic setting [45, 46]. Although some studies have reported that up to 10% of ER− BC are PR+ (ER−/PR+) [55, 56], recent evidence has indicated that this percentage is much lower when more sensitive immunohistochemical detection methods for ER are used or when analysis of ER and PR mRNA levels by quantitative real-time PCR is used [52, 57–59]. The higher frequency of ER-/PR+ tumours in some studies may be due to a false-negative ER assay, very low level ER or to variant ERs not recognised by the antibody, but still capable of stimulating PR expression [60]. In a study of 155,175 women with known joint ER/PR receptor status using data from the NCI's SEER program in the United States, Dunnwald and colleagues [26] reported that the proportion of ER−/PR+ tumours declined over the study period (1990–2000). In a central immunohistochemical analysis of ER and PR from 6,291 patients enrolled in the BIG198 clinical trial, Viale et al. reported that 0.2% of patients displayed an ER−/PR+ profile [61]. In our hands, the percentage of ER−/PR+ tumours reported recently in our routine practice is between 1 and 2% of BC [62 and unpublished data].

When the combinatorial expression of ER and PR are considered, four subgroups are recognised: double HR+ (ER+/PR+), single HR+ (ER+/PR− and ER−/PR+) and double HR− (ER−/PR−). The double positive group, which comprises the majority of tumours (55–65%) [26, 33, 63], shows the best prognosis and a good response to hormonal therapy, and has been used as a feature of the Luminal A class in some of the recent GEP classification systems of BC [22, 23, 64]. It has been reported that 75–85% of tumours with ER+/PR+ phenotype respond to hormonal therapy, whereas less than 10% of ER−/PR− tumours respond [32, 65, 66]. Compared to other subtypes, the double HR+ tumours are also associated with older age, lower grade, smaller size and lower risk of mortality. Dunnwald et al. [41] demonstrated that the associations between ER/PR subgroups and mortality risk are independent of tumours stage, age or grade and that the magnitudes of these relative risks vary amongst different tumour stage and grade.

The double HR− group which comprises the second largest group (18–25%), are more likely to be of grade 3 (approx 85%) and associated with a higher recurrence rate, decreased overall survival and unresponsiveness to endocrine therapy [45, 46, 48, 63, 67–69]. It has also been reported that HR− status is the most important predictive marker concerning response to a preoperative taxane/

anthracycline-based regimen. However, despite the high pathologic complete response rate, survival of patients with this phenotype was reported in several studies to be shorter than for those with HR+ tumours [11]. However, some types of invasive carcinoma that are typically HR−, e.g. adenoid cystic carcinoma and secretory carcinoma, have an excellent prognosis with minimal regional recurrence [70, 71]. This, in addition to other evidence, points towards the heterogeneous nature of the HR− subgroup of BC [12, 72]. This group of tumours corresponds to the vast majority of basal-like, normal breast-like and HER2+ classes in the GEP molecular subtype classification [22, 23].

The significance of BC with a single HR+ phenotype that includes ER+/PR− tumours, which comprise the third largest group of BC (12–17%) [26, 33, 63], and ER−/PR+ (1–2%) is still poorly understood. These tumours may correspond to the Luminal B class in the GEP classification [21–23, 64] and may show frequent expression of other features characteristic of poor prognosis. Interestingly, these tumours are more often of high histological grade, large size, more likely to be aneuploid and show higher expression of proliferation-related genes, EGFR and HER2 than ER+/PR+ cancers.[33, 54] Clinical data regarding metastatic and adjuvant treatment responsiveness suggest that hormone therapy is less effective in the single HR+ tumours than in the ER+/PR+ class [45, 48, 73], with only about 40% responding to hormonal manipulation [32, 65]. However, one study reported that the response rate of ER+/PR− tumours to an aromatase inhibitor is similar to that of ER+/PR+ cancers [74]. In addition, some studies have demonstrated that both single HR+ groups are similar in that they both might have biological characteristics somewhere in between ER+/PR+ and ER−/PR− [33, 75]. Moreover, Dowsett et al. [32] have demonstrated that ER−/PR+ cancers can benefit from endocrine therapy in contrast to ER−/PR− tumours. They concluded that measurement of PR status in ER− patients defines a group of patients that benefit from tamoxifen, but would be excluded from tamoxifen therapy on the basis of ER status alone. As discussed by the authors [32], it is plausible that the ER−/PR+ tumours derive benefit from tamoxifen because they result from false negative ER assessment results.

