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The only widely used breast cancer molecular assay is in situ hybridisation (ISH) for HER2 gene amplification and we highlight key issues with the interpretation of this assay with particular attention to the difficulties of the equivocal category. New molecular assays such as ISH for the Topoisomerase II alpha (TOP2A) gene and for the aberrations in the copy number of the centromeric region of chromosome 17 are readily performed in a standard histopathology laboratory, but there is to date insufficient data to support their routine use. We also review the current data on two commercially available multigene expression assays, Oncotype DX and MammaPrint and discuss their potential use. Overall, while new molecular assays have significant potential to improve patient selection for therapy, well-performed histopathology with reliable interpretation of standard hormone and HER2 assays provides the most important predictive and prognostic information in early breast cancer.

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Abstract

Recent advances in understanding the molecular pathology of breast cancer offer significant potential to identify patients who may benefit from adjuvant therapies. To date few of these advances are utilised in a routine setting. We review molecular assays that are currently in use or are in the advanced stages of development, which may be used as predictive or prognostic biomarkers in breast cancer.

The only widely used breast cancer molecular assay is in situ hybridisation (ISH) for HER2 gene amplification and we highlight key issues with the interpretation of this assay with particular attention to the difficulties of the equivocal category. New molecular assays such as ISH for the Topoisomerase II alpha (TOP2A) gene and for the aberrations in the copy number of the centromeric region of chromosome 17 are readily performed in a standard histopathology laboratory, but there is to date insufficient data to support their routine use. We also review the current data on two commercially available multigene expression assays, Onco*type* DX and MammaPrint and discuss their potential use.

Overall, while new molecular assays have significant potential to improve patient selection for therapy, well-performed histopathology with reliable interpretation of standard hormone and HER2 assays provides the most important predictive and prognostic information in early breast cancer.

Key words: Breast cancer, molecular assays, prognostic biomarkers, in situ hybridisation, HER2, TOP2A, chromosome 17, Oncotype DX, MammaPrint.

Introduction

There have been significant improvements in outcome from breast cancer over the past two decades[1] due to earlier diagnosis as well as to the use of targeted therapies especially hormonal therapy for oestrogen receptor expressing breast cancer[2]. Despite these advances, there are still women with breast cancer who have a poor outcome and a key research and clinical question is how to select the right treatment for the right patient. This requires the use of biomarkers; candidate genes in a breast cancer patient that can predict outcome (prognostic biomarkers) or response to therapy (predictive biomarkers). It has been known for some time that breast cancer is a heterogeneous disease, first recognised many years ago with the discovery of hormone receptor positive breast cancer[3] and later HER2 amplified disease[4, 5]. More recently, gene expression microarray (GEM) studies have refined our understanding that based on the genetic profile of a breast cancer, the biology and clinical behaviour varies significantly[6]. Although molecular biology techniques have significant potential to improve the selection of optimal breast cancer therapy for individuals, only one molecular biomarker, HER2 gene amplification, is in routine use. The aim of this review is to highlight recent developments and controversies in HER2 molecular testing and to discuss the most promising molecular markers in breast cancer pathology that are currently in practice or closest to introduction in a routine setting – specifically Topoisomerase II alpha (TOP2A), and two multigene assays; Onco*type* DX and Mammaprint assays. These assays will also be compared to routine immunohistochemical markers for their predictive potential.

Current issue in testing for HER2 gene amplification

The Human Epidermal Growth Factor Receptor 2 (HER2) gene is located on 17q12-q21 and encodes a 185kd protein that is part of the epidermal growth factor family. The HER2 protein is a transmembrane tyrosine kinase receptor that forms either homodimers or heterodimers with other members of the HER family (EGFR, HER3 and HER4). Activation of HER2 results in activation of the RAS-MAPK pathway stimulating cell proliferation, while interaction with the phosphatidylinositol 3'-kinase (PI3K) pathway inhibits cell death (reviewed in [7]. The net effect of this is promotion of an aggressive tumour phenotype, reflected in the association of HER2 amplification with larger, higher grade tumours and a poor outcome [8].

Reports of the incidence of HER2 gene amplification vary widely, with earlier studies suggesting as high as 30% of breast cancers were HER2 amplified [4, 5]. More recent studies suggests that around 15% of newly diagnosed invasive breast cancers are HER2 positive, although higher grade and node positive tumours which are more likely to receive adjuvant chemotherapy have a higher incidence of HER2 positivity of around 25% [9]. Our own figures from Royal Prince Alfred Hospital's recently commenced HER2 in situ hybridization (ISH) testing programme show around 17% of 325 early invasive breast cancers are amplified while St George Hospital has reported a very similar proportion of HER2 amplified cases of 16.2% in 1708 patients tested to date (unpublished data).

HER2 targeted treatments are making an impact in this otherwise poor prognosis breast cancer. The first HER2 specific therapy was a monoclonal antibody, trastuzumab,

directed against the juxtamembrane portion of the extracellular domain of the HER2 receptor [10]. A number of trials suggest that trastuzumab improved the disease free survival (DFS) and overall survival (OS) of women with early stage HER2 positive breast cancers by as much as 50% [11-13]. A recent meta-analysis of randomised control trials of trastuzumab in early breast cancer has confirmed a highly significant reduction in breast cancer deaths, recurrence and metastasis (all $p<0.00001$) [14]. Trastuzumab also improves survival in metastatic breast cancer, with a recent study showing a 44% reduction in the risk of death compared to non-HER2 metastatic breast cancer [15]. More recently a dual tyrosine kinase small molecule inhibitor (with activity against EGFR and HER2) lapatinib is also proving to be an effective therapy in metastatic breast cancer in combination with capecitabine [16], with a 51% reduction in the risk of disease progression. Taken together, these data emphasise the need to accurately identify those patients who may benefit from these targeted therapies.

