Genetic characterization of breast cancer and implications for clinical management

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Abstract

Breast cancer is a genetic disease caused by the accumulation of mutations in neoplastic cells. In the last few years, high-throughput microarray-based molecular analysis has provided increasingly more coherent information about the genetic aberrations in breast cancer. New biomarkers and molecular techniques are slowly becoming part of the diagnostic and prognostic armamentarium available for pathologists and oncologists to tailor the therapy for breast cancer patients. In this review, we will focus on the contribution of breast cancer somatic genetics to our understanding of breast cancer biology and its impact on breast cancer patient management.

Keywords: microarray ● comparative genomic hybridization ● gene expression ● biomarker ● therapeutic target

Introduction

Breast cancer is a genetic disease caused by the accumulation of mutations in neoplastic cells [1–3]. Genetic analyses have for long been performed in breast cancer research in order to unravel the molecular aberrations leading to tumour initiation and progression [1, 3, 4]. In the last few years, increasingly more coherent information about genetic aberrations in breast cancer has been generated and molecular techniques are slowly becoming part of the diagnostic and prognostic armamentarium available for pathologists and oncologists to tailor the therapy for breast cancer patients.

Despite the advancements, it should be noted that breast cancer management still relies on clinico-pathological features (*i.e.* tumour size, histological grade and presence of axillary lymph node metastasis) and three immunohistochemical markers (oestrogen receptor [ER], progesterone receptor [PR] and HER2) for treatment decision-making) [5, 6]. However, it has become clear in the last years that these parameters are not sufficient to tailor therapy for individual patients and a more predictive model is needed.

Since the development of approaches for high-throughput molecular analysis in the 90s, studying the whole genome and transcriptomic of cancers in a single experiment has become a reality [5, 6]. This technological advancement has led to a paradigm shift in cancer research: from a reductionist approach where single genes/ proteins could explain complex phenotypes, to a model where phenotypic characteristics are explained by the interaction of multiple genetic, epigenetic and transcriptomic aberrations. Furthermore, these methods have provided a unique opportunity to unravel the molecular underpinning of the histological characteristics of cancers and their clinical behaviour. In fact, seminal high-throughput genetics and transcriptomic studies have brought to the forefront of breast cancer research the concept that breast cancer is a heterogeneous disease and provided some tantalizing evidence to suggest that there is a high degree of phenotypic–genotypic correlations in breast cancer [7–10].

Several reviews have addressed the contribution of high-throughput expression profiling to our understanding of breast

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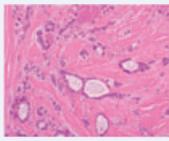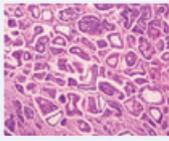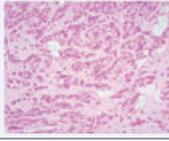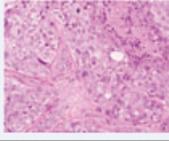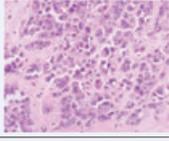E-mail: Jorge.Reis-Filho@icr.ac.uk

Molecular Subtype	ER	PR	HER2	Basal Markers[†]	Other Markers	Proliferation	Molecular Pathways	Genomic Pattern	Histological Grade*	Histological type°	Morphology
Luminal A	+++	+++	-	-		Low	Luminal Cks ↑;ER-pathway ↑↑↑ (e.g. *CCND1, TFF1, GATA3*)	Simplex	1 or 2	Tubular carcinoma, Cribriform carcinoma, IDC-NST, Classic Lobular carcinoma	
Luminal B	+	+/-	+/-	+/-		High	Luminal Cks ↑; ER-pathway ↑ (e.g. *CCND1, TFF1, GATA3*); *TP53* mutations	Mainly complex, sawtooth or firestorm	2 or 3	IDC-NST, Micropapillary carcinoma	
HER2+/ER-	-	-	+	+/-		High	HER2 amplicon genes ↑ (e.g. *GRB7, TOP2A, GATA4*); *TP53* mutations	Complex firestorm	Mainly 3	IDC-NST	
Basal-like	-	-	-	+		High	Basal Cks ↑; DNA repair ↓ (BRCA1 pathway); *TP53* mutations	Complex sawtooth	Mainly 3	IDC-NST, Metaplastic carcinoma, Medullary carcinoma, Adenoid Cystic carcinoma	
Molecular apocrine	-	-	+/-	+/-	AR+	High	Androgen signalling ↑; Fatty acid and lipid synthesis ↑	Complex, sawtooth or firestorm	2 or 3	Apocrine carcinoma, IDC-NST, Pleomorphic Lobular carcinoma	
Claudin-low	-	-	-	+/-	E-cad-	High	Cell-cell adhesion ↓ (e.g. *E-cadherin*); EMT and stem cell function ↑	Complex, sawtooth or firestorm	Mainly 3	Metaplastic carcinoma, IDC-NST	
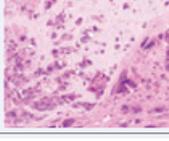
Fig. 1 Molecular subtypes of breast cancer. [†] Basal markers: epidermal growth factor receptor (EGFR) and Cytokeratins 5/6, 14 and 17; * Nottingham grading system; ° Most prevalent; Ck: cytokeratin; E-cad: E-cadherin; EMT: epithelial-mesenchymal transition; ER: oestrogen receptor; PR: progesterone receptor; AR: androgen receptor; IDC-NST: invasive ductal carcinoma of no special type.

cancer and its impact on breast cancer patient management [5, 6, 11–15]. This review focuses on the characterization of genetic aberrations in breast cancer cells (*i.e.* somatic genetics) rather than germline DNA and how studying these genetic aberrations is leading to a paradigm shift in the way breast cancer is perceived and how breast cancer patients are treated.