In a different approach, instead of using positive and negative categories, Goldhirsch and colleagues [76] have used the level of expression of both ER and PR to predict response to endocrine therapy. They reported two categories of HR+ BC; those that express high levels of both ER and PR (ER and PR $> 50\%$) and are highly endocrine responsive, and those that express low levels of either/both receptors (ER or PR $< 50\%$ and ER $> 10\%$) and are incompletely endocrine responsive. A third group which shows negative expression for ER and PR (both $<10\%$) do not benefit from endocrine therapy. Stendahl et al. [43] have reported that adjuvant tamoxifen improved survival for premenopausal patients with tumours showing $>75\%$ PR positivity at which point PR was also independently associated with favourable overall survival. Tumours with lower percentage of PR positivity showed that no similar effect, whilst a gradually increasing tamoxifen effect was observed in tumours with $>10\%$ ER+ nuclei. Based on their findings, they concluded that a fractioned rather than dichotomized immunohistochemical evaluation of both ER and PR should be implemented in clinical practice. Furthermore, a meta-analysis of tamoxifen trials showed that women with ER+ tumours derive significant benefit from 5 years of tamoxifen in reducing the odds of recurrence and death, and this benefit is directly proportional to the level of ER, with patients with higher tumour ER levels deriving the greatest benefit from therapy [18].

In summary, there is sufficient evidence to demonstrate that joint ER/PR assessment defines phenotypic groups that have different biological characteristics, including tumour size, grade, stage, patient's outcome and response to therapy. Breast cancers can be ranked from good to worse for ER+/PR+ to ER+/PR− to ER−/PR+ to ER−/PR− and that joint ER/PR expression identifies BC variants better than either independent ER or PR expression [26, 33, 45, 46, 48, 50, 53, 54, 63].

Most GEP studies [20–23] emphasise the importance of HR expression in BC and showed that HR+ tumours constitute a distinct group of tumours that are different from HR− BC or HER2 over-expressing tumours [12]. GEP support the existence of at least two luminal-like subclasses (A and B), and recent studies have implied that rather these differences more probably represent a biological continuum [12, 77, 78] which includes the double positive and single HR+ tumours and also relates to the level of expression of HR as well as other biomarkers within the HR+ tumour class. At one end of the ER+ spectrum, there are the so-called Luminal A tumours which are characterised by high levels of ER and downstream transcriptional targets of ER, other luminal associated markers in addition to low levels of expression of proliferation-related genes, whereas the Luminal B group is characterised by low to moderate expression of ER and other luminal specific genes, but is further distinguished by high expression of proliferation-related genes [22, 23, 79]. It has been reported that Luminal A tumours respond better to hormonal therapy, whilst Luminal B tumours are more often resistant to this therapeutic modality and may benefit from combined endocrine treatment and chemotherapy [80]. However, it is important to mention that to date, there is neither internationally accepted single definition for the luminal subgroups/classes or spectrum [12] nor has the use of ER and PR alone to define them been widely adopted and additional markers including HER2 [12, 64, 81] and

proliferation markers, e.g. ki-67 (MIB1) [80, 82] or genomic-grade index [83], have been adopted by some groups.

HER2

The clinical importance of amplification of *HER2* gene in BC was recognised in 1987 [84]. Numerous subsequent studies found that *HER2* gene amplification/protein overexpression is a predictor of poor prognosis and response for systemic chemotherapy [11, 85–88]. HER2 protein expression and gene amplification occurs in 13–20% of invasive ductal BC and more than half (~55%) of these cases are HR− [84, 89]. HER2 expression shows an inverse relationship with both ER and PR expression [90]. The prognostic impact of HER2 positivity is higher in node-positive compared with node-negative patients. Following the development of a humanised monoclonal antibody against HER2 (trastuzumab; Herceptin; Genentech, South San Francisco, CA, USA) and clinical trials demonstrating benefit of the use of anti-HER2 agents in patients with HER2+ BC [91–93], the reasons for establishing the HER2 status in routine clinical practice has changed, since it is a prerequisite for clinical use of trastuzumab in patients with HER2+ advanced disease [94] as well as in the adjuvant setting for HER2+ early stage BC [92]. In addition to trastuzumab, HER2 continues to be an important target in the development of a variety of other new cancer therapies, which include small-molecule drugs directed at the internal tyrosine kinase portion of the HER2 oncoprotein (i.e. lapatinib), and vaccines.

