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Chapter 2

Molecular profiling of breast cancer

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INTRODUCTION

Traditionally, breast cancer has been classified by tumour stage. Morphological features, namely histological tumour type, tumour grade, proliferation status and lymphovascular invasion, have also been recognised as important prognostic variables [1,2].

We now know that breast cancer is caused by a heterogeneous group of tumours, and that tumour behaviour and response to therapy is determined by underlying biological features. The widespread use of mammographic screening, the greater availability of systemic therapy options and the move towards precision medicine drove the transition from purely prognostic classification systems to those predict the response to various therapeutic modalities. The current morphological surrogates based on the anatomical and histological properties of the tumour do not, however, reflect the biological and molecular heterogeneity of breast cancer, nor do they fully explain the genetic abnormalities found in tumours. Therefore, the importance of finding molecular biomarkers that predict behaviour and response to therapy is increasingly recognised.

Gene expression profiling

The first molecular classification systems employed a limited number of biomarkers, namely hormone receptors and HER2 which predicted the response to hormone therapy and anti-HER2-targeted therapy respectively. Although these markers showed prognostic significance, their clinical application was based on their predictive value. With the introduction of high-throughput molecular technology, it became possible to conduct simultaneous analyses of the expression of thousands of genes in a single experiment. Using unsupervised clustering techniques, breast cancer was grouped into clusters or intrinsic subtypes based on the quantitative expression of several genes (transcriptome profiles). These molecular subtypes were recognised to be associated with the outcome of therapy [3,4].

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Although genome-wide microarray-based gene expression profiling (GEP) studies provided further evidence to support the hypothesis that oestrogen receptor (ER) and HER2 are the key determinants of the molecular profiles of breast cancers, the studies also showed that these two biomarkers alone were unable to explain the heterogeneity of breast cancer. This technology in fact provided a molecular explanation for breast cancer heterogeneity observed at the clinicopathological level. For instance, the luminal ER-positive class, which comprises approximately 70% of breast cancers, shows a heterogeneous clinical outcome and a variable response to systemic therapy. It has been documented in ER-positive breast cancer that hormone therapy reduces a 10-year recurrence rate by approximately 50% and reduces a 15-year mortality by a third [5]; however, a proportion of ER-positive tumours did not respond [6], indicating that other genes and pathways are involved in determining the response and behaviour of these tumours.

Currently available variables have limited predictive value, emphasising the need to identify molecular assays and gene signatures that determine the outcome and response to therapy more precisely. Therefore, the introduction of GEP techniques has provided hope that the molecular profiling of breast cancer can be used to predict outcome and response to treatment. Subsequent studies demonstrated not only an association between molecular intrinsic subtype classification and outcome and response to therapy, but they have also indicated that different pathways are active in certain subtypes, making them potential candidates for targeted therapy.

Although GEP-based molecular subtyping shows promise, its application in routine practice has been less successful because of the cost, access to the technology and the need for fresh tumour material. Subsequently, replicating microarray-based molecular 'intrinsic subtype' classification systems have been developed. These use more available technology, including reverse transcription polymerase chain reaction (RT-PCR), and immunohistochemistry (IHC) which utilises a limited number of biomarkers. More recently, molecular intrinsic subtyping that uses formalin-fixed paraffin-embedded (FFPE) material has become available [7].

Despite the limited clinical use of molecular intrinsic subtyping and class discovery studies, GEP technology has opened new avenues for the molecular prognostication of breast cancer and the prediction of response to therapy. Importantly, it led to the introduction of molecular multigene assays that aim to classify subgroups of breast cancer based on the outcome or response to different therapies [8]. These supervised class-prediction studies were based on the idea that various molecular subgroups of breast cancer can be categorised by the aggregation of genes rather than individual genes. This approach involves the identification of a set of genes (a gene signature) that can be used collectively to identify tumours with specific biological or clinical features. The term 'genomic signatures' was used to refer to the expression of a set of genes in a biological sample using microarray technology, while the term 'metagene' refers to the aggregate measure of the expression of a group of genes that show coordinated expression in a set of samples. Most of these multigene assays were used in breast cancer to stratify prognostically clinically relevant groups into low and high risk subgroups to guide further treatment.

In addition to prognosis and prediction use, GEP technology has proved useful in other applications. For instance, microarray-based gene expression tests have been developed to identify the cancer tissue of origin. These include the Pathwork Tissue of Origin Test that was

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developed using a 2000-gene classification model for identification of tumour tissue of origin with an overall accuracy of up to 90% [9], and the THEROS Cancer TYPE ID which is a RT-PCR-based test that uses 92 genes and FFPE samples [10].