Contribution of gene expression analysis

Microarray-based gene expression profiling studies can be performed in multiple ways. One of the approaches, pioneered by the Stanford group [16, 17], focused on the use of unsupervised methods to test whether microarrays would provide biologically

and/or clinically meaningful information about breast cancer diversity. Unsupervised hierarchical clustering using an 'intrinsic gene list' led to the identification of five molecular groups according to their expression pattern, namely: luminal A, luminal B, HER2, basal-like and normal breast-like (Fig. 1).

The most obvious distinction observed by microarray analysis is between the transcriptome of ER-positive and ER-negative breast cancers. The cluster enriched for ER-positive tumours displays an expression pattern to some extent reminiscent of that of normal luminal epithelial cells of the mammary gland, including consistent high-level expression of low molecular weight cytokeratins 8/18, ER and genes associated with an active ER pathway [12, 13, 16–18]. The luminal subtype is further sub-classified into at least two subgroups: luminal A and luminal B. Luminal B tumours are more often of higher histological grade and have a significantly worse prognosis. Expression of proliferation-related

genes appears to be the major difference between each subgroup (low in luminal A and high in luminal B). Although the separation of luminal tumours in two subgroups of prognostic significance is appealing, a recent large meta-analysis of published available expression data suggested that luminal tumours form a continuum and that the separation of these tumours into two subgroups based on proliferation is arbitrary [19].

The ER-negative cluster appears to be substantially more heterogeneous. In the studies carried out by the Stanford group, three different subtypes were identified: normal breast-like cancers, HER2 and basal-like. Normal breast-like cancers are rather poorly characterized and their clinical significance is yet to be determined [12, 13, 16–18]. Some have suggested that this subgroup may be a mere artefact of expression profiling (*i.e.* a disproportionally high content of stromal cells) [20], given that identification of this group of tumours by microarrays is less stable when fine needle aspiration biopsies are used [21] or when microdissected samples are subjected to expression array analysis (JS Reis-Filho and R Natrajan, unpublished observations). The HER2 and basal-like subtypes have in common an aggressive clinical behaviour. HER2 tumours are characterized by overexpression of HER2 and genes associated with HER2 pathway and/or *HER2* amplicon on 17q12. Although the vast majority (>80%) of HER2 cancers as defined by microarrays harbour *HER2* gene amplification or HER2 3+ immunohistochemical expression [13, 21], not all tumours that are *HER2*-amplified fall into the HER2 cluster by expression arrays analysis. There is also evidence to suggest that some *HER2*-amplified, ER-positive cancers fall within the luminal B subtype rather than the HER2-microarray subtype [20, 21]. Basal-like subtype is the third group in the ER-negative cluster and is so named because the neoplastic cells of this tumour type consistently express genes usually found in normal basal/ myoepithelial cells of the breast, including high molecular weight cytokeratins (5/6 and 17), P-cadherin, caveolins 1 and 2 [22–29], nestin [30] and epidermal growth factor receptor (EGFR) [24] and, in a minority of cases, harbour *EGFR* gene amplification [12] or aneusomy [31]. These tumours are usually of high histological grade and characterized by high mitotic indices, the presence of central necrotic zones, pushing borders, conspicuous lymphocytic infiltrate and typical/ atypical medullary features [32, 33]. Moreover, metaplastic elements are not uncommonly found [34]. In addition to these three subgroups, recent studies have led to the identification of at least three additional molecular subtypes of ER-negative cancers, namely a 'molecular apocrine' subgroup, which shares some features with the HER2 subtype and appears to have activation of the androgen receptor signalling [35], an interferon subtype, which is characterized by high expression of interferon regulated-genes, including *STAT1* [36] and a 'claudin-low' subgroup, which comprises tumours that have transcriptomic features suggestive of a 'stem cell-like' phenotype [37, 38] (Fig. 1). It should be noted that the clinical and biological significance of tumours pertaining to these newly described classes remains to be determined.

Despite the interest that this molecular taxonomy has generated, it should be remembered that this is a working model and that further classes may be identified. Furthermore, several authorities in breast cancer molecular profiling have postulated that this molecular taxonomy would have histogenetic implications [17, 18, 39]. For instance, basal-like cancers would originate in basal cells, whereas luminal cancers would stem from luminal epithelial cells. It should be noted, however, that direct evidence in support of this concept is scant and that there is evidence to suggest that specific genetic aberrations may lead to phenotypic changes during breast cancer progression (*e.g.* BRCA1 loss of function leads to down-regulation of ER pathway) [8, 40, 41].

Another controversial issue related to this molecular taxonomy is related to the terminology 'basal-like', given that unlike basal cells of normal mammary gland, basal-like tumours express low molecular weight cytokeratins 8/18 and, on the other hand, expression of basal (high molecular weight) cytokeratins 5/6 and 17 is not restricted to basal cells [42]. Although there is a substantial overlap between basal-like cancers and triple negative phenotype (ER-negative, PR-negative and HER2-negative), the terms basal-like and triple negative should not be used as synonymous [43–46]. There is direct evidence to demonstrate that only 70–80% of tumours classified as basal-like by microarray analysis are of triple negative phenotype and that only 70–80% of triple negative cancers are basal-like cancers [21, 44, 45, 47, 48]. In fact, triple negative tumours encompass the majority of basal-like and claudin-low cancers, and a subgroup of molecular apocrine and normal breast-like tumours. Furthermore, the clinical behaviour of triple negative cancers expressing or lacking basal markers appears to differ. Retrospective studies have suggested that triple negative cancers that express basal markers have a worse outcome than triple negative cancers lacking cytokeratins 5/6, 14, 17 and EGFR [43, 49].