There are a number of modalities to assess the HER2 status. One of the first techniques used to assess HER2 status was immunohistochemistry (IHC) – early trastuzumab metastatic breast cancer trials enrolled patients with 2+ or 3+ expression of Her2 protein. Subsequent retrospective analyses showed that only patients with 3+ IHC or gene amplification by FISH benefited [17-20]. Subsequently, a common approach has been to triage patient eligibility for trastuzumab via IHC; those with no or weak staining are termed negative and no further testing is performed [20]. Her2 protein positive patients (3+ uniform strong membranous staining in >30% of tumour cells) may receive trastuzumab in many studies while patients with equivocal staining on IHC (2+) are referred for FISH testing. This approach is endorsed by the most recent ASCO /CAP guidelines on HER2

ISH testing shown in Table 1. Briefly, a FISH result of more than 6 HER2 copies per nucleus or a ratio of the number of copies of HER2 to the centromeric probe for chromosome 17 (CEP17) of >2.2 is reported as a positive, amplified result. Cases with a mean HER2 copy number per nucleus of <4 or a HER2/ Chromosome 17 ratio of <1.8 are negative, and cases with copy number between 4 and 6 or a ratio of 1.8-2.2 are considered equivocal and require further investigation to determine their status.

In Australia, Pharmaceutical Benefit Scheme (PBS) funded use of trastuzumab requires confirmation of gene amplification via ISH. This testing of all early breast cancers is supported through the Roche Australian ISH Testing Program for Breast Cancer. All breast cancer irrelevant of their IHC status may be tested through this program. This approach ensures that only patients with HER2 gene amplification are eligible to receive HER2 targeted therapy. Initially FISH was the only modality to do this, but newer bright field modalities of ISH are now being widely utilised. The two most utilised bright field techniques are chromogenic in situ hybridization (CISH) and silver in situ hybridisation (SISH) [21]. While these new techniques are not specifically addressed in the ASCO guideline, the guidelines state that any new assay should show >95% concordance with an established assay and several studies have confirmed the utility and accuracy of these techniques [21-24]}. Advantages of these techniques are the durability of the signal which does not fade appreciably with time and the ability to be interpreted with a standard light microscope with easier interpretation of tissue morphology rather than requiring an expensive fluorescence microscope. SISH is an automated system (Ventana, Roche Diagnostics), while CISH is a two day manual procedure utilising a kit (Invitrogen, SPotLight). Disadvantages of these bright field methods in our experience are a lower

sensitivity compared to FISH and they are less likely to work on suboptimally fixed tissues or those that have undergone decalcification (eg biopsy of a bony metastatic site). At Royal Prince Alfred and St George Hospitals we have also found that CISH signal tends to be weaker in archival blocks of an age of >5 years. They are generally used as single probe tests for HER2; those cases that have <4 (negative) or those with >6 (positive) copies of HER2 per nucleus require no further testing. Cases in the equivocal range (between 4 and 6 copies of HER2 per nucleus) require a second probe applied to a parallel section from the same tissue area for CEP17, which enables calculation of a HER2/ Chromosome 17 ratio. As for FISH, cases with a ratio of >2.2 are positive and <1.8 are negative. Around 2% of cases fall within the equivocal range (1.8-2.2 copies) [25] and are usually subject to FISH [20].

There is also some debate about the utility and clinical significance of the equivocal category of HER2/ Ch 17 ratio 1.8-2.2[18]. Some argue that it is unnecessary and creates diagnostic and therapeutic dilemmas [25]. Instead, in cases with a HER2/ Ch 17 ratio in the "equivocal" range, the authors argue that an additional 20 nuclei should be scored by the primary scorer while a second independent scorer counts a minimum of 40 nuclei. When these two ratios are in agreement, this result is reported. If there is no agreement, the entire assay should be repeated and the specimen be rescored [25].

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these two ratios are in agreement, this result is reported. If there is no agreement, the entire assay should be repeated and the specimen be rescored [25]. An alternative approach might be to select another tumour block if available for testing.

However Dowsett and colleagues [26] report that even in experienced laboratories, borderline FISH cases can be difficult to interpret [26]. Twenty breast cancer cases were FISH tested by 5 large reference laboratories in this concordance study which reported HER2/Ch17 ratios in the range 1.7 (i.e. negative) to 2.3 (i.e. positive) , with an overall discordance rate of 20%. There is a deficiency in the literature regarding the clinical significance of cases that fall in the equivocal range and further studies are required to clarify this issue [20].

The use of a chromosome 17 centromeric probe may also contribute to the difficulties involved in assessing equivocal cases. Chromosome 17 polysomy has been reported to occur in around 2-9% of breast cancer [25]. However, recent studies based on Comparative Genomic Hybridisation (CGH) arrays which assess the copy number of multiple genes along the entire chromosome suggest that some cases of so called Ch17 "polysomy" are not true increases in the number of copies of the whole chromosome 17, but in fact reflect co-amplification of the centromeric region [27]. This could result in cases where HER2 is truly amplified, but because there is co-amplifciation of the centromeric region reflected in an increased Ch17 probe count, the ratio may incorrectly be reported at less than 2 [28] not reflecting the true amplified status of the tumour [29].