Gene expression profiling (GEP) studies have also demonstrated that HER2 is one of the key markers in BC as a high proportion of HER2+ tumours cluster together in a class which is distinct from HR+/HER2− and HR− (basal and normal breast-like) tumours [22]. HER2 status may also be predictive for other systemic therapies [86]. It has been reported that HER2 positivity is associated with relative, but not absolute, resistance to endocrine therapies in general [95]. However, this effect may be specific to tamoxifen, but not to oestrogen depletion therapies, such as aromatase inhibitors [96, 97]. Similar to ER, the HER2 status of BC narrows the pool of candidates eligible for HER2-directed therapies, but it does not definitively select those who will respond. Several studies have reported that HER2 may be a predictive marker of response to anthracycline-based chemotherapy [98–101]. It has also been suggested that HER2 positivity is predictive of better response to higher dose anthracycline-containing regimens compared with standard regimens [102, 103] and to taxane compared with non-taxane-containing regimens [104, 105], however, the predictive value of HER2 remains a complex subject and further validation is still required [98].

Combinatorial expression of ER, PR and HER2

The results of GEP studies demonstrate that BC is composed of distinct molecular classes largely characterised by well-defined patterns of expression of HR, HER2 in addition to few other key molecular variables, such as proliferation and basal cell type-related gene alterations [12]. Importantly, these molecular classes showed that potential prognostic and predictive utility. The results of these studies, in addition, the availability of treatment option have emphasised the importance of studying the molecular portraits of BC in concert. Therefore, several attempts to validate and translate these molecular classes into defined groups that can be identified in routine practice have been carried out. Most studies have used a combination of various immunohistochemical (IHC) markers including ER, PR and HER2 with or without additional markers, such as basal marker and proliferation markers (see below), as IHC surrogates to define the molecular classes initially identified by GEP and to improve our understanding of the prognostic and predictive value of studying these markers in combination. It should be noted that there is a paucity of data on direct comparisons between GEP and immunohistochemical surrogates to define the molecular subgroup of a given case. Furthermore, the stability of some of the molecular subgroups as defined by GEP has also been called into question [106].

Most studies have considered ER or HR positivity regardless of expression of other markers as the most important feature for a tumour to be classified as of Luminal type, whilst HR- and HER-negative (triple negative; TN) phenotype was used to define the basal-like class [64, 107–110]. HR+ luminal tumours which comprise the largest proportion of BC phenotypes are a heterogeneous group where molecular subtyping and consideration of expression of other markers could be of utmost importance and of clinical relevance. Several studies have classified HR+ tumours that are also HER2+ as the Luminal B subclass [64, 81, 107, 109, 111], which constitutes approximately 6% of HR+ tumours (3–11%) [64, 82, 112]. This approach is supported by the fact that some Luminal tumours identified in GEP express HER2 and that HR+/HER2+ show poorer outcome than HR+/HER2− tumours. However, other authors have classified all HER2+ tumours in the HER2 subclass independent of their HR status [108]. The later approach is also supported by the fact that HR+/HER2+ tumours are candidates to specific systemic therapy targeting HER2. Importantly, there is evidence to suggest that *HER2*-amplified cases have similar genetic changes [113] and outcome [64, 82, 114] regardless of their HR status.

It has also been reported that some forms of ER+ BC are resistant to chemotherapy [27, 28, 115] and that a

significant proportion of cases do not respond to hormone therapy. Therefore, the addition of other predictive biomarkers, such as HER2 and proliferation markers to ER and PR may help predict response to chemo and endocrine therapy in HR+ tumours regardless of the terminology of Luminal subtypes. This hypothesis was utilised in the construction of the gene set that constitutes the Oncotype DX assay [13]. Preclinical and clinical data suggest that HER2 overexpression confers intrinsic resistance to hormonal treatment in HR+ tumours. This in addition to the adverse prognostic effect of HER2 overexpression may indicate that patients with HR+/HER2+ BC might not derive a benefit from single-agent hormone therapy. Results from randomised clinical trials that combined hormone treatment with targeted anti-HER2 therapy in postmenopausal women with HR+/HER2+ advanced BC indicate that this novel dual-targeting strategy significantly improves outcomes compared with hormone therapy alone. Other studies also suggest that HR+/HER2+ BC might benefit more from anti-HER2 therapy plus chemotherapy [116]. Darb-Esfahani and colleagues [117] have reported that HR+/HER2+ tumours show a good response rate to neo-adjuvant chemotherapy and a favourable prognosis. HR+/HER2− tumours have a good prognosis irrespective of achievement of a pathological complete response, whereas patients with HR−/HER2− and HR−/HER2+ tumours show the worst prognosis, particularly if they do not achieve a pathological complete response. Hayes et al. [105] have also demonstrated that a difference in response to chemotherapy based on HER2 and HR where HER2+ tumours are associated with a benefit from the addition of paclitaxel after adjuvant treatment with doxorubicin plus cyclophosphamide in node-positive BC, whilst HER2−, ER+ tumours, may gain little benefit. Konecny et al. [95] found an inverse correlation between HER2 expression and the level of expression of ER and that in patients with HR+ tumours, HER2+ tumours had statistically significantly lower ER/PR levels than HER2− cancers. Therefore, they suggested that the relative resistance of HER2+/HR+ tumours to hormone therapy is due to reduced ER/PR expression or high proliferation rates rather than positivity of HER2. In fact, there is evidence to support the conclusion that lower ER, lower PR and positive HER2 are associated with lower responsiveness to any type of endocrine therapy and HR+ tumours overexpressing HER2, therefore, require the blockage of the HER2 pathway in addition to oestrogen deprivation.