This molecular classification has attracted the attention of both scientific and medical communities due to its prognostic significance and potential predictive power. Luminal tumours are amenable to treatment with endocrine therapy, whilst HER2 cancers may respond to novel tailored therapies using either humanized monoclonal antibodies against HER2 or HER2 tyrosine kinase inhibitors [12, 13, 16–18, 50–54]. Despite the interest in applying this taxonomy to patient management, the use of microarrays for patient decision-making has proven challenging due to their poor performance when RNA from formalin-fixed paraffin-embedded tissues is used. Several groups have endeavoured to generate surrogate markers for the 'intrinsic gene list' molecular taxonomy [20, 24, 43, 49, 55, 56] and although approaches have been put forward, there is no internationally accepted definition for the molecular subgroups. In fact, even in the microarray-based studies, different intrinsic gene lists and statistical methods were employed to identify the molecular subgroups rendering the comparison of their clinical and biological features challenging.

Gene expression studies can also be undertaken in a supervised fashion aiming to devise gene signatures that accurately predict the class membership of a new sample on the basis of the expression levels of key genes. Such predictors can be used for many types of clinical decision-making, including diagnosis,

prediction and, most often, prognostication. Using this supervised approach, numerous microarray-based prognostic gene signatures have been developed [6, 11] and are described as being able to provide prognostic information beyond standard clinical assessment. Signatures for prognostication [57–59], prediction of response to Tamoxifen [60, 61] and chemotherapy, classifiers based on histological grade [62–64] and expression pattern of the stromal compartment [65–67], predictors specific for ER-negative tumours [68] have all been published and described as statistically independent prognostic or predictive factors. However, none of these signatures has been formally subjected to an independent validation in a clinical trial context.

These classifiers attracted great attention in the oncology community as they may, in fact, be useful for clinical decision-making, particularly in the increasingly common situation of a node-negative patient with a small ER-positive invasive carcinoma. Comprehensive reviews on these signatures have already been published [6, 11] and are beyond the scope of this review. It should be noted, however, that some of these signatures have already been introduced in the market. The 70-gene signature [58, 69] is offered under the name of Mammaprint and is currently being tested in the MINDACT (*i.e.* Microarray In Node-negative Disease may Avoid ChemoTherapy), a prospective randomized study comparing the 70-gene signature with the common clinical-pathological criteria in selecting patients for adjuvant chemotherapy in node-negative breast cancer. Given that Mammaprint is a microarray-based assay, it depends on RNA-preserving tissue collection, complicating its implementation in the clinical practice. In a pilot study for the MINDACT trial, Mook *et al.* [70] addressed this issue directly and described a 72% success rate under optimal experimental conditions, indicating that close collaboration between surgeons, clinicians, pathologists and researchers is needed for a successful implementation of this technique in the daily practice. In the RASTER (*i.e.* MicroarRAy PrognoSTics in Breast CancER) study, which also evaluated the use of the Mammaprint signature in a clinical setting, out of 585 eligible patients, 31.6% (158) of patients were excluded because of sampling failure ($n = 128$) and incorrect procedure ($n = 30$) [71]. Furthermore, although the 70-gene signature was devised to increase the number of patients who could be safely spared the cytotoxic effects of chemotherapy, adjuvant chemotherapy was more often administered to patients whose therapy was guided by the results of the prognostic signature than to patients who were managed solely according to the Dutch CBO clinical-pathological guidelines [71].

The Recurrence Score, also named Oncotype Dx, is a quantitative reverse transcriptase-based assay which has been developed to be applied in early-stage, endocrine-responsive, Tamoxifen-treated patients, is based on the mRNA expression levels of 21 genes [72] and makes use of formalin-fixed paraffin-embedded tissue. OncotypeDx has already been introduced in clinical practice, given that it was validated in prospectively collected clinical trial samples that allowed to its validation [73, 74]. This signature identifies three groups: low, intermediate and high recurrence scores. Low recurrence score patients appear not to benefit from adjuvant therapy and to benefit more from endocrine therapy; on the other hand, patients with high recurrence score cancers derive significantly greater benefit from chemotherapy. The management of patients with tumours with intermediate recurrence scores is currently being prospectively addressed in the TAILORx (*i.e.* Trial Assigning Individualized Options for Treatment [Rx]) trial, which will determine whether patients with intermediate score should be treated with Tamoxifen only or with a combination of Tamoxifen and chemotherapy.

Given the fact that a multitude of signatures are available and that the overlap between the gene lists is negligible [75], one faces the challenge of which signature should be used. Several studies have been performed to address the concordance between the signatures and concluded that most of them showed significant agreement in their outcome prediction for the individual samples [19, 75–79]. Wirapati *et al.* [19] have not only confirmed that different prognostic signatures identify similar groups of breast cancer patients, but also that the assignment of patients into good or poor prognosis is largely dependent on the expression levels of genes pertaining to the 'proliferation cluster'. Some signatures performed even better when only the proliferation-related genes were used to predict prognosis [19]. Another conclusion drawn from these analyses is that the prognostic power of most classifiers is limited to the subgroup of ER-positive/ HER2-negative subgroup [19, 76], providing another line of evidence that proliferation is the major determinant of prognosis in this subgroup of patients.

High-throughput genetic analysis

The concept that breast cancer encompasses a plethora of entities with distinctive biological characteristics and clinical behaviour is also underpinned at the molecular genetic level by a complex array of genetic alterations that affect the function and control of individual genes and cellular processes [1, 80]. Not only expression profiling analysis, but also the study of cancer genetics has had a profound impact in our understanding of the evolutionary pathways and causative factors in the initiation, development and progression of breast cancer. Furthermore, the use of microarray-based comparative genomic hybridization (aCGH) has proven to be a very useful tool for the identification of potential therapeutic targets [81].