Bartlett and colleagues argue that conversely, cases where Chromosome 17 is not used may falsely underestimate the incidence of HER2 amplification [30]. They comment that due to nuclear transection in thin cut sections and incomplete hybridisation of DNA

probes the observed mean chromosomal copy number in reality falls significantly below the theoretical threshold of 2 copies per cell. A previous study in normal breast by this group identified a range for disomy of chromosome 17 of 1.3-1.85 copies per nucleus, arguing that polysomy is better defined as >1.85 copies per nucleus [31]. One consequence of these calculations is that theoretically a tumour with a HER2 copy number of 3 and monosomy for Ch17 (<1.3 copies) would have a ratio of >2 and be amplified. Using dual colour FISH for HER2 and Chromosome 17 they assessed 1711 cases of breast cancer for HER2, including 593 cases with 2+ Her2 IHC enriching for this borderline group. The authors found that using a dual probe (HER2 and Chromosome 17) theoretically 16.4% of cases with a HER2 copy number of 3 to 4 were amplified for HER2. They also observed that 3.28% of cases with an observed HER2 copy number of between 2 and 3 were also "amplified". The authors argue that therefore Chromsome 17 signal should be assessed in all breast cancer cases with a HER2 copy number of >2 and that the current guidelines result in underdiagnosis of HER2 "amplification". However, whether these technically amplified cases represent true amplification in terms of their biology and response to trastuzumab was not determined by this study, and it is clear that such technically amplified ratio are mostly generated through loss of Chromosome 17. Further study is required to determine the outcome and response to treatment of this group of "technically" amplified low HER2 copy number cancers.

The Australian approach of requiring ISH confirmation of HER2 gene amplification has been supported by a recent review addressing issues raised by the 2007 ASCO/CAP HER2 testing guidelines [25] which argues strongly that for primary FISH testing of breast cancers. Immunohistochemistry for HER2 has a number of well recognised problems –

the assay is significantly affected by tissue fixation, edge and crush artefact which is a particular problem in core biopsies and there is no internal positive control. Although FISH may be affected by fixation, DNA in formalin fixed, paraffin processed (FFPE) material is relatively stable and there is an endogenous internal control in the nucleus of every cell, which should have up to 2 copies of the HER2 gene. Furthermore IHC is subjective, requiring interpretation of intensity of membranous expression. In contrast, FISH is semi-quantitative, relying on counting signals within tumour nuclei. The greater reliability and reproducibility of FISH as an HER2 assay is supported by data showing much greater concordance in external quality assurance programs (United Kingdom National External Quality Assurance Scheme: UKNEQAS Immunocytochemistry journal: http://www.ukmeqasicc.ucl.ac.uk/neqasicc.shtml) [32], compared to IHC, where up to 20% of HER2 assays performed in routine laboratories are incorrect [20]. The College of American Pathologists (CAP) also published findings from its proficiency testing program and found that 100% of participating laboratories correctly classified unknown samples for HER2 status by FISH[33].

While there are well recognised problems with Her2 IHC, the technique is still valuable in assessing HER2 status of breast cancers. We find it useful as an additional internal quality control measure – it is reassuring that 3+ IHC cases are in the large majority (>80%) amplified, and if the ISH signal is weak in a 3+ IHC, CISH negative case we will often repeat the assay with increased pretreatment or go on to perform FISH to ensure we are not missing an amplified case due to technical problems. IHC is also very valuable for detecting heterogeneity of HER2 amplification. While this phenomenon is not widely recognised Associate Prof Morey Director of the National HER2 Reference Laboratory at

St Vincent's Hospital reports that this occurs in around 0.4% (33 of 9035) diagnostic HER2 ISH cases [34]. This clonality was reflected by the immunohistochemistry and was easier to detect as it was readily apparent at low power in comparison to ISH where the signal is only easily seen at high power magnification, increasing the risk of missing a small amplified clone (example shown in Figure 1). Many of their reported cases showed background polysomy and a merging of amplified and non-amplified components. The majority also showed a mixture of amplified and non-amplified DCIS [34]. Interestingly, although forming a small minority of a largely non-amplified tumour, the nodal metastasis contained amplified tumour cells. This issue of heterogeneity also reinforces the caution that is needed when interpreting HER2 assays (whether IHC or ISH) on core biopsies.

A critical issue for HER2 testing, whichever method is selected, is the need for strict quality control and quality assurance of HER2 testing, with >95% concordance with another validated test. A recent study from the North Central Cancer Treatment Group (NCCTG) Intergroup trial N9831, a randomised phase III clinical trial evaluating trastuzumab as adjuvant therapy for patients with HER2 positive early breast cancer has highlighted the need for regulated testing. A preliminary protocol specific review of the first 119 patients showed only 67% of samples classified as HER2-positive by FISH performed by the local laboratory were confirmed as FISH HER2 amplified at the central laboratory. Criteria for the trial were subsequently altered to require central re-testing for HER2 and concordance was only 88.1% for FISH and 81.6% for immunohistochemistry. Interestingly, most of the local-central discordant cases were re-tested at a reference laboratory, and there was good concordance between the central and reference laboratory

(95.2%) – within the suggested ASCO/CAP guidelines. These data emphasise that HER2 testing is best performed in relatively high volume laboratories.

While HER2 is best recognised as a predictive marker for response to trastuzumab therapy, there is accumulating evidence that is may also predict response to a number of other breast cancer therapies. It has been reported to contribute to endocrine therapy resistance [35-38], possibly taxane response [39] and a number of clinical trials suggest that patients with HER2 amplified tumours may derive benefit from anthracyclines [40, 41] also seen in a recent meta-analysis [42].

Anthracyclines such as doxorubicin and epirubicin are widely used as chemotherapeutic agents in breast cancer, but are also associated with a variety of serious adverse effects, particularly cardiotoxicity, which is probably under-reported but is becoming more apparent with longer term survivors and in older patients [43]. While a clear benefit is derived from anthracycline chemotherapy in the adjuvant setting [2, 44, 45], the effects overall are quite modest, which when coupled with the higher risk of adverse effects and toxicity highlights the need to accurately identify those patients with the greatest potential benefit. A number of studies have suggested a link between HER2 and anthracycline benefit, (reviewed in [46]) but this link has no known biological basis [47]. In contrast, topoisomerase II alpha (encoded by TOP2A), which is closely located to the HER2 gene on Ch17 and frequently co-amplified with it, is a direct molecular target of anthracycline chemotherapy.