Current evidence indicate that ER+/PR+/HER2− tumours have the best prognosis and show the best response to hormone therapy. Single HR+ HER2− tumours show worse outcome and response to treatment whilst HR+ HER2+ tumours show the worst outcome and response to hormone therapy amongst the HR+/luminal tumours [32, 118]. Although HR− tumours are usually poorly differentiated (i.e. 85% of HR− tumours are grade 3 in the Nottingham series), show aggressive behaviour and poor outcome and least likely to respond to hormone therapy, these tumours constitute a heterogeneous group that includes two main subclasses: HER+, and HER2− (TN) tumours. TN class can be further divided into basal-like (core basal phenotype) and TN non-basal (null phenotype) [33, 64, 107–110]. These subclasses show significant difference in behaviour and outcome (Fig. 1). The behaviour, outcome and response to therapy of these TN tumours are discussed in detail elsewhere [12, 119, 120]. Other classes that have been described amongst HR− tumours include the molecular apocrine subtype [12, 34], interferon-rich group and the Claudin low group [121].

Combinatorial expression and prediction of endocrine therapy resistance

Currently available antihormone-treatment strategies for ER+ BC consist of either targeting the ER itself through the use of HR selective ER modulators, such as tamoxifen, or by depriving cancer cells of their oestrogenic stimulus through (i) gonadal suppression (in premenopausal women) or (ii) aromatase inhibitors (in the postmenopausal setting). Although the absence of ER expression can predict lack of response to endocrine therapy, the predictive power of positive ER expression is limited by the phenomenon of hormone resistance. This resistance can be either de novo (present before hormone therapy) or acquired during the course of treatment. Two patterns of acquired resistance have also been documented; patients who initially respond and relapse quickly and patients with a sustained response followed by a late relapse. For example, it has been reported that approximately 50% of metastatic breast cancers will display de novo resistance to hormone therapies despite being HR+ and more than one-third of patients with endocrine-responsive, early stage BC, and almost all of those with metastatic disease, will develop hormone resistance during the course of their disease (acquired resistance) despite an initial response to the therapy resulting in disease relapse and progression [122, 123]. Several key mechanisms involved in the process of hormone resistance have been identified [124, 125]. There is, however, evidence that specific markers can be used to identify tumours that exhibit resistance to specific antihormone therapy agents. These markers include both conventional markers of endocrine responsiveness (ER and progesterone receptor, PR), receptor tyrosine kinases, such as the HER family of receptors and, in particular, HER2 and EGFR, CDK10 and the insulin-like growth factor-1 receptor (IGF1R), which might be through cross-talk with the ER itself [126–131]. Clinical evidence for the role of