Although the clinical use of molecular classification of breast cancer remains limited, the introduction of the next generation sequencing (NGS) or massively parallel sequencing (MPS) [11] appears to open new avenues for decoding breast cancer molecular complexity, refining molecular classification and identifying new therapeutic targets. These techniques hold promise for improving diagnosis, prediction of outcome and behaviour, and in aiding selection of therapies for individual patients [12]. Circulating tumour cells (CTCs) have also received attention as biomarkers that may refine detection of metastatic disease and burden of metastatic tumour and monitor cancer progression. However, its clinical utility is still under investigation [13].

Although these molecular classification systems provide complimentary prognostic and improved predictive power than conventional variables, we still have a long way to go in terms of delivering truly personalised medicine. Here, we present a review of current and emerging molecular classification systems of breast cancer with emphasis on the molecular intrinsic subtype and multigene signatures.

MOLECULAR CLASSIFICATION OF BREAST CANCER USING INTRINSIC SUBTYPES

The first use of GEP to stratify breast cancer into molecularly distinct classes was introduced by Perou and colleagues in 2000 [3]. In their pioneering study, they demonstrated that breast cancer at the transcriptome level is not a single disease. Despite the fact that each individual tumour possesses a unique genetic profile, tumours clustered together to produce distinct reproducible classes based on transcriptomic profiles with common overlapping features that have biological and clinical significance.

The classification of breast cancer was based on the expression of a subset (n = 496) of differentially expressed genes termed the 'intrinsic' gene set. This intrinsic gene set was identified using a supervised analysis algorithm to select genes that show little variance within repeated samplings of the same tumour, but show high variance across tumours. Although no knowledge of outcome was used to derive this gene set, the distinct tumour subtypes identified showed prognostic significance.

In Perou's study [3], two main tumour clusters were identified according to their ER expression. The ER-positive cluster was enriched with ER, ER-related genes and other genes characteristic of the luminal epithelial cells, and this class was termed 'luminal' in reference to its molecular similarity to luminal mammary epithelial cells. The other cluster was ER-negative, and it exhibited three distinct subclasses, which were termed HER2-positive, basal-like and normal breast-like. The HER2 subgroup was characterised by overexpression of HER2 and other genes pertaining to HER2 amplicon. The basal-like class was largely characterised by the lack of expression of ER and HER2, by positive expression of genes characteristic of basal-like cells of the breast, and by a high proliferative activity. The normal breast-like class displayed a triple-negative phenotype, but it did not cluster with the basal-like centroid and was characterised by expression profiles similar to those found in normal breast tissue.

Importantly, these classes showed different clinical outcomes: the luminal subtype had the most favourable response whereas the HER2-positive class had the worst, and the

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basal-like subclass had a poor prognosis although it was relatively better than the HER2-positive, trastuzumab-naive cancers. Despite the identification of novel molecular classes and the prognostic significance of the 'intrinsic subtype' classification, this classification system showed strong concordance with the current classification systems that use ER, progesterone receptor (PR) and HER2, and the evidence for changing treatment of the few discordant cases based on intrinsic subtype remains insufficient. For instance, membership of the HER intrinsic subclass does not necessarily imply HER2 gene amplification and protein overexpression, and patients with HER2 gene amplified tumours remain candidates for anti-HER2 targeted therapy regardless of the intrinsic subtype of the tumour.

Subsequent GEP studies indicated that the luminal class, which comprises the majority of breast cancer, is heterogeneous with respect to the expression of other genes and outcome [14]. Many studies report at least two subclasses: luminal A and B subtypes. The former is identifiable by high levels of expression of ER and ER-activated genes, low levels of proliferation related genes, negative HER2 and having the best outcome. The luminal B class, on the other hand, was not clearly defined. Most studies indicate that luminal B tumours are associated with a worse prognosis, but the molecular definition was variable and not reproducible. In general, it was characterised by ER expression but with higher proliferation rates and/or HER2 expression and low or absent PR expression [15]. Other luminal subclasses have been described, for example luminal C [16] and luminal N [17], but luminal A and B remain the most validated subtypes despite its limitations.