Topoisomerase II α (TOP2A)

TOP2A is located on chromosome 17q21-q22 and encodes a 170 kDa enzyme

Topoisomerase II alpha. Located close to and frequently co-amplified with HER2 gene, TOP2A, plays a key role in fundamental nuclear processes including DNA replication, transcription, recombination, chromosome structure, condensation and segregation [48]. The prevalence of TOP2A aberrations differs widely in the literature ranging from 9% in unselected breast cancer to 46% to 90% of HER2 amplified breast tumours [49,50] whilst it is only rarely detected in HER2 non-amplified cancers [51, 52]. Our own unpublished data in 69 HER2 amplified patients (manuscript in preparation) found a high frequency of TOP2A aberrations; TOP2A was amplified in 21 cases (34%) and deleted in 9 cases (15%). Other studies have reported TOP2A deletion rates in HER2 amplified tumours of 16 to 43% (reviewed in [53]). There are a number of likely reasons for this variability, including differences in study populations as well as inconsistent definitions of what constitutes amplification or deletion. TOP2A may be assessed via FISH [54] or bright field techniques such as CISH [55]. Cut-points for TOP2A gene aberrations have also varied considerably in the literature with studies using gene copy numbers or TOP2A/Ch17 ratios. For those who used copy numbers, amplification ranged from greater than five signals per nucleus in more than 50% of cells [56] to six or more gene copies when detected in at least 20% of screened malignant cells [57]. In contrast, TOP2A was also considered amplified when the *TOP2A*/Ch17 ratios were 1.5, 2.0 or 2.1 and deleted when the TOP2A/Ch17 ratio was less than 0.67, 0.7, 0.8 or 1.0 [49, 58-63]. Therefore it is important to standardise the methodology particularly the scoring criteria used to define amplification and deletion. This would help to eliminate inconsistencies in results and make reporting more uniform.

One of the major mechanisms of anthracycline action is via inhibition of the TOP2A

enzyme [51], by impairing DNA replication and repair [57] via p53 DNA damage sensors and caspase mechanisms, thereby promoting apoptosis [43]. In view its direct interaction with anthracycline chemotherapy, TOP2A has been proposed as a likely candidate biomarker for the beneficial effect of anthracycline therapy and this is supported by a number of studies. Knoop *et al* [64] retrospectively analysed 805 tumours for HER2 and TOP2A gene aberrations from the Danish Breast Cancer Cooperative Group trial 89D comparing a CMF regimen (cyclophosphamide, methotrexate and fluorouracil) to CEF (cyclophosphamide, epirubicin and fluorouracil). They found that while no predictive value for anthracycline (epirubicin) benefit was seen for HER2 amplification, TOP2A amplification (TOP2A/Ch17 ratio of >2.0) or deletion (ratio <0.8) was associated with increased recurrence-free survival and overall survival (hazard ratio of 0.57 for TOP2A amplification and 0.63 for TOP2A deletion). In contrast, patients who had a normal TOP2A genotype had a similar outcome with both regimens.

An interim report (San Antonio Breast Cancer Research Symposium Dec 14-17, 2006, San Antonio Texas abstract 52) on the Breast Cancer International Research Group (BCIRG) randomised phase III trial 006 in 4943 patients comparing 3 chemotherapy regimens 1) doxorubicin and cyclophosphamide followed by docetaxel (AC/ET) with 2) doxorubicin and cyclophosphamide followed by docetaxel and trastuzumab (AC/ETH) and 3) docetaxel, carboplatin and trastuzumab (TCH) in HER2 positive early breast cancer patients also suggested that deletion or amplification of TOP2A was indicative of a poor outcome and predicted a greater benefit of regimens containing anthracycline.

Most recently retrospective analysis of TOP2A gene amplification by CISH in 391 patients of Trial 9401 from the Scandanavian Breast Group of anthracycline-based

chemotherapy dose escalation demonstrated that HER2 did not predict specific response to therapy, but found that TOP2A amplification was associated with a better relapse free survival in patients treated with a tailored and dose escalated epirubicin containing regimen (FEC) [55].

However, as outlined in two recent reviews [39, 46], other trials have not identified such a clear-cut role for TOP2A. The Cancer and Leukaemia Group B (CALGB) trial 8541-150013 retrospectively evaluated whether TOP2A amplification could predict benefit from intensive dose cyclophopshamide, doxorubicin and fluorouracil in 687 cases of HER2 amplified early breast cancer using a triple FISH probe for TOP2A, HER2 and chromosome 17 but found no association with outcome [62]. Another large study using FISH to retrospectively assess HER2 and TOP2A status in 2123 patients with early stage breast cancer treated with doxorubicin based adjuvant chemotherapy found no association with outcome for TOP2A, although high level HER2 amplification was a prognostic marker in anthracycline treated patients [54].

Thus the data on the predictive benefit of TOP2A amplification and are conflicting and there are a number of reasons for this, including differing methods of assessment of TOP2A status, but in particular all these trials relied on retrospective analysis of TOP2A and HER2 genomic status and were statistically underpowered to reliably assess their capacity as a predictive biomarker. Furthermore, many utilise pre-trastuzumab regimens, and so the role of anthracyclines in trastuzumab treated patients is not yet clear. The role of TOP2A deletion is even more unclear, with studies showing conflicting associations with sensitivity or resistance to anthracycline therapy [53, 60, 64]. The issue is further complicated by a small study (n=81) showing that dissimilar to HER2, there is no

association between TOP2A amplification by FISH and expression of the protein by immunohistochemistry [65]. This finding is supported by a recent study showing no association between TOP2A deletion and loss of protein expression [66].

The issues have all contributed to suggest that assessment of TOP2A gene aberrations is not yet ready for the clinic [67], with a need to design prospective trials that are adequately powered to address the predictive potential of this gene for anthracycline therapy response with rational and uniform criteria for defining gene aberrations.