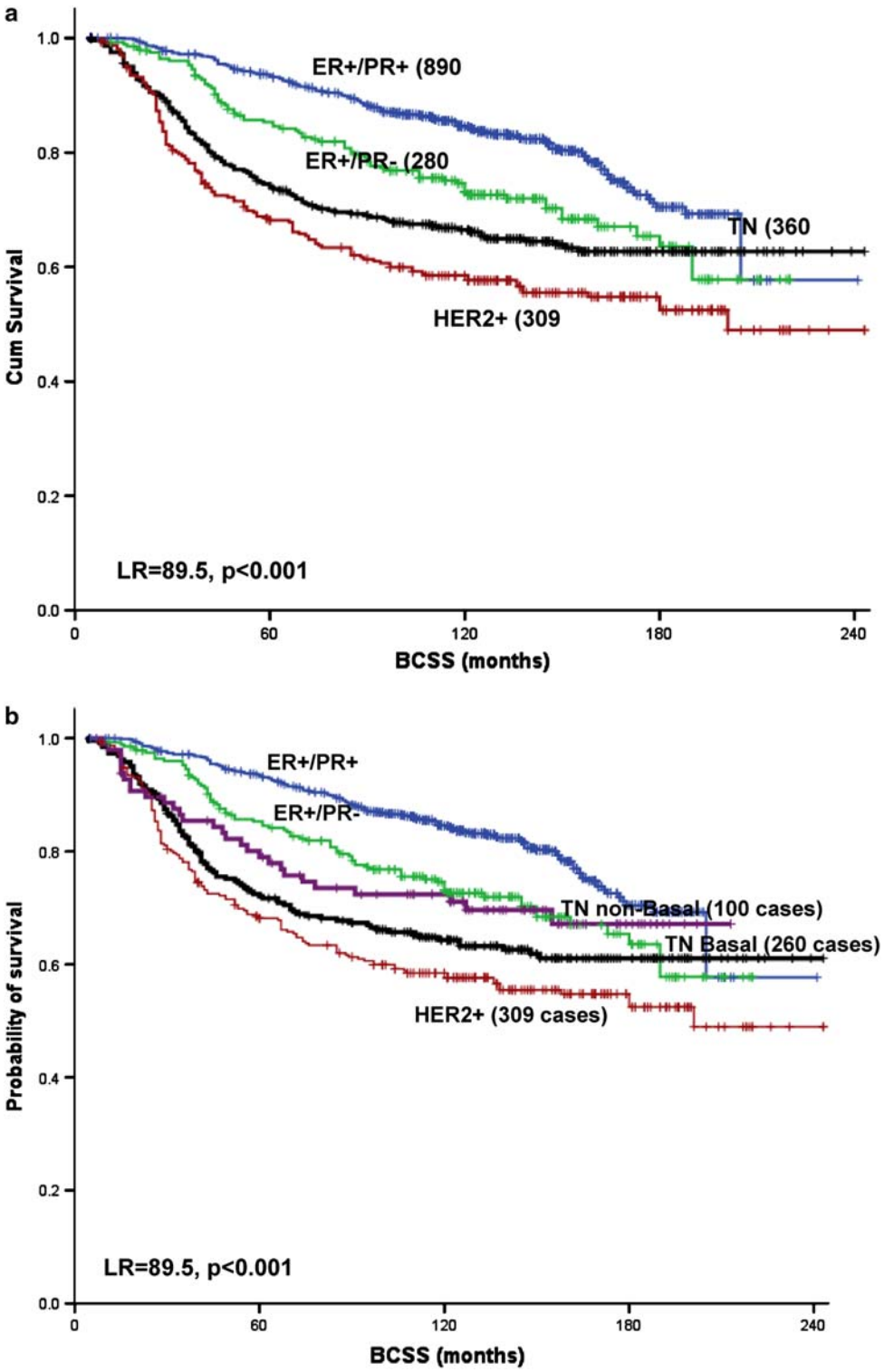

Fig. 1 Correlation between biomarker expression (ER, PR, HER2 and basal marker (**b**) Ck5/6 and EGFR) and outcome (breast cancer specific survival; BCSS) in the Nottingham series [110, 136]. BCSSP = breast caner specific survival TN = triple negative (ER/PR/HER2−) TN = triple negative basal (ER/PR/HER2− and Ck5/6 and/or EGFR positive) TN = triple negative non-basal (ER/PR/HER2/CK5/6/EGFR negative)

EGFR (HER1) and HER2 in tamoxifen resistance has come from neoadjuvant trials where EGFR and HER2+ patients have significantly greater response to aromatase inhibitors than to tamoxifen [96]. Tovey et al. [126] have demonstrated that EGFR/HER2/HER3-positivity and/or PR-negativity comprise a high-risk group within ER+ BC patients that was more likely to show early relapse on tamoxifen. However, this predictive value of de novo resistance lost its significance after 3 years of tamoxifen. In the ATAC trial [50], PR− patients derived greater benefit from initial aromatase inhibitor treatment compared with tamoxifen. However, in the IES trial [132], PR status had no effect on

response when aromatase inhibitors were given as delayed treatment to patients who had been disease free on tamoxifen for 2–3 years. The recent results of the ATAC trial [118] demonstrate that quantitative expression of PR and HER2 status did not identify patients with differential relative benefit from anastrozole over tamoxifen and that time to recurrence is longer for anastrozole than for tamoxifen in all subgroups. The results of the BIG 1–98 clinical trial demonstrated that Letrozole improved disease-free survival compared with tamoxifen regardless of HER2 status [97, 133] or PR status [61]. Therefore, although initial data indicated that PR, EGFR and HER2 expression might be used to predict resistance to hormone therapy in the ER+ BC [48, 50, 126, 132], the evidence available suggest that PR and HER2 are prognostic rather than predictive markers, and that these biomarkers cannot be used to determine which patients would benefit most from aromatase inhibitors or tamoxifen [61, 97, 133, 134].