Similarly to the luminal class, a number of studies have classified basal-like tumours into subgroups. In a study of 587 triple-negative breast cancers, Lehman and colleagues [18] reported six subtypes, each displaying a unique gene expression profile. These were basal-like I, basal-like II, an immunomodulatory, a mesenchymal, a mesenchymal stem-like and a luminal androgen receptor (AR) subtype. The differential response of these subtypes to neoadjuvant chemotherapy has been investigated [19]. Other studies report four subtypes of triple-negative breast cancer (luminal AR, mesenchymal, basal-like immune-suppressed and basal-like immune-activated) [20].

Despite the different microarray platforms and the different intrinsic gene lists which varied from several hundred to 1300 genes with limited overlap [21], most studies have consistently reported at least four classes, namely luminal A and B, HER2-positive and basal-like subtypes. Other novel molecular classes have also been described, such as the claudin-low subtype which exhibits reduced expression of genes involved in tight junctions and intercellular adhesion and increased expression of immune response genes [22]. This class is located using hierarchical clustering near the basal-like tumours, and both share some characteristic gene expression such as low expression of HER2 and luminal gene cluster. Other classes include the molecular apocrine subtype, characterised by the expression of androgen receptor and related genes. Both claudin-low and apocrine subtype have a triple-negative phenotype and a poor clinical outcome.

A genomics-driven classification of breast cancer based on an integrative analysis of GEP and genome-wide CNAs was reported by the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) [23]. This study of 2000 breast cancers reported that the number of molecular subtypes is likely to be 10, which are called 'integrative clusters', and that these subtypes showed distinct clinical behaviours. The METABRIC study also confirmed that triple-negative tumours are characterised by complex patterns of copy number gains and losses throughout the genome.

Although identification of these 10 integrative clusters was based on multiplatform molecular subtyping system using GEP and CNAs, in a subsequent study of 7544 breast

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cancers, Ali et al [24] reported that these integrative clusters can be identified by a gene-expression based approach alone, and that this classification system may be more informative in some contexts than the intrinsic subtype system. The Cancer Genome Atlas (TCGA) network [25] has analysed 466 breast cancers across five platforms: genomic DNA copy number arrays, DNA methylation, exome sequencing, mRNA arrays, microRNA sequencing and reverse phase protein arrays. The integrated findings (1) demonstrated the existence of four main breast cancer classes, (2) identified two novel protein expression defined subgroups related to stromal/micro-environmental elements and (3) provided key insights into previously defined gene expression subtypes. TCGA hypothesised that much of the clinically observable heterogeneity and plasticity occurs within, and not across, these major molecular subtypes. Research into molecular portraits of breast cancer is active, with many ongoing large scale studies utilising multiple platforms that will probably lead to further refinement of the molecular classification of breast cancer over the coming years.

To overcome the problems of fresh tissue, the availability of microarray-based technology, cost and assay reproducibility, other techniques such as RT-PCR and IHC coupled with tissue microarrays have been introduced to replicate this molecular taxonomy and to identify intrinsic subtypes in routine practice. Two approaches have been identified. The first involves the identification of a minimum gene set from microarray-based studies, which is then used to identify the GEP-defined classes. One successful example is the PAM50 (prediction of microarray using 50 classifier genes plus 5 reference genes) classifier [26]. PAM50 categorises breast cancer into the four intrinsic subtypes: luminal A, luminal B, HER2-enriched and basal-like, and it provides independent prognostic significance. It has been reported that a three-gene model, ESR1, ERBB2 and AURKA (SCMGENE), can also identify the four major breast cancer intrinsic subtypes. Although both classifiers provide independent prognostic information, other studies have reported that PAM50 provides greater prognostic information than the 3-genes SCMGENE assay and that the PAM50 assay but not the SCMGENE provides independent predictive information of pathological complete response in multivariate models. This may be explained by the greater amount of gene expression diversity of PAM50, and it also further supports the idea that identification of the major and clinically relevant molecular subtypes of breast cancer are best captured using larger gene panels [27].

The second approach for identifying intrinsic breast cancer subtypes in routine practice uses tissue microarrays and IHC. Utilising a large panel of biologically relevant biomarkers, these techniques apply unsupervised clustering techniques to identify molecular classes with and without comparison with GEP defined molecular subtypes. In a previous study, we applied 25 IHC biomarkers to 1950 unselected breast cancer series [28] and identified seven molecular classes. For practical reasons, we limited the number of genes used to identify those classes in routine practice to ten [17]. As the performance of clinicopathological factors varies among the seven molecular classes, the concept of refining the Nottingham Prognostic Index (NPI) applied equally to breast cancer regardless of the molecular features. NPI Plus (NPI+) was based on classifying breast cancer into seven distinct molecular classes using those ten biomarkers, after which we incorporated clinicopathological variables to identify distinct prognostic groups within each of the seven classes [29]. Using the NPI+ formulae, through incorporating molecular features and clinicopathological parameters, a patients' outcome stratification superior to the traditional NPI was achieved [29].