Chromosome 17 aberrations as a marker of anthracycline benefit

In view of this uncertainty regarding TOP2A, an intensive search is underway to identify and validate alternative markers in this region of chromosome 17 that may explain the overall association of HER2 amplified tumours with greater benefit from anthracycline chemotherapy. There has been speculation that polysomy of chromosome 17, rather than the specific genes HER2 or TOP2A may in fact be a marker of an unknown gene that predicts anthracycline response. Chromosome 17 is the second most dense human chromosome in terms of genes, conatining many genes important in cancer such as HER2, TP53, and BRCA1 as well as TOP2A [46].

To date there is relatively little published data regarding the utility of chromosome 17 in this setting. Reinholz and colleagues presented in abstract form at the San Antonio Breast Cancer Symposium in 2007 [68] their study examining whether chromosome 17 could predict outcome in 1888 patients in the HER2 positive N9831 intergroup adjuvant trastuzumab trial. Chromosome 17 was not associated with tratuzumab response but the data indicated that patients on standard chemotherapy who did not receive trastuzumab

with chromosome 17 polysomy benefited more than those with a normal Ch 17 count. Bartlett and colleagues have identified in the UK National Epirubicin Adjuvant Trial (NEAT) that in 1762 patients who were assessed for HER2, TOP2A and Ch 17 aberrations using a triple FISH probe, the most powerful predictor of anthracycline benefit was seen with Ch17CEP (the chromosome 17 *CE*ntromeric enumeration *P*robe) duplication [58]. As discussed earlier, these workers argue that counting of signals in thin tissue sections is likely to result in relative under-counting of signals [31], thus they define Ch17CEP duplication as greater than 1.86 observed signals per cell (in contrast to the standard definition of "polysomy" as >3 signals per nucleus [29]). Although HER2 and TOP2A were predictive of relapse free and overall survival in this cohort, there was no interaction with anthracycline benefit. Interestingly around two thirds of patients with Ch17CEP duplication were not HER2 amplified, suggesting that anthracycline benefit may not be confined to HER2 amplified patients as described in some studies [49,51]. The authors conclude that assessment of Ch17CEP duplication is the most powerful predictor described to date of anthracycline chemotherapy benefit and suggest that validation in a larger meta-analysis would be helpful in leading to introduction of this predictive biomarker into routine practice. Clearly further investigation into candidate genes for this effect in the centromeric region of Ch17 is required. The observed changes in Ch17CEP copy number may reflect unbalanced translocations, subchromosomal amplification or deletion or whole chromosomal duplication (which as discussed above is a rare event in breast cancer [27]).

Molecular Classification of Breast Cancer

Traditionally, pathological determinants of tumour size, lymph node status, endocrine receptor status, grade, lymphovascular invasion and HER2 status have driven prognostic predications and, ultimately, adjuvant therapy recommendations for women with early breast cancer. A large meta-analysis of adjuvant chemotherapy has shown an improvement of 24% in disease free survival (DFS) and 15% in overall survival (OS) in women receiving adjuvant chemotherapy [2]. This analysis did not include taxane based regimens which show an even greater benefit, providing up to 30% improvement in both DFS and OS in hormone receptor negative tumours, although their role in hormone–receptor positive tumours is still not clear[69]. Current guidelines recommend chemotherapy be considered for the majority of women even including tumours that are hormone receptor positive, HER2 negative if they are larger than 1cm [70]. However, these prognostic and predictive factors are relatively crude measures and many patients are over-treated or undertreated as a result. In the last decade there has been considerable interest in developing assays that may help select patients for adjuvant therapies – both endocrine and chemotherapy. With the development of new technologies which allow for screening of the relative abundance of messenger RNA transcripts in the cancer tissue, representing the entire genome, there has been much research directed at developing assays to answer this key issue in breast cancer management.

Perou et al [6] used cDNA microarrays representing 8,102 human genes to characterise gene expression patterns in a set of 65 surgical specimens of human breast tumours from 42 different individuals. A subset of 456 genes, termed the "intrinsic" gene subset, consisted of genes with significantly greater expression variation between different tumours than paired samples from the same tumour. Using this subset, the authors were

then able to identify different molecular subtypes of breast cancer: luminal A, luminal B, HER2 enriched, basal-like and normal breast-like. These 5 molecular subtypes have been confirmed to show distinct differences in behaviour in a number of independent data sets [71-73]; Sorlie *et al* [72] examined a subset of 49 patients with locally advanced breast cancer who were treated with doxorubicin and found that the recurrence –free survival (RFS) and OS differed significantly among the breast cancer subtypes, with the luminal A having the longest survival times, the basal-like and HER2-positive subtypes the shortest survival times, and the luminal B tumours having an intermediate survival time. Importantly these gene expression subtypes appear stable between primary and subsequent metastatic lesions occurring years later [74]. While gene expression array studies provide a large amount of useful prognostic and predictive data it is clearly not practical or possible to perform these studies on all patients with breast cancer. Consequently there is an ongoing search for reliable immunohistochemical surrogate markers of these subtypes for application to routine diagnostic pathology laboratories, particularly to identify basal-like cancers and the high-risk, hormone-receptor positive luminal B subgroup. Current biomarker panels use a combination of ER, PR, HER2, cytokeratins 5 & 6 (CK5/6) and the epidermal growth factor receptor (EGFR), although debate still exists as to which is the best combination of markers, with recent publications (Carey et al, 2006 [75]; Cheang et al, 2008 [76], Hugh et al, 2009 [77], Livasy et al, 2007 [78] , Rakha et al, 2009 [79]) all proposing different methods of defining the basal-like and luminal B subtypes in particular.