Genomic profiling of invasive breast tumours with high-resolution aCGH has shown that breast cancers can be classified into three groups according to the pattern of genetic aberrations they harbour [82–85]. Hicks *et al.* [83] profiled a series of 243 breast tumours with oligonucleotide arrays and described three patterns of genomic profiles, significantly associated with prognosis. The genomic profiles are first classified as 'simplex' or 'complex'. The 'simplex' pattern is characterized by broad segments of duplications and deletions, usually comprising entire chromosomes or chromosomes arms, with occasional isolated narrow peaks of amplification. This

group is associated with good outcome and is typical of low-grade cancers, frequently displaying concurrent 1q gain and 16q loss. On the other hand, the complex pattern is associated with poor outcome and comprises two distinct categories: 'sawtooth' and 'firestorm'. The 'sawtooth' category is characterized by many narrow segments of duplication and deletion, often alternating and affecting all chromosomes. Although most of the genome harbours copy number changes, the events typically do not involve amplifications. The second complex pattern, called 'firestorm', resembles the simplex type, however displays at least one localized region of clustered, relatively narrow peaks of amplification, each one confined to a single chromosome arm. Similar genomic patterns were described by Chin *et al.* [82], under the terms '1q/16q' or 'simple', 'amplifier' and 'complex'. Aspects of the genome abnormalities, such as the fraction of the genome altered and number of amplified arms, were significantly associated with distinct histopathological features. A strong association of high-level amplification with poor outcome was also described. For instance, amplification of any of the recurrent amplicons found in this cohort (8p11–22, 8q24, 11q13–14, 12q13–14, 17q11–12, 17q21–24, 20q13) was an independent predictor of reduced survival and distant recurrence.

It is thought that some of the variety in the complexity of the copy number profiles seen in breast tumours may be attributed to the underlying DNA repair defects present in the tumours. For instance, in the firestorm/ amplifier pattern, the clustering of multiple amplicons on a single arm may reflect a concerted mechanism of repeated recombination on that arm rather than a series of independent amplification events [83]. Certain arms are more prone to undergo this process, such as 11q and 17q, which harbour genes already identified as amplicon drivers, including *CCND1* and *HER2*, respectively. The 'sawtooth'/ complex pattern, on the other hand, may indicate a different type of genetic instability acquired during tumourigenesis.

Several studies have addressed the genome-wide copy number alterations of the molecular phenotypes of breast cancer, either defined by gene expression profiling [82, 86–89] or immunohistochemical surrogates [85]. Those studies revealed that the molecular subtypes of breast cancer harbour different patterns of genetic aberrations and distinct recurrent changes (Fig. 1). For instance, the luminal subtype is more often of simplex pattern, the HER2 subtype displays typically a firestorm pattern and the basal-like tumours are usually of 'sawtooth' pattern [85]. Those results indicate that at least some of the phenotypic diversity of breast cancers may be driven by the pattern of genetic aberrations they harbour.

Luminal A cancers are characterized by few copy number changes, displaying 'simplex' profiles. They typically harbour the changes characteristic of the 'low nuclear grade breast neoplasia family' [90, 91]; that is, 1q gain, 16p gain and 16q loss [82, 87, 88]. Luminal B tumours are characterized by more complex genomes and display high-level amplifications more frequently. Gains of 1q, 8q, 17q and 20q, losses of 1p, 3q, 8p, 13q, 16q, 17p, and 22q, and amplifications involving 8p11–12, two regions of 8q,

11q13–14, 19q13 and 20q13 have been described to be prevalent in luminal B tumours [82, 87].

As expected, the HER2 subtype displayed the highest prevalence of high-level amplification of 17q12 [82, 85, 87], but amplifications of 7p15, 7p14, 8q21-q24, 11q13.2-q13.3, 14q11.1-q11.2, 14q12-q13.1, 14q23.2-q23.3, multiple loci on distal regions of 17q, 19q13.41, 21q22.3, and 22q11 were also reported [82, 85]. Gains of 1q, 7p, 8q, 16p and 20q and losses of 1p, 1q, 8p, 8q, 13q, 17p and 18q were also recurrent in this subgroup of breast cancer [82, 85, 88].

Expression profiling analyses have suggested that *HER2*-amplified tumours can be subdivided into two subgroups: a luminal B, ER-positive group and an ER-negative/ HER2-positive group. Our group performed a genomic analysis of *HER2*-amplified tumours stratified according to ER expression and demonstrated that *HER2*-amplified ER-positive and ER-negative cancers are remarkably similar at the genetic level, with only 1.43% of the genome differentially gained or lost between the two subgroups. Importantly, though, gains of 17q23–24, losses of 1p, 7q, 9p21.3 and 11q13.5 and amplifications of 11q13.3 and 17q21.2 were shown to be significantly associated with the *HER2*-amplified ER-positive subgroup, whilst ER-negative tumours were associated with gains of 5p15-p12 [92]. Taken together, these findings provide circumstantial evidence to suggest that *HER2*-amplified tumours constitute a distinct entity regardless of the ER status. On the other hand, analysis of an extended cohort by CISH has shown that *TOP2A* was preferentially amplified in the ER-positive subset of *HER2*-amplified tumours, indicating that distinct therapeutic targets may be specific to distinct subgroups of *HER2*-amplified tumours [93].