In a more simplified approach, some authors have used the three routinely available markers (ER, PR and HER2) plus KI67 to classify breast cancer into four molecular classes,

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with KI67 being used to stratify luminal tumours into two subclasses [2]. In a previous meta-analysis of more than 10,000 breast cancers, Blows et al [30] reported six intrinsic subtypes using five IHC markers (ER, PR, HER2, EGFR and CK5/6). In the recently published ESMO Clinical Practice guidelines, breast cancer is classified into five molecular subtypes based on the expression of those four markers with or without the addition of molecular gene signatures:

- Luminal A tumours are defined as ER- and PR-positive, HER2-negative, KI67 low (≤10%) and low-risk molecular signature (if available).
- Luminal B tumours are subclassified into luminal B HER2-positive and luminal HER2-negative but with either high proliferative activity (KI67 ≥ 30%), PR low or showing poor prognostic gene expression signature if available.
- Basal-like is defined as triple negative.
- HER2-positive is defined as HER2 overexpression/gene amplification with absence of ER and PR [31].

Similarly, the international expert panel at the St Gallen International Breast Cancer Conference in 2013 endorsed the use of PR expression in defining luminal A breast cancer for those cases having substantial PR expression (≥20%). They have also recommended a Ki67 threshold of ≥20% as an indicative of high Ki67 status in defining luminal B cancers [32].

However, it remains unknown how many molecular classes or intrinsic subtypes exist, and more importantly how many classes can be reliably identified with the currently available data and how accurate current molecular classification systems are. Thus, this molecular classification system remains a model in development.

MULTIGENE PROGNOSTIC AND PREDICTIVE SIGNATURES

In addition to intrinsic subtype classification, microarray-based GEP has been expanded to identify relevant classes (class prediction) using supervised clustering techniques. These class prediction GEP studies attempted to forecast outcomes for individual patients with breast cancer. This class prediction approach was pioneered by van't Veer et al [33] who selected a 70-gene signature based on the association of the expression of each gene with the likelihood of metastasis within 5 years. In summary, out of the 25,000 genes on the microarray, 5000 genes significantly regulated in >3 tumours were selected. Out of those 5000, 231 genes were found to correlate with outcome. Further refinement of the genes revealed that the minimum optimal marker genes that predict outcome were 70 genes. This 70-gene signature was validated on a group of 295 patients [33] and was later commercially marketed as MammaPrint assay (Agilent, Amsterdam, the Netherlands) (Table 2.1). The poor prognostic signature was characterised by up-regulation of genes involved in cell cycle, angiogenesis, signal transduction, proliferation, invasion and metastasis.

As the indications for adjuvant chemotherapy in breast cancer are more complex, a common application of gene signature assays is the identification of breast cancer patients with a sufficiently good prognosis that adjuvant chemotherapy can be safely omitted. Despite the effort invested in developing prognostic gene signatures, most signatures are reduced to two or three risk categories (i.e. low, intermediate and high). Chemotherapy is sufficient for patients with low-risk tumours, while those with high-risk tumours are candidates for such treatment. In clinical settings where adjuvant chemotherapy is indicated, gene signatures appeared less useful and omitting chemotherapy based on gene signatures in these circumstances require validation in large randomised trials.

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The most frequent application of prognostic gene signatures in breast cancer remains in the clinical indeterminate group of ER-positive, HER2-negative and lymph node-negative or with low nodal burden disease. In fact, most studies have demonstrated that combining molecular signatures with the conventional factors provides the most useful prognostic information. Other gene signatures have been developed to predict the response to specific therapies.

Initial class prediction studies that attempted to identify gene signatures that predicted outcome were empirical, and based on profiling of tumours from patients with good or poor outcomes and collections of genes that discriminated between them [8]. Despite the minimal overlap between gene signatures and the fact that they were identified purely on their correlative and predictive value without consideration for the underlying mechanisms, most of them showed clinically significant risk stratification [41].