Hugh and colleagues [77] discriminate luminal A and B patients on the basis of Ki67 expression in tumours (Luminal A; hormone receptor positive, HER2 negative and Ki67

≤13%, Luminal B; same pattern but Ki67 > 13%). They studied tumours from more than 1,300 patients participating in the Breast Cancer International Research Group (BCIRG) 001 trial comparing FAC (5-fluorouracil, doxorubicin and cyclophosphamide) to TAC (docetaxel, doxorubicin cyclophosphamide). In this study TAC improved relapse free and overall survival compared to FAC among patients with luminal B class, HER2 class and triple negative tumours but not for tumours belonging to the luminal A class (receptor positive, HER2 negative and Ki 67 ≤13%). The hazard ratio for a relapse among patients treated with the TAC versus FAC regimen was 0.50 for triple negative patients, 0.46 for HER positive and 0.66 for patients with luminal B tumours. Thus the addition of a simple proliferation index resulted in a highly effective separation of ER-positive patients into two intrinsically different luminal A and luminal B populations underscoring the importance of proliferation and suggests that incorporation of a proliferation score into therapy decisions may complement histological grade.

Our own study investigating panels of immunhistochemical/ in situ hybridisation surrogate markers for intrinsic molecular subtypes revealed differences in recurrence and breast cancer specific death between subtypes [80]. We defined five different subgroups luminal A = ER+ and/or PR+, HER-2- ; Luminal B = ER+ and/or PR+, HER-2+; HER-2 = ER- and PR-, HER-2+; basal-like = ER-, PR-, HER-2-, CK 5/6 + and/or EGFR+; unclassified = negative for all five markers using criteria similar to those recently described by Cheang et al [76] but using FISH to determine HER-2 status [81]. Using these surrogate panels we found there was a markedly shorter recurrence time for the more aggressive basal-like, HER2 and unclassified subtypes. Critically, however, while these surrogate were able to provide useful information regarding recurrence, they were not as

powerful predictive markers as standard clinicopathologic variables such as tumour size, lymph node status, lymphatic invasion, histological tumour grade and hormone receptor expression suggesting that well performed histopathological examination of breast cancer is still the gold standard for providing prognostic and predictive data.

Gene Expression profiling assays as predictive biomarkers

The benefit of adjuvant chemotherapy has been demonstrated in a number of clinical trials, reducing overall risk of recurrence by up to 25%, however the absolute benefit for individual patients is small (1-5%) [82]. The NSABP trials B14 and B20 showed women with lymph node negative, hormone receptor positive tumours treated with endocrine therapy alone had a low recurrence rate of 15% over 5 years, meaning 85% of ER positive patients do not require adjuvant chemotherapy [82]. The problem is how to identify those low risk patients, who can safely be spared chemotherapy who are currently not reliably identified using standard clinicopathological factors.

There is considerable interest in developing assays that may better help select patients for adjuvant therapies – both endocrine and cytotoxic chemotherapy. Traditional biomarker research has relied on assessing the expression of single or small numbers of genes at a time mostly using IHC and ISH as diagnostic tools. Analysis of single genes has been so far successful for a handful of markers (such as ER, PR, HER2 and more recently Ki67), with their widespread adoption into routine practice. However, cancer often involves aberrations in many genes and multiple pathways can be defective. Gene expression profiling using RNA microarrays or PCR technology is an efficient way of taking a snapshot of the gene expression signature of tumours. With the development of new

technologies which allow for relatively affordable screening of the relative abundance of messenger RNA transcripts in cancer tissue, representing the entire genome, there has been much research directed at developing assays to answer this key issue in breast cancer management.

An increasing number of diagnostic tools/tests that make use of gene expression signatures are now available to assess patient risk and survival as well as the benefit of adjuvant therapy (reviewed in [83]). These tools promise improved identification of patients who will benefit from treatment and those patients who could be spared unnecessary treatment. Many large microarray studies have controversially differed in the relative abundance of top genes involved in breast cancer with relatively little overlap between them, however this is thought to be due to differences in array platforms and the complexities and differing methodologies of data analysis. A way to resolve this is to use standardised methods, a feature that commercialised diagnostic testing can provide. Two assays in particular have been validated with clinical trials and are in current clinical use to varying degrees: Oncotype DX and Mammaprint.

Onco*type*DX ™

Onco*type*DX ™ is a diagnostic assay that employs quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using FFPE breast cancer specimens, to measure the expression of a panel of 21 genes primarily in ER positive, node negative patients. These genes comprise groups related to ER (ER, PR, Bcl2 & Scube2), proliferation (Ki67, STK15, Survivin, Cyclin B1 and MYBL2), invasion (Stromelysin3, Cathepsin L2), HER2 (HER2 and GRB7), the macrophage marker CD68, the antiapoptosis gene

BAG1 [81] and GSTM1, as well as 5 reference "housekeeping" genes. They are given relative weighting by a scoring algorithm (with the heaviest weighting for ER and proliferation related genes) to develop a recurrence score (RS). There are three categories based on this score originally defined as low risk (<18), intermediate risk (18-30) and high risk (≥31).

This 21 gene signature was tested prospectively in the National Surgical Adjuvant Breast and Bowel Trial (NSABP) B-14, comprising 2644 patients with ER positive, histological node negative tumours [84]. The randomly allocated groups were tamoxifen only or placebo, with the trial showing that tamoxifen reduced recurrence over 15 years follow up. In a subset of 668 patients for whom paraffin tissue blocks were available, comprising, the 21 gene signature revealed a 5 year distant recurrence rate of 22.1% for patients with a high RS score, compared to 2.1% for the low RS score, and 30.5% and 6.8% respectively at 10 years. Furthermore, the majority of patients with high or intermediate RS scores relapsed within 5 years, compared to around one third of recurring patients with low RS scores. In multivariate analysis of distant recurrence, the RS score was independent of age and tumour size. Further analysis revealed it performed better than Adjuvant! (www.adjuvantonline.com) which uses standard clinicopathological variables in predicting recurrence [85].