Basal markers

The potential poor outcome associated with the expression of high molecular weight or 'basal' Cks in BC has been known for over two decades [135], and this has been confirmed by numerous other studies that have also demonstrated that the poor prognosis of basal Cks is independent of the expression of HR or HER2 [112, 136–138]. However, the results of GEP studies, which showed that the majority of basal-like class of tumours are characterised by the TN phenotype in addition to expression of basal-associated markers, have emphasised the use of HR and HER2 expression in addition to basal markers to define a class of tumours that has a poor prognosis, lack the benefit of available targeted therapy and shows distinct molecular features (basal-like BC). In addition, it has been reported that there is a relationship between basal Cks expression and BRCA1-associated tumours and that basal Cks expression can predict BRCA1 status, and hence, may benefit from a similar therapeutic strategy [110, 139]. Tumours expressing basal Cks show specific pattern of distant metastasis [110]. The relationship between tumour size and proportion of lymph node positivity and outcome is also less clear amongst tumours that express basal markers [140, 141]. These findings may emphasise the importance of expression of basal markers in BC irrespective of controversial notion of their relationship to their potential cell of origin or their expression pattern in normal breast epithelial cells [142].

Although the number of basal IHC markers used to define basal-like tumours is large and expanding, the most widely used and generally accepted basal markers are Ck5/6 and EGFR; in addition to these markers, Ck17 and Ck14 have also been used in some studies [64, 108, 110, 112, 119, 143]. It has been demonstrated that using basal markers (Ck5/6 and EGFR) in the TN tumours identifies a biologically and clinically distinct subgroup of TN tumours (core basal phenotype/basal-like tumours; 60–90%), which could justify their use in the TN tumours [110, 144, 145]. This is also supported by the findings of some studies which reported a difference in response to chemotherapy between basal-like tumours and TN non-basal tumours (five negative phenotype or the so-called normal-breast like class in GEP studies) [115].

A small percentage of HR+ tumours show basal markers expression (average 8% of HR+ (range 1–18%) [64, 112, 144]) prompting the question whether this group of patients belongs to the luminal-like or basal-like cancer subclass. We have observed that HR+ tumours with basal marker coexpression exhibit a poorer prognosis when compared to HR+ basal marker negative tumours (unpublished observation by EAR and IOE). However, it is not clear whether this effect is independent of level of HR expression or expression of proliferation markers or this could represent a possible link between Luminal B and basal-like BC as indicated in gene expression studies [22, 23]. Although a proportion of HER2+ tumours express basal markers, the number of these cases is small, and no difference in survival has been identified between HER2+/basal marker positive and HER2+/basal marker negative tumours, although it has been reported that HER+/basal marker positive BC less frequently respond to Herceptin [146], large scale study of HR+ and HER2+ tumours with basal markers expression is needed to clarify these points.

Proliferation markers

In early stage BC, it has been estimated that chemotherapy can achieve 20–30% improvement in disease-free survival and around 15% or greater increase in overall survival rates [147, 148]. However, de novo and acquired resistance to therapy is observed in a significant subset of patients, leading to subsequent disease progression [149]. In contrast to predictive factors for targeted therapy, predictive markers for chemosensitivity are less well-defined [150, 151]. Chemotherapy agents including CMF, taxanes and anthracycline-based chemotherapy affect cell division or DNA synthesis and function in some way. It is also now acknowledged that increased cell proliferation is a key determinant of clinical outcome in patients with BC [152, 153]. The most widely used proliferation marker in BC is Ki-67 (MIB1) which is present exclusively in cycling cells [154] and can be used to predict response to neoadjuvant [155, 156] or adjuvant [157] chemotherapy of BC and to neoadjuvant [158, 159] or adjuvant [160] endocrine therapy of ER+ tumours.