Although these first-generation gene signatures were composed of different gene lists, they mainly related to the expression of proliferation-associated genes. Given that the majority of ER-negative tumours have high expression levels of proliferation-related genes, these first generation signatures showed the highest discriminatory power in ER-positive disease. Subsequent studies have attempted to generate multigene predictors based on hypotheses derived from in vivo or in vitro experiments, or based on genes characteristic of a biological process [42,43]. Examples of the latter include genes associated with host immune responses, wound healing and other stromal gene signatures that carry prognostic value independent of ER status and proliferation [44]. Other gene signatures have been developed to classify breast cancer into subgroups based on response to specific therapies or prediction of outcome of patients treated with a particular type of therapy [45]. **Table 2.1** shows some of the most common gene signatures reported in breast cancer. Some examples are provided below.

MammaPrint score

MammaPrint is a microarray-based prognostic score that utilises fresh tissue or tissue that has been treated with a special solution. It is approved by the US Food and Drug Administration (FDA) for breast cancer patients >61 years with stage I/II, lymph node-negative or 1–3 node-positive disease and is performed by a central laboratory [4]. MammaPrint stratifies patients into low-risk or high-risk prognostic groups [33]. Although it is applicable to breast cancer regardless of ER expression, the prognostic risk discrimination is mainly seen in the ER-positive tumours, while ER-negative breast cancers are almost always classified as high risk, limiting its clinical value in ER-negative tumours. The MammaPrint test has been validated in retrospective studies, but its clinical utility is under investigation in a large prospective phase III trial: MINDACT (microarray in node-negative and 1–3 node-positive disease may avoid chemotherapy) that accrued 6600 patients between 2006 and 2011. Results are yet to be published.

Oncotype DX assay

The Oncotype DX is a RT-PCR-based assay performed on FFPE breast cancer samples. Oncotype DX uses 21 genes (16 cancer-related genes and 5 reference housekeeping genes) to compute a recurrence score from 0 to 100, which can be categorised into low risk (score <18), intermediate risk (score 18–30) or high risk (score ≥31) groups [34]. The Oncotype DX was developed for the purpose of splitting hormone therapy-treated patients who did not receive chemotherapy into two risk groups, and it has been validated in similar

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Table 2.1 Prognostic and predictive gene signatures in early stage invasive breast cancer

Test name/vendor	Component gene(s)	Intended clinical utility	Biological material/ technology	Classification
Microarray and RT-PCR based assays				
Oncotype DX (recurrence score) (Genomic Health) [34]	21 genes (16 cancer-related and 5 controls)	ER+, HER2-, node-negative breast cancer Predicts the likelihood of chemotherapy benefit as well as recurrence in hormone therapy treated patients Test may extend to node-positive patients	FFPE, RT-PCR	3 categories: low risk (RS < 18), intermediate risk (RS 18–30), or high risk (RS > 31)
MammaPrint/ Amsterdam Signature (Agendia) [33]	70 genes (first prognostic gene signature to be identified)	Node-negative ER+ or ER– Estimates the recurrence risk Test may extend to node positive patients	Fresh or FFPE, microarray	2 categories: low risk and high risk
Genomic Grade Index (GGI; MapQuant Dx) (Ipsogen)[35]	97 genes (related to tumour grade). A modified version using 8 genes and RT-PCR	Test can reclassify grade 2 ER+ breast cancer into high or low molecular grade	Fresh tissue (microarray) or FFPE (RT-PCR)	
Molecular grade index (MGI) (Biotheranostics) [36]	5 genes (proliferation related)	ER+ node-negative patients treated with hormone therapy.	FFPE, RT-PCR	2 categories: low and high risk of recurrence
Two-Gene Expression Ratio (H/I) (Quest Diagnostics) [36]	Ratio of the relative mRNA expression of 2 genes (HOXB13 : IL17BR)	ER-positive, node-negative patients	FFPE, RT-PCR	2 categories: low and high risk
THEROS Breast Cancer Index (BCI) (Biotheranostics) [36]	A combination of MGI (5 genes) and the 2 gene ratio (H/I) (HOXB13:IL17BR)	Risk of distant recurrence in ER+ node-negative breast cancer Risk of late distant metastasis and benefit from extended (>5 years) endocrine therapy	FFPE, RT-PCR	
The Rotterdam Signature (Veridex) [37]	76 genes (60 genes for ER+ and 16 genes for ER- patients)	Node-negative patients. Predicts recurrence in ER+ treated with tamoxifen	Fresh tissue, microarray	
EndoPredict (Sividon Diagnostics) [38]	11 genes (8 cancer-related and 3 controls)	Predicts distant and late recurrences in ER+/HER2- node-negative and node-positive patients treated with endocrine therapy alone	FFPE, RT-PCR	Can be combined with tumour size and nodal status to produce clinical score

Table continued...