Further studies confirmed the utility of this 21 gene assay, now called Onco*type*DX ™, including a retrospective case control study [86] which identified its role as a predictive biomarker for hormonal therapy in the NSABP B14 trial as well as for chemotherapy in NSABP trial B-20 [87]. The trial in which the 21 gene assay was performed in a subset of 651 patients, (227 who received tamoxifen only and 424 who also received

chemotherapy, either methotrexate and fluorouracil (MF) or cyclophosphamide, methotrexate and fluorouracil (CMF)) showed a large benefit of chemotherapy for patients with a high RS, and minimal benefit for those with a low score [88]. A benefit for node positive patients with a high RS has also been shown [89]. The data for the intermediate RS group was not clear, but currently there appears to be no clear cut benefit in this group [88].

The Onco*type* DX™ assay has had fairly wide uptake, at least within North America, with over 6000 physicians requesting the test for over 40 000 patients and there is accumulating evidence that the test is altering patient management [90], primarily in the low RS group, increasing the number of patients who receive hormonal therapy only [91]. The test is generally ordered for those patients who ultimately prove to have low (48%) and intermediate (37%) risk scores, with lower number of high risk score patients (15% of assays performed) who are usually identified using standard clinicopathologic variables and are less likely to be referred for testing as decisions regarding chemotherapy are usually more straightforward. The testing process consists of pathologist-guided selection of a representative block of tumour. Fifteen 5μm sections are cut, with recommendations to minimise contamination (utilising a new section of the microtome blade or a new blade between cases, cleaning the water bath between cases and wearing clean gloves for cutting and mounting process.) These sections are ultimately sent to Genomic Health in the USA for in house performance of the assay in which a report outlining and explaining the RS score is provided The current cost to Australian patients for whom there is no rebate is just under $4000 with turn around time of around 2 weeks.

Despite the data showing the prognostic and predictive potential of the Oncotype DX

assay, there is emerging evidence that routinely, well performed immunohistochemical markers may provide just as much information to aid therapeutic decision making. Preliminary data from the Translational arm of the Arimidex, Tamoxifen, Alone or in Combination (TransATAC) trial [92] presented at the 2009 San Antonio Breast Cancer Symposium compared the prognostic power of Onco*type* DX recurrence score, with a formula utilising four standard immunohistochemical markers ("IHC4" - combined ER, PR, Ki67, HER2). Quantitative IHC scores were obtained for ER, PR and Ki67and HER2 in 1,125 of women on the TransATAC trial with Onco*type* DX results and for whom FFPE sections were also available. The IHC4 score showed reasonable correlation with the RS (Pearson coefficient 0.7) and provided a similar amount of prognostic information as the RS score. These results suggest that 4 standard IHC assays performed in a high quality laboratory can provide similar prognostic information for endocrine treated ER positive breast cancer patients as the Onco*type*DX Recurrence Score. Measurement of ER and PR has been performed by IHC rather than by ligand binding assay (LBA) since the early 1990's. However, there is a well recognised problem with reliability and reproducibility of testing. There can be a large discordance in measurement of these key receptors between laboratories, with the Australasian RCPA Quality Assurance Program (QAP) reporting ER positive rates ranging from 26 to 100% of breast cancers in a multi-laboratory audit [93]. It is possible that since a significant component of the 21 gene signature relies on expression ER associated genes that any accurate measure of ER status may provide just as useful data to aid in therapeutic clinical decision making. Despite yielding potentially informative assessments of risk in patients considered indeterminate by routine clinical variables, Onco*type*DX ™ still returns 40-66% of cases

as intermediate risk [94] with no clear data to suggest a benefit of chemotherapy. A large clinical trial called TAILORx (Trial Assigning Individualised Options for Treatment Rx) conducted by the North American Breast Cancer Intergroup is currently underway to prospectively determine address whether ER positive, node negative patients with an intermediate score may benefit from chemotherapy and aims to recruit at least 10000 patients. The cut offs for each category have been altered from their initial descriptions (see above), to low (<11), intermediate (11-25) and high (>25) risk categories to minimise potential for under-treatment in the high risk and intermediate group. Patients with a low RS score receive hormonal therapy only, while high risk patients receive standard chemotherapy. The intermediate RS group are randomised to receive either hormonal therapy or hormonal therapy and chemotherapy. The outcome of this trial will not be known until at least 2013, and until then although the assay has significant benefit in identifying low risk ER positive node negative patients who can be spared chemotherapy, it offers little benefit for intermediate risk patients, who often also have equivocal clinicopathologic features.

There is preliminary data to suggest that addition of standard clinicopathological variables to the Onco*type* DX RS can help reduce the number of cases that fall into the intermediate risk group. At the 2010 American Society for Clinical Oncology meeting (ASCO) Tang et al [95] examined both pathologic and clinical factors such as tumour size and grade, and patient age, in combination with the RS to assess whether the RS may achieve more prognostic power. All patients in the NSABP trial B-14 and the ATAC study with ER-positive tumour specimens and a successful Onco*type* DX RS assay were included. The meta- analysis included 647 B-14 patients and 1088 ATAC patients; B-14

patients were node-negative and were treated with tamoxifen while the ATAC patients were node-positive or node-negative, and were treated with tamoxifen or anastrozole. Meta-analysis assessed the risk of distant recurrence combining the individual study multivariate risk assessments using recurrence score and pathologic and clinical (RSPC) information. RSPC prognosis combining clinical and pathology information with RS was significantly more powerful than using RS alone. Furthermore, compared with the Onco*type* DX RS alone, fewer patients were classified as intermediate risk using the RSPC index (18% vs 26%; p=0.001), and 72% of pts with intermediate RS 18-30 were pushed in to either high or low risk categories. The RSPC index combining RS with pathology and clinical information with RS supplied more powerful prognosis for early stage breast cancer patients than RS alone and it was estimated that its use would reduce the number of patients with intermediate risk by 30% and enhance individualised treatment decisions.