Ki-67 has been used in combination with other markers in BC to provide prognostic and predictive value [159,

161]. In a recent study, Cheang et al. [82] used Ki-67 in addition to ER, PR and HER2 to define molecular classes of BC. They reported that Ki-67 and HER2 expression can divide HR+ tumours into three prognostically distinct classes; Luminal A, which is HER2− and Ki-67 proliferation index low, Luminal B, which is HER2− and Ki-67 proliferation index high and Luminal/HER2+ tumours that showed HER2+ and high proliferation. Using Ki-67 to divide HR+ HER− tumours into two subclasses, it was demonstrated that HR+ tumours, that are Ki-67 high, are associated with poor outcome regardless of systemic therapy [82]. In a different approach demonstrated that the importance of assessment of proliferation rate in HR+ tumours, Loi et al. [83] have used genomic grade index to classify HR+ tumours into two subtypes with difference in prognosis in both systemically untreated and tamoxifen-treated populations. It should be noted, however, that before proliferation assessment is introduced in clinical practice, methods (e.g. mitotic counting, Ki-67 and genomic grade) and cutpoints need to be standardised. Importantly, proliferation markers, akin to histological grade, are of limited value in the TN and HER2+ tumours [77], as the majority of these tumours are poorly differentiated with a high proliferation index.

Ellis et al. [158] have reported that in patients with ER+ BC, who have received neoadjuvant endocrine treatment, Ki-67 level and ER status were independently associated with both RFS and BCSS in multivariate analysis together with lymph node status and tumour size. Similar results were demonstrated in a retrospectively collected series of patients with ER+ tumours, who received adjuvant endocrine treatment [160].

Moreover, recent data presented at San Antonio Breast Cancer Symposium show that the standard immunohistochemical tests (ER, PR, HER2 and Ki-67), when used in combinatorial manner can provide similar information as expensive molecular assays such as the GHI Recurrence Score [162].

Additional markers

Other markers that have been used as a predictor of outcome and response to therapy include Topoisomerase II alpha expression and (TOP2A) gene amplification (molecular target for anthracyclines) [98, 150, 163], Bcl2 and p53 expression [164, 165]. TOP2A aberrations (amplification, deletion) are found in up to approximately 30–90% of HER2-amplified BC, and amplifications are more common than deletions. Although *TOP2A* amplification has been reported in cases devoid of *HER2* amplification, this is an infrequent (3–9%) event [163] and even rarer in studies where *HER2* and *TOP2A* copy numbers were determined by high-resolution microarray-based comparative genomic hybridisation studies [37, 166]. Bcl2 is positively regulated by HR in BC and its expression has been reported to provide an independent predictor of BC outcome [164, 167]. Bcl2 has been reported to predict pathological response to a neoadjuvant anthracycline/docetaxel-based regimen [168]. In a previous study of Bcl2 and p53 expression in BC, we have demonstrated that a combination of both markers provides independent prognostic value with p53+/Bcl2− phenotype was independent predictor of a worse prognosis in multivariate analysis. Mauri et al. [169] used Bcl2 and p53 in HR+ tumours to predict outcome and response to therapy. They showed that ER+/p53+ phenotype was at higher risk of relapse/death as compared with ER+/p53− phenotype, whilst the worst prognosis was observed in ER−/p53+ tumours. Similar results were obtained when Bcl2 was combined with ER [169]. Yamashita et al. [170] have assessed the expression of p53, HER2 and Ki-67 in 506 invasive ductal carcinoma using IHC, and showed that the coexistence of HER2 and p53 expression is a strong prognostic marker in BC better than each marker individually. In another study, combination of Bcl2 and HER2 appears to be a useful in predicting prognosis in curatively resected stage III BC patients [167].

Multi-gene assays

Genomic prognostic tests are highly complex compared with more traditional tests used in routine practice (e.g. IHC). They require quantitative measurements of multiple candidate genes rather than the measurement of a single analyte. The Oncotype DX and MammaPrint assays are a prototype for an alternative type of genomic diagnostic test. Other multiparameter gene expression tools have also been developed [161, 171, 172]. For detailed reviews, the readers are referred to Sotiriou and Pusztai [173] and Weigelt et al. [12].

Oncotype DX

It was developed on the basis of a prospectively chosen 250-candidate gene set. Statistical analysis and modelling of these genes led to the selection of the 21 genes (16 cancer-related and 5 references) constituting the Oncotype DX assay panel to predict the likelihood of distant BC recurrence for individual patients. The expression levels of these genes are measured by using RT-PCR. A quantitative algorithm has been developed to produce a number between 0 and 100, the 'recurrence score' (RS) [13, 174]. RS is categorised into three risk strata: low (score < 18), intermediate (score > 18, but < 30) or high (score > 30). Oncotype DX is considered as a clinically validated, high-complexity, multianalyte RT-PCR genomic test that predicts the likelihood of BC recurrence in early stage,

node-negative, ER+ BC. Multiple studies have demonstrated that RS provides an accurate, reproducible measure of BC aggressiveness and therapeutic responsiveness [175–177]. In summary, Oncotype DX assay is prognostic for HR+, lymph node-negative patients. A low RS is predictive of tamoxifen benefit in HR+, node-negative cases. A high RS is predictive of chemotherapy benefit over hormonal therapy in HR+ patients. It should be noted, however, that the information provided by Oncotype DX is complementary to that provided by clinicopathological parameters, including tumour size, lymph node metastasis and even histological grade, as multivariable survival models demonstrate that all of these variables provide independent prognostic information [174].