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Test name/vendor	Component gene(s)	Intended clinical utility	Biological material/ technology	Classification
Prosigna (PAM50) Kit (risk of recurrence assay; ROR) (NanoString Technologies) [39]	50 genes (used in the PAM50 molecular classification assay) and 5 control genes	ER+ node-negative and node-positive patients treated with hormone therapy. Evaluates distant recurrence-free survival at 10 years	FFPE, RT-PCR and by the nCounter Dx analysis system	3 categories: low, intermediate and high risk
BreastOncPx (Lab Corp)	14 genes (not including ER or HER2)	Risk of distant metastasis (metastasis score). Helps identify higher-risk patients who might benefit from additional therapy	FFPE, RT-PCR	
Celera Metastasis Score (Lab Corp)	14 genes	ER+, node-negative patients treated with hormone therapy	FFPE, RT-PCR	2 categories: low and high risk
Invasiveness Gene Signature (IGS) (OncoMed Pharmaceuticals) [38b]	186 genes	Both ER and node-positive and node-negative patients	Fresh tissue, microarray	
Immunohistochemistry (IHC) and in situ hybridisation (ISH)-based tests				
IHC4 score [39]	4 genes (ER, PR, Ki67 and HER2	ER+ patients treated with hormone therapy	FFPE and IHC	
Insight Dx Mammostrat (Clarient Diagnostic Services)	5 genes	ER+, node-negative patients treated with tamoxifen alone Predicts benefit from additional cytotoxic chemotherapy	FFPE and IHC	3 categories: low, intermediate and high
Insight Dx Mammostrat Plus (Clarient Diagnostic Services)	9 genes (As above plus ER, PR, KI67 and HER2)	As above, plus hormone receptor/HER2 status	FFPE, additional genes evaluated by IHC and FISH	2 categories
ProEx Br (The BD/ TriPath)	5 genes (5 antibody /5 separate slide IHC assay that uses an image analysis system)	Overexpression of ≥2 markers (score ≥2) has been associated with disease relapse in node-negative and node-positive patients	FFPE, IHC	2 categories
eXagenBC (eXagen Diagnostics)	3 genes (DNA Copy number) for ER-positive and 3 genes for ER-negative tumours	Node-negative and node-positive patients	FFPE and FISH	2 categories: low and high risk
Signatures based on a biological process				
Wound-response signature [40]	442 genes	Node-negative and node-positive patients	Fresh, microarray	2 categories
Immune signatures	14 genes related to immune function	Predictive for relapse in trastuzumab-treated HER2+ patients		2 categories
Invasiveness Gene Signature (IGS)	186 genes	Predicts 10-year distant metastasis free survival in node-negative patients	Fresh, microarray	2 categories

FFPE, formalin-fixed paraffin-embedded; ER, oestrogen receptor; PR, progesterone receptor; RT-PCR, reverse transcription-polymerase chain reaction; FISH, fluorescence in situ hybridisation.

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independent studies. These studies indicated that the Oncotype DX score predicts the likelihood of distant recurrence or breast cancer death in ER-positive patients treated with hormonal therapy alone and demonstrated benefit from adjuvant chemotherapy primarily in patients with high recurrence score. Currently, Oncotype DX is the most widely used prognostic assay for ER-positive node-negative breast cancer in the UK and the USA. The clinical utility of Oncotype DX is currently under further investigation in large prospective randomised clinical trials: TAILORx (patients with ER+, HER2–, node-negative breast cancer) and RxPONDER [patients with ER+, HER2– breast cancer with 1–3 positive nodes (N1)].