Basal-like and triple negative tumours are characterized by a sawtooth genomic pattern; therefore, they are particularly enriched for low-level copy number gains across all chromosomes, whilst amplifications are less frequently found in these subgroups than in luminal B and HER2 cancers. Chromosome arms described to harbour increased copy number included 1q, 3q, 6p, 6q, 7q, 8q, 9p, 10p, 11q, 12p, 12q, 13q, 16q, 17q, 18q and 21q, and reduced copy number was frequent in 1p, 1q, 3p, 4p, 4q, 5q, 8p, 7p, 10q, 12q, 13q, 14q, 15q, 16p, 17p, 17q, 20q, 22q and Xq [82, 85–87, 94, 95]. Andre and Pusztai [94] have described that gains of 6p21.2–6p12 are particularly associated with triple negative tumours, a region also described as harbouring gains or amplifications by other groups [85, 86]. Although rare, recurrent amplifications in basal-like cancers were found at 1q21, 3q25, 3q26, 8q24, 12p13, 15q26, 19q11–12 [85, 86]. Basal-like and triple negative carcinomas commonly display a high degree of genetic instability; however, by performing an oligo-array-based copy number analysis of 171 tumours of relatively small size, Chin *et al.* [89] have described a new subtype of breast cancer, characterized by low genetic instability and enriched for high histological grade, ER-negative and basal-like tumours, which can also be observed in the data presented by Natrajan *et al.* [85]. The authors could further derive a gene expression signature for this low genetic instability subtype, which, when applied to independent cohorts, could identify a cluster enriched for ER-negative tumours.
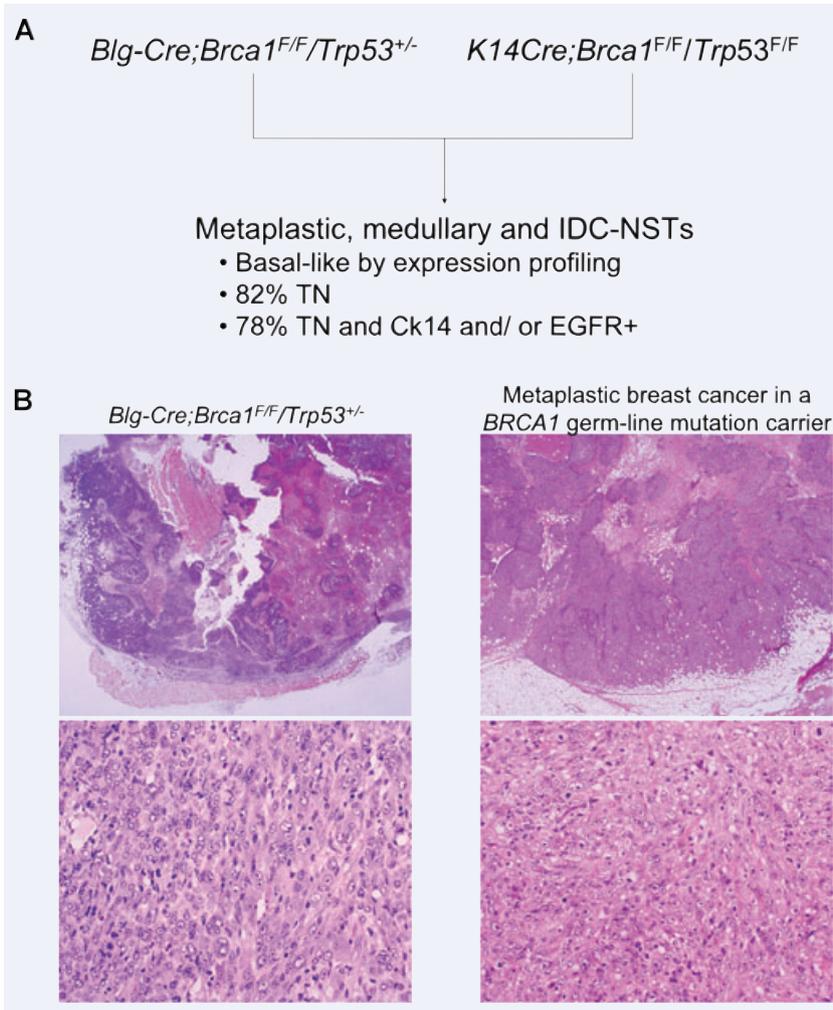
Fig. 2 Conditional mouse models of basal-like and triple negative breast cancers. (**A**) *Brca1* and *Trp53* inactivation in different cellular compartments of the mouse mammary gland leads to the development of basal-like and triple negative breast cancers. In the *Blg-Cre;Brca1$^{F/F}$/Trp53$^{+/−}$* mouse model, Brca1 was inactivated in luminal epithelial cells of the mouse mammary gland and all cells harboured only one functional copy of *Trp53*. In the *K14cre;Brca1$^{F/F}$/Trp53$^{F/F}$* mouse model, *Brca1* and *Trp53* were inactivated in the basal/ myoepithelial cells of the mouse mammary gland. Histopathological and immunohistochemical analysis of tumours developing in mouse models where *Brca1* and *Trp53* were inactivated in different lineages of the mouse mammary gland revealed that tumours had morphological and immunohistochemical features that recapitulated those of human basal-like breast cancers [8, 9] (*i.e.* a convergent phenotype driven by the type of genetic hits). (**B**) Representative scanning and medium power magnification micrographs of a tumour developing in *Blg-Cre;Brca1$^{F/F}$/Trp53$^{+/−}$* mouse model and in a patient with a pathogenic *BRCA1* truncating germline mutation. Ck: cytokeratin; EGFR: epidermal growth factor receptor; IDC-NST: invasive ductal carcinoma of no special type; TN: triple negative.

Phenotypic–genotypic correlations

The study of the molecular genetics and the phenotypic diversity of breast cancers has revealed interesting genotypic–phenotypic correlations [10, 96–101]. This is perhaps best exemplified by secretory carcinoma, a rare special type of breast cancer. This histological type consistently harbours a t(12;15) (p13;q25) chromosomal translocation [10, 102, 103], involving the genes *ETV6* and *NTRK3*. Furthermore, it has been demonstrated that this translocation is specific of tumours with the typical secretory morphology [102, 104], not being present, for example, in acinic cell carcinomas, a histological type once considered to be a variant of the secretory carcinoma [105].