MammaPrint

It is based on the 70-gene signature derived from the analysis of the microarray-based gene expression profiles of 78 retrospectively accrued young BC patients (<55 years), with tumours <5 cm and lymph node negative. The end point for test development was 5-year distant recurrence. Patients are classified by calculating the correlation coefficient between a patient's expression levels of the 70 genes and an average good-prognosis expression profile. Tumours are classified into good prognosis class if the correlation coefficient exceeds 0.4, and poor prognosis class if less [14, 178–180]. Interestingly, the 70-genes comprised those regulating cell cycle, invasion, metastasis, signal transduction and angiogenesis with omission of previously identified individual genes associated with outcome, e.g. ER, HER2 and cyclin D1. This supports the power of a collective genetic signature over individual genes. In fact, MammaPrint, Veridex (the 76 gene signature) and most of the first generation prognostic signatures mainly identify the poor prognostic highly proliferative ER+ BC and ER− cancers (regardless of proliferation [77]). Hence, their contribution for prognostication of breast cancers when other markers are used in conjunction is rather limited [181]. It is also important to mention that these multiparameter gene expression tools are not widely available, costly and as stated by Pusztai et al. [106] that there is substantially less experience with these emerging technologies than with the more established methods, the accuracy of which is often overestimated.

Combinatorial biomarker expression and pattern of survival

Consistent with the relevant biological and clinical role of these key molecular markers on BC behaviour, an important association with the pattern of survival is noted. First, HR+ tumours in general show distinct pattern of survival that is different from HR− tumours. In HR+ tumours, the mortality rate is appears constant overtime from diagnosis, whilst the rate for HR− tumours is high during the first 3–5 years and then declines showing a plateau curve of survival. However, not all HR+ or HR− tumours show the same patterns. HR+ tumours with high proliferation (Luminal B) show response to neoadjuvant chemotherapy and survival pattern similar to HR− tumours with early frequency of events and a later decline [82]. Results from multiple studies indicate that the prognosis of HR+/HER2+ show high mortality rate and poorer outcome during the first few years after diagnosis, but this difference decreases with time to and the two survival curves converge after 7–9 years. A similar pattern was observed for tumours expressing basal markers including HR+/basal markers positive when compared to HR+/basal negative and basal-like tumours when compared to TN basal markers negative. Furthermore, tumours with a high proliferation index behave in a similar way to HR− BC and basal markers positive tumours with early frequent events [64, 82, 112].

In conclusion, although biomarker expression in BC provides prognostic and predictive information, this can be improved by considering their combinatorial expression. ER, PR, HER2 and proliferation markers in addition to basal-associated markers, Bcl2, p53 and TOP2A are key molecular biomarkers in BC and provide prognostic and predictive value, however, their significance varies in the different molecular classes and in particular, in relation to HR status of BC. The results of GEP studies in BC have led to the understanding that BC is a genetically complex disease involving multiple molecular mechanisms and biological pathways that distinct molecular subtypes of BC may, in fact, constitute different diseases, and that proliferation is not only a prognostic marker but also a predictor of response to endocrine therapy and neoadjuvant chemotherapy [12]. Based on the current lines of evidence, the inclusion of a proliferation marker (i.e. mitotic counting and/or Ki-67 assessment) to the current panel of predictive markers (ER, PR and HER2) would be desirable. It should be noted, however, that standardisation of methods for proliferation assessment and the cut-offs for the different clinical contexts still need to be standardised before proliferation can be introduced in routine clinical practice.

The increasing number of treatment options has further increased our need to improve classification and clinical management for individual BC patients and emphasises the need for improved understanding of the significance and added value of combinatorial expression of key biomarkers in BC. Despite the requirement of stringent standardisation, quality control and the use of antibodies extensively validated [85, 134], IHC can provide excellent assessment of biomarker expression in BC. It should be emphasised that

IHC overcomes one of the most important limitations of GEP (i.e. the contamination of tumour samples with stromal cells and inflammatory infiltrate), as the distribution of the biomarker of interest can be determined in situ with direct morphological control. However, pathologists should strive for optimising pre-analytical and analytical parameters to ensure reproducible results.

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