PAM50 risk of recurrence score (Prosigna kit)

Although PAM50 test was developed to identify breast cancer molecular intrinsic subtypes using FFPE tissue on a RT-PCR platform, the assay has been modified into a simple molecular test for predicting outcome by generating a numerical risk score (abbreviated to 'ROR,' or risk of recurrence) which parallels that of Oncotype DX [39]. The PAM50 ROR assay has been adopted as the 'Prosigna Breast Cancer Prognostic Gene Signature' by a commercial partner (Nanostring Technologies, Seattle, WA, USA). The Prosigna assay utilises the nanotechnology-based nCounter digital gene expression platform, allowing it to be performed in any pathology laboratory. The Prosigna kit quantifies mRNA expression of 50 genes used in the PAM50 molecular classification algorithm and 5 housekeeping genes and computes the ROR, which reflects the intrinsic breast cancer subtype of the case. Prosigna can be used to estimate short- and long-term recurrence-free survival for stage I/II (including 1–3 positive nodes) ER-positive breast cancer in postmenopausal women treated with adjuvant hormone therapy [39]. The Prosigna Kit was approved by FDA and has received clearance in the EU. Prosigna has also been selected as the genomic test in the OPTIMA main trial, which follows on from the preliminary phase of this study, OPTIMA prelim. The OPTIMA prelim study used a five-multigene genomic test and included 301 patients. OPTIMA (Optimal Personalised Treatment of early breast cancer using Multi-parameter Analysis) is a UK-based clinical trial that aims to reduce the use of chemotherapy in hormone response HER2-negative breast cancer patients with lymph node-positive status (pN1–2 or pN0 and pT > 30 mm) who are currently offered chemotherapy as standard treatment [46].

Breast cancer index

Breast cancer index (BCI) consists of two independently developed gene signatures: molecular grade index (MGI), a five-gene predictor that recapitulates tumour grade/proliferation; and the two-gene ratio (*HOXB13/IL17BR*) signature (H/I), which was developed independent of tumour grade/proliferation. Compared to Oncotype DX and IHC4, BCI is a predictor of early distant recurrence and is the only significant prognostic for risk of late recurrence [47].

EndoPredict

The EndoPredict test (Sividon Diagnostics GmbH, Koln, Germany) is an RT-PCR-based assay using FFPE tissue, and it measures the expression of eight breast cancer-related genes and three housekeeping genes. It stratifies ER-positive breast cancer patients treated with adjuvant endocrine therapy alone into low or high risk of recurrence. The EndoPredict

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score has also been combined with clinical variables, namely lymph node stage and tumour size, to compute a clinical risk score termed EPclin that shows predictive value for identification of ER-positive patients at risk for late recurrence [48]. The assay is marketed in Germany and Europe as a prognostic kit that can be performed by local laboratories.

IHC4 assay

The IHC4 assay uses IHC and FFPE tissue and is based on the assessment of ER, PR, HER2 and Ki67. It takes the semiquantitative expression values of these four markers and produces a ROR score.

IHC4 has provided prognostic stratification comparable to the other costly multigene signatures such as Oncotype DX. Although IHC4 is inexpensive and can be performed in local laboratories, standardisation of IHC4 and quality assurance programs are required before its use becomes widespread [39].

Most currently available multigene signature assays provide significant independent prognostic value in ER-positive HER2-negative breast cancer, particularly in the node-negative or low burden disease. Although comparative studies of these molecular assays indicate that discordant risk prediction occurs when different prognostic assays are applied to the same tumour, each provide independent prognostic signature when considered alone. In a previous study, six-gene signature assays, including Oncotype DX, PAM50/ROR and MammaPrint, were applied to the same patient cohort, and although each test provided significant prognostic value, individual risk assignments were not often concordant [49], therefore the authors suggested that combining several multigene tests may offer more accurate outcome prediction [49].

Although most gene signatures available have little or no prognostic value in HER2-positive and ER-negative tumours, some studies have reported prognostic stratification power for specific sets of genes. For instance, a prognostic predictor based on 158 genes [HER2-derived prognostic predictor (HDPP)] has been found to provide more accurate stratification of tumours into good and bad prognosis [50]. Similarly, prognostic signatures linked to genes involved in immune, inflammatory and/or chemokine pathways have been developed for ER-negative breast cancer. GEP has also been used to subdivide triple-negative breast cancer into distinct groups, and a prognostic significance of a ratio of B-cell and interleukin-8 (IL-8) metagenes has provided a prognosis value in triple-negative disease. Although there is a near consensus that multigene prognostic assays provide useful complementary information to well-established traditional clinicopathological variables in ER-positive tumours, it is not surprising that clinically useful prognostic signatures for HER2 positive ER-negative breast cancer are still non-existent.