The study of germline genetic aberrations and breast cancer phenotypic diversity has also revealed another example of genotypic–phenotypic correlations in breast cancer. Tumours arising in *BRCA1* germline mutation carriers display a rather specific constellation of morphological features and these features are strikingly similar to those described in basal-like breast cancers [28], including high histological grade, atypical medullary features, high proliferation indices, pushing borders and conspicuous lymphocytic infiltrate [98, 106, 107]. Over 75% of these cancers display a triple negative phenotype, express basal markers and display *TP53* mutations [17, 108, 109]. In addition, tumours from *BRCA1* mutation carriers cluster predominantly in the basal-like group by gene expression profiling [18, 110]. Taken together, there is evidence to suggest a strong genotypic–phenotypic correlation between *BRCA1* and the basal-like phenotype [28, 41, 98, 111–113]. This genotypic–phenotypic correlation has been further confirmed by the development of two conditional mouse models, where *Brca1* and *Trp53* were inactivated either in the basal or luminal cells of the mouse mammary gland [8, 9]. These transgenic animals [8, 9] developed tumours whose histopathological, immunohistochemical and transcriptomic characteristics recapitulated the cardinal features of human basal-like breast cancers (Fig. 2).

The analysis of lobular neoplasia and invasive lobular cancers has also revealed an important genotypic-phenotypic association in breast cancer [114, 115]. Despite the similarities between low-grade ductal and lobular carcinomas at the immunohistochemical and genetic levels, several lines of evidence have suggested that these two histological subtypes differ by the target gene of 16q deletions [115–118]. Whilst in low-grade ductal cancers, the target gene remains to be identified, in lobular proliferations, it has been shown to be the *CDH1* gene, which encodes E-cadherin, an adhesion molecule that mediates homophylic-homotypic adhesions [119–121]. Loss of E-cadherin expression has been shown to be associated with the characteristic histological features of lobular carcinoma (*i.e.* discohesive cells) and some have hypothesized that E-cadherin loss of function would drive the clinical behaviour of this special type of breast cancer [114, 122] and the peculiar metastatic pattern, characterized by deposits in unexpected anatomical sites, such as the peritoneum, meninges, gynaecological and gastrointestinal systems [123]. Direct evidence in support of this concept was provided by a conditional mouse model ($Cdh1^{F/F};Trp53^{+/+}$) [7].

Pure micropapillary carcinoma is another rare special type of breast cancer, which constitutes a distinct entity at the morphological, transcriptomic [124] and genetic levels [100], indicating genotypic–phenotypic correlation. Morphologically, micropapillary carcinomas display a characteristic growth pattern of cell clusters with inverted polarity and are associated with a higher prevalence of lympho-vascular invasion and lymph node metastatic deposits. Unsupervised hierarchical clustering of gene expression profiles of distinct special types of breast cancer revealed that micropapillary carcinomas formed a separate cluster [124]. In addition, immunohistochemical and aCGH analyses [100] revealed that these cancers display a luminal B phenotype and have a distinct pattern of genetic aberrations when compared to that of ER- and grade-matched invasive ductal carcinomas, providing strong circumstantial evidence that micropapillary morphology is not a mere histological pattern but actually identifies a discrete molecular entity [100, 101]. Interestingly, the analysis of mixed tumours with areas of micropapillary differentiation admixed with other growth patterns revealed remarkably similar patterns of genetic aberrations and immunohistochemical profiles between the papillary and non-papillary components of each tumour [101]. This is in agreement with previous studies suggesting that the presence of a micropapillary component, even in the form of a small focus in an invasive ductal carcinoma, is associated with a pattern of metastases and clinical behaviour similar to those of pure micropapillary carcinomas [101].

Biomarkers and therapeutic targets

Despite the translational research efforts of 1980s and 1990s, only three biomarkers have been introduced in pathology laboratories to define the therapy of breast cancer patients: ER, PR and HER2.

Interestingly, all of these biomarkers have optimal negative predictive values (*i.e.* patients with ER-negative breast cancer are highly unlikely to respond to endocrine therapy; HER2 negative breast cancers fail to respond to humanized monoclonal antibodies against HER2). However, their positive predictive value is rather limited, with a substantial proportion of patients with HER2-positive disease either harbouring *de novo* resistance or developing resistance to trastuzumab over time [125]. Approaches targeting DNA-damage defects in triple negative/ basal-like cancers have been described, however they have not been completely implemented in the clinical practice. Therefore, new biomarkers and therapeutic targets, associated with robust and reliable companion diagnostics, must be identified.

Molecular studies have confirmed the importance of these three biomarkers and there are several lines of evidence to suggest that breast cancer comprises at least two distinct diseases (ER-positive and ER-negative). Studies aiming to identify new biomarkers and therapeutic targets, and/or evaluate response to therapy should take into account the distinctive molecular features of the different subtypes, otherwise they risk under- or overestimating their performance [94, 126, 127]. Specific molecular pathways and networks may be important in one particular molecular subtype, such as proliferation-related genes in ER-positive cancer, but not in others (*e.g.* proliferation-related genes in ER-negative cancers) [76].

Following the multitude of profiling studies on breast cancer, several new biomarkers and potential therapeutic targets have been identified. It is likely that in the future new predictive markers specific for each one of the molecular subtypes of breast cancer will be added to the routine panel; however, prospective validation of the markers identified so far is still missing for their implementation in clinical practice. Here, we will present advances in some of the available markers/ targets and will also discuss others which may soon become available.

HER2 assessment and resistance to trastuzumab

HER2 status is currently assessed at the protein level by immunohistochemistry or at the DNA level by *in situ* hybridization, either fluorescence (FISH) or chromogenic (CISH), which has been recently approved for use in North America by the Food and Drug Administration (FDA) organization. Clinical guidelines for HER2 assessment are constantly being reviewed and current protocols follow the latest American Society of Clinical Oncology/ College of American Pathologists recommendations published in 2007 [128]. When assessed by FISH, most frequently two locus-specific probes for the *HER2* gene and chromosome 17 centromere (CEP17) are used. CEP17 probe is used to correct the absolute *HER2* gene copy number with the number of chromosomes 17 (*HER2*:CEP17 ratios). When more than three copies of CEP17 are detected, the case is presumed to harbour chromosome 17 polysomy. Recent data [129, 130], however, derived from aCGH studies, have shown that increased copies of CEP17 are rarely due to true chromosome 17 polysomy and may actually stem from gains and/or amplification of CEP17, regardless of copy number gains of

the short and long arms of chromosome 17 [130]. Furthermore, correction with CEP17 probes in some cases may provide misleading *HER2* gene status assessment [130], resulting in patients being denied of effective anti-HER2 therapy.