NEXT GENERATION SEQUENCING

NGS has revolutionised breast cancer genetics and genomics, and it is expected to pave the path for personalised treatment of breast cancer patients. Common uses of NGS include whole-genome sequencing, whole-exome sequencing, targeted exome sequencing (target-enrichment methods to capture genes of interest) and hotspot (sequences selected regions/regions with recurrent mutations of selected genes of interest) sequencing.
NGS has been used to characterise genomic alterations such as copy number changes, insertions/deletions and mutations; to facilitate sequencing at a greater depth (at the base-pair level); to enable the identification of subclonal mutations; and to help differentiate

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'driver' mutations that contribute to cancer development from 'passenger' mutations that do not appear to play a significant role in cancer development.

In addition to providing information about the genomic landscape of breast cancer, NGS has confirmed both intertumour and intratumour heterogeneity and showed that each breast cancer is largely unique.

NGS has been used to show that mutation frequencies in breast cancer are lower than in other cancers, such as lung squamous cell carcinoma or bladder urothelial carcinomas, but are similar to those of ovarian and renal clear cell carcinomas. In a NGS study of 100 breast cancers [51], the number of somatic mutations was reported to vary markedly between individual tumours, and to correlate with patient age and histological grade. Multiple mutational signatures were observed and driver mutations were identified in at least 40 cancer genes including new cancer genes [51]. Somatic driver point mutations and/or copy number changes were identified in at least 40 cancer genes. There was a maximum of 6 mutated cancer genes in an individual breast cancer, and 28 cases of the 100 showed a single driver. Seven of those 40 cancer genes (TP53, PIK3CA, MYC, ERBB2, FGFR1, CCND1 and GATA3) were mutated in >10% of cases and these contributed 58% of driver mutations [51]. Overall, the study sample had a mean of 56.9 (range 5–374) somatic mutations per cancer.

TCGA network reported different genetic abnormalities associated with the molecular intrinsic subtypes. 40% of luminal A tumours had a mutated PIK3CA gene. PIK3CA and TP53 were mutated in 29% of luminal B tumours. TP53 was mutated in 80% of basal-like classes which exhibited a more consistent pattern and the highest amount of mutations compared to luminal tumours. The HER2-enriched tumours showed HER2 amplification in 80% of cases [25]. The vast majority (76%) of breast cancers are characterised and driven by recurrent CAN (C class), while tumours characterised and driven by recurrent mutations (M class) are almost exclusively of luminal subtype (92%), and 99% of basal-like tumours are of C class [52].

NGS has also been used to demonstrate the spatial and temporal intratumour heterogeneity of breast cancer. Various degrees of intratumour genetic heterogeneity have been found, even in the absence of overt histological phenotypic heterogeneity. Triple-negative and basal-like tumours tend to have greater intratumour heterogeneity than nonbasal-like tumours. Mutations in common driver genes such as TP53, PIK3CA and PTEN are usually found in high clonal frequencies and several somatic mutations are present in only a fraction of cancer cells. NGS also showed that the constellations of somatic mutations found between a primary breast cancer and its metastases (temporal heterogeneity) and between distinct areas within the primary tumour (spatial heterogeneity) are not identical providing further evidence to indicate that breast cancer evolve over the course of the disease. This clonal genetic heterogeneity may explain resistance of some breast cancer to selective environmental pressures and therapy.

CONCLUSION

Molecular testing is becoming increasingly important in the prevention, diagnosis and treatment of breast cancer. Despite the enormous amount of work that has been carried out to develop and refine breast cancer molecular classification, it is still evolving. With the increasing use of more sophisticated high-throughput techniques such as MPS, large amounts of data will continue to emerge, which may lead to the identification of novel therapeutic targets and provide the basis for more precise classification systems.

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Key points for clinical practice

- Breast cancer is a diverse spectrum of diseases featuring distinct histological, biological, molecular and clinical phenotypes.
- Studies indicate that traditional classification systems are insufficient to reflect the biological and clinical heterogeneity of breast cancer because tumours of similar clinicopathological features exhibit dissimilar behaviour and responses to specific therapy, but advances in high-throughput molecular techniques and bioinformatics have improved our understanding of breast cancer biology, refined molecular taxonomies and led to the development of novel prognostic and predictive molecular assays.
- Molecular testing is becoming relevant to the prevention, diagnosis and treatment of breast cancer. Molecular prognostic and predictive assays are still evolving, but there are only a small number relevant to routine clinical practice and these are focussed on identifying patients at high risk of relapse, or patients who have potential to benefit from adjuvant chemotherapy in the context of lymph node negative, oestrogen receptor positive breast cancer.
- With the increasing use of more sophisticated molecular techniques, such as next generation sequencing, novel therapeutic targets will be identified, and also to support more precise classification systems.

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