HER2 gene copy number assessment, albeit constituting a marker with high negative predictive value (*i.e.* patients lacking *HER2* gene amplification are very unlikely to benefit from anti-HER2 agents), its positive predictive value is rather limited, given that a substantial number of patients are either *de novo* resistant or acquire resistance over time to anti-HER2 agents. In the last years, several mechanisms of *de novo* and acquired resistance to trastuzumab have been identified. It is currently accepted that *PIK3CA* activating mutations, PTEN loss of function, IGF1R overexpression or expression of p95 HER2 isoform may all play a role in both *de novo* or acquired resistance to therapies that target HER2 [125, 131]. Furthermore, it has been recently suggested that *HER2*-amplified breast cancers that harbour a basal-like transcriptome may be less sensitive to anti-HER2 therapies [132]. Therefore, one can anticipate that additional molecular testing of HER2-positive cancers is likely to be incorporated in clinical practice to increase the positive predictive value of predictors of response to anti-HER2 agents.

Topoisomerase IIα

The function of topoisomerases is to separate the strands of the DNA double helical structure and, therefore, these enzymes are fundamental for all cell processes that need to access the information stored in the DNA, such as transcription, recombination and replication [133]. Topoisomerase IIα is encoded by *TOP2A* gene which maps to 17q12, in close proximity to *HER2* gene, is co-amplified in 22–55% of *HER2*-amplified tumours [93, 134–140] and its amplification seems to be limited to *HER2*-amplified tumours [93, 135, 136, 139, 140]. HER2 status has been reported to predict response to anthracycline-based chemotherapy [141–144], however, topoisomerase IIα is the direct molecular target of anthracyclines, which bind covalently with topoisomerase IIα after double-strand breaks have occurred, inducing lethal cellular damage by inhibition of relegation [133]. Based on the fact that topoisomerase IIα is the molecular target of anthracyclines, it has been hypothesized that tumours with *TOP2A* gene amplification may display an exquisite sensitivity to anthracycline-based chemotherapy and that *TOP2A* gene copy number assessment could be used as a predictive marker for this type of systemic therapy. Several retrospective analyses have indeed described increased benefit with anthracyclines-based therapies in patients harbouring *TOP2A* amplification [93, 137, 139, 145]. However, data are still contradictory and *TOP2A* deletions have also been shown to be associated with 13–43% of *HER2*-amplified tumours [134–138] and potentially to mediate sensitivity to anthracyclines [137]. Recent studies have been published with discordant results. O'Malley *et al.* [138] surprisingly found considerable number of cases harbouring *TOP2A* amplification without *HER2* amplification and described a non-significant interaction between anthracycline treatment and the simultaneous presence of *TOP2A* alteration (a combined category of deletion + amplification) and *HER2* amplification for both recurrence free survival and overall survival. Harris *et al.* [134] compared regimens with different doses of anthracyclines and concluded that *TOP2A* amplification does not predict benefit from increased doses of anthracyclines in *HER2*-amplified breast cancer. Further prospective analyses are needed to clarify the prevalence of *TOP2A* amplification in tumours lacking *HER2* gene amplification and the role of *TOP2A* amplifications and deletions in relation to response to anthracycline-based chemotherapy in breast cancers.

PPM1D

PPM1D maps to 17q23.2 and encodes a member of the PPM serine/ threonine phosphatase family, which has been shown to have oncogenic properties and to relate with multiple cancer types, including breast cancers [146]. PPM1D activation results in a negative regulation of p53 function and other tumour suppressor pathways, by selective inactivation of p38 kinase [147, 148]. Furthermore, additional functions of PPM1D contributing to its oncogenic effect include regulation of the base excision pathway of DNA repair [149], progesterone receptor function [150] and the regulation of the cell cycle and DNA repair-associated CHK1, CHK2 and ATM kinases [149, 151–153]. PPM1D has also been linked with regulation of NFκB signalling [154] and loss of PPM1D function has been shown to sensitize cells to stress- and DNA damage-induced apoptosis [155]. Recently, PPM1D has been identified as a potential therapeutic target in a subset of HER2 and luminal tumours. *PPM1D* gene amplifications were found in 8% of invasive breast cancers (20% and 8% of HER2 and luminal tumours, respectively) [85]. Functional analysis revealed that breast cancer cell lines harbouring amplification and overexpression of *PPM1D* require both PPM1D expression and phosphatase activity for their survival and that PPM1D chemical inhibition is selectively lethal in cell lines displaying *PPM1D* gene amplification [85]. These results warrant the design of clinical trials to test the use of PPM1D inhibitors in patients with HER2 and luminal cancers harbouring *PPM1D* amplification and overexpression.

8p11-p12 amplicon

The 8p11-p12 region is reported to be amplified in 10–15% of breast cancers and is correlated with histological grade, proliferation rates and poor prognosis [156–159]. The expression and signalling of at least two genes in this amplicon have been shown to be required for the survival of cancer cells harbouring 8p11.2-p12 amplification: *FGFR1*, a tyrosine kinase receptor [120], and *PPAPDC1B*, a transmembrane phosphatase [160]. Although these genes constitute promising therapeutic targets, further clinical validation is required to establish whether inhibition of these genes in 8p11.2-p12-amplified cancers is a suitable therapeutic strategy for a subgroup of breast cancer patients.

BRCA1, basal-like tumours and PARP inhibitors

BRCA1 and BRCA2 function is required for competent DNA double-strand breaks repair by homologous recombination, which is an accurate mechanism of DNA repair [112, 161]. Tumours with BRCA loss of function have been shown not to be able to elicit homologous recombination in the presence of DNA double strand breaks and have to resort to error prone mechanisms, such as non-homologous end joining and single strand annealing, to correct these DNA defects [35, 161, 162]. It is believed that the defective homologous DNA repair is the cause of the high levels of genetic instability observed in BRCA deficient tumours [98, 112, 161]. Several groups have hypothesized that the defective homologous recombination DNA repair in BRCA tumours could be exploited from a therapeutic angle [98, 112, 161]. Pioneering work from Ashworth's group has provided direct evidence to suggest that BRCA deficient cells show an exquisite sensitivity to cross-linking agents, which cause DNA double strand breaks, and to inhibitors of the poly-ADP ribose polymerase (PARP) [163].

PARP is an enzyme, which also has a role in DNA repair processes. Its best-known function is in base excision repair, by detecting and binding single-strand DNA breaks. The inhibition of PARP causes an increase in persistent single-strand DNA breaks [164], which evolve to double-strand breaks during DNA replication. This agent has been shown to be selectively lethal in cells harbouring homologous recombination defects as those cells need to make use of error-prone mechanisms for DNA-damage repair, leading to chromosomal instability and cell death [35, 162, 163, 165]. This approach seems to be more specific and with fewer side effects than traditional therapies, leading to the development of clinical trials. Results from a phase I clinical trial, which has investigated the response to a novel PARP inhibitor AZD2281 (olaparib) in patients with tumours from distinct organs (*e.g.* breast, pancreas, prostate, ovary), have shown objective anti-tumour activity only in confirmed carriers of *BRCA1* or *BRCA*2 independently of the organ of origin. However, not all BRCA mutation carriers displayed benefit from the drug, possibly explained by different sensitivity to PARP inhibitors and acquisition of resistance [166].

Interesting mechanisms of resistance to agents targeting homologous recombination defect have been described. Edwards *et al.* [167] and Sakai *et al.* [168] developed models of BRCA2-deficient cells resistant to PARP inhibitors or cisplatin, respectively, and identified a novel mechanism of drug resistance. In both studies, resistance was shown to be caused by intragenic deletions of varying sizes spanning the original mutations in *BRCA2* and restoring its open reading frame. Importantly, the mechanism that rendered the cells sensitive in first place (*i.e.* lack of homologous recombination DNA repair) was the likely cause of the drug resistance. As BRCA deficient cells have a high degree of genetic instability, they easily acquire secondary genetic events, resulting in the presence in a given tumour/ cell line of multiple sub-clones. Edwards *et al.* [167] have demonstrated that the intragenic deletions encompassing the *BRCA2* mutations found in

PARP inhibitor resistant *BRCA2* mutant cells were flanked by sequences of microhomology, suggesting that these intragenic deletions were likely to be caused by the use of single strand annealing DNA repair, which was, ironically, prompted by the lack of competent homologous recombination. Treatment with PARP inhibitors leads then to selection of clones harbouring specific mutations, which restore homologous recombination and thus the cancer cells' ability to survive when exposed to these agents. The idea that sensitivity to a given therapy, which depends on a specific mutation for efficacy, can be abolished by a revertant mutation (*i.e.* intragenic deletion) that restores the activity of a gene inactivated by a mutation has direct clinical implications and raise considerations for new drug-design strategies and the whole field of pharmacogenomics [169]. Regarding the clinical setting, there are potential biomarkers, which could, theoretically, be used to investigate homologous recombination capacity and DNA damage in cancer cells. One of these biomarkers is Rad51, a protein that in response to DNA damage locates to the double-strand breaks. Rad51-containing foci (aka, RAD51 foci) can be visualized in the nuclei of cells with competent homologous recombination in the presence of DNA damage, whereas homologous recombination-deficient cells cannot produce them. It should be noted, however, that the presence of RAD51 foci may not necessarily equate with competent homologous recombination in 100% of cases. Furthermore, there is evidence to suggest that RAD51 foci may be formed in a BRCA2-independent fashion [170]; however, the biological significance of this observation remains to be determined. Another potential biomarker, which could be used in combination with Rad51, is H2AX, a histone that is phosphorylated by different PI3K family kinases shortly after double-strand breaks formation, indicating the cellular response after DNA damage [171, 172]. Similar to Rad51, foci of phosphorylated γH2AX can be detected by inmunohistochemistry in the nuclei of cells after the DNA damage has occurred and decreases as long as double-strand breaks are repaired. Banuelos *et al.* [172] have examined cervical cancer biopsies before and after the treatment with cisplatin and ionizing radiation and demonstrated that phospho-γH2AX foci could be detected in formalin-fixed paraffin-embedded samples and that increased production of phospho-γH2AX foci was associated with a better response to treatment. This marker has potential for clinical use but several steps of validation are still required.

Conclusion and future perspectives

High-throughput microarray-based technologies have changed the way breast cancer is perceived and highlighted its molecular heterogeneity. A comprehensive characterization of the genetic aberrations driving breast cancer has demonstrated a high degree of genotypic–phenotypic correlation and led to the discovery of new biomarkers and therapeutic targets for distinct subtypes of breast cancer. The advent of the next generation sequencing technology and the possibility to characterize numerical and structural

DNA changes, as well as single base pair aberrations, in a genome-wide fashion in a single experiment will certainly revolutionize breast cancer genetics and somatic genomics. Results from the International Breast Cancer Genome Consortium, where 2000 breast cancers will be entirely sequenced using high-throughput, massively parallel methods, will certainly lead to the identification of molecular drivers, 'addictive oncogenes', and additional genetic aberrations that can be exploited using a synthetically lethal approach. Most importantly, this new type of analysis will provide a unique opportunity of defining the molecular underpinning of the morphological features of specific subgroups of breast cancer and may lead to the development of a novel taxonomy for the disease.

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