MammaPrint [R]

The Netherlands Cancer Institute (NKI) developed the '70-gene' signature using gene expression microarrays on tissue from a retrospective series of node-negative breast cancer patients who were under 55 years of age, with tumours smaller than 5cm and who were treated with loco-regional therapies only [96]. This initial study found 213 genes that could identify patients with a high risk of developing distant metastases. This signature was subsequently refined to 70 genes was validated on a set of 295 patients where it was able to distinguish patients at high and low risk of distant metastasis based on 10 year survival figures. This 70-gene signature was developed into an FDA-approved diagnostic

test named MammaPrint using the Agilent microarray platform, and is recommended for node-negative patients under 61 years of age, with stage I or II disease with tumour size < 5cm. It can also be used for patients with 1-3 positive nodes, although currently only outside the USA as it has not been FDA-approved yet for this indication. This assay requires either fresh frozen or tissue collected at room temperature into a RNA preserving solution and is currently not suitable for FFPE tissue. MammaPrint uses the 70-gene signature to discriminate patients with high or low risk of recurrence and encompasses genes associated with proliferation, metastases, stromal invasion, and angiogenesis. MammaPrint does not directly assess ER, PR or HER2 mRNA, although a modified assay TargetPrint does. The MammaPrint assay dichotomises patients into low or high risk groups, with no intermediate group, in comparison to the Onco*type* DX assay which generates a continuous score and unlike the Onco*type* DX assay, ER negative patients can be assessed.

Compared to the St Gallen and NIH consensus criteria, the 70-gene signature is equally as effective at predicting patients who would benefit from adjuvant treatment (van 't Veer et al, 02) and was able to identify patients with a higher risk of developing distant metastases than by traditional methods. However, with 70-80% of breast cancer patients receiving unnecessary treatment (EBCTCG 98a, EBCTCG 98b), the greatest value of MammaPrint is in its ability to identify patients who could be spared unnecessary adjuvant therapy in the “low risk” group who show greater than 90% chance of being disease free for a minimum of 5 years. At the moment, the MammaPrint assay is largely a prognostic, rather than predictive assay, although a large prospective trial to assess its

predictive capability is underway called the MINDACT trial (Microarray In Node-negative Disease may Avoid ChemoTherapy). Patients with node negative, and more recently some lymph node positive patients are eligible for the trial whether ER positive or negative. Patients who was classified as high risk using standard clinicopathological factors as assessed by Adjuvant!Online and via MammaPrint receive chemotherapy, while patients identified as low risk by both methods receive hormonal therapy as appropriate. However, any discord between standard criteria and the MammaPrint assays results in randomisation to receive either adjuvant chemotherapy or hormonal therapy as clinically appropriate.

A validation cohort was analysed using MammaPrint, which included both node-negative and node-positive patients and also patients who received systemic adjuvant treatment. The 70-gene signature was found to be the strongest predictor for distant metastasis-free survival, independent of adjuvant treatment, tumour size, lymph node status, histological grade and age [97]. In addition, the prognosis signature significantly improved identification of patients at high risk and low risk, reducing potential clinical under-treatment or overtreatment of these patients. Another independent validation cohort using 307 node-negative breast cancer patients who did not receive systemic adjuvant treatment also confirmed significant benefit of the prognostic categories identified by MammaPrint [98].

Although these assays seem to show superior performance to aid clinical decision making than standard clinicopathological variables in particular groups of patients, there is significant criticism about the overall utility of "gene signatures", especially in regards to

the selection of genes within them. There are very few genes in common between the many gene signatures published for example there is only a single gene in common between the 21 genes of the Oncotype DX assay and the 70 genes of the Mammaprint assay, (SCUBE 2 which is an oestrogen regulated gene). A major contributor to this problem is the varying different composition of patients within the discovery patient cohorts [99]. Michiels and colleagues repeatedly (500 times) generated signatures of the top 50 prognostic genes from the Van't Veer dataset (on which the MammaPrint assay is based) and found that by manipulating patient selection, entirely different signatures were generated. After 500 repeats, only 20% genes in the original published 70 gene signature were seen in more than half of the new signatures generated by changes in patient selection and an additional 10 genes were frequently identified that were not present in the 70 gene signature [100]. These data suggest that these signatures are not stable and will likely vary significantly within different groups of patients. This is reflected in the studies of Fan and colleagues [101] who compared the predictions of five published breast cancer gene signatures on the same dataset. In regards to the 21 (Onco*type*DX) and 70 gene (MammaPrint) signatures, outcome prediction agreement was only 80% and the analysis also revealed that 50% of patients with an intermediate risk score by the Oncotype DX assay were classified as high risk by the 70 gene Mammaprint signature [100].

Conclusions

The recent advances in molecular biology are yet to have a significant impact on the routine diagnosis and management of breast cancer, with the exception of HER2. This review has described a number of promising tests that may have a role to play in specific subsets of breast cancer. While it seems that the ISH assays for TOP2A or Chromosome 17 may be readily adapted to a routine pathology laboratory setting, there is currently insufficient clinical data to support their use. Current information on the multigene assays such as Onco*type* DX or MammaPrint in our opinion also does not yet support their routine use, at least until the outcome of the TAILORx and MINDACT trials are known in view of the cost and limitations of the assays. Finally, our review of the literature still highlights the importance of well performed routine histopathology and accurate assessment of hormone receptor and HER2 assays in leading to optimal patient outcomes.

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Table 1 Interpretation of HER2 ISH testing in Breast Cancer from ASCO/CAP guidelines on HER2 testing [20]

Result	Single probe (eg CISH or SISH)	Dual Probe (eg FISH or C/SISH with CEP17 probe)
Negative	Mean HER2 copy number <4 signals per tumour cell nucleus	HER2/ CEP17 ratio <1.8
Positive	Mean HER2 copy number >6 signals per tumour cell nucleus	HER2/ CEP17 ratio >2.2
Equivocal	Mean HER2 copy number 4-6 signals per tumour cell nucleus	HER2/CEP17 ratio 1.8-2.2

Table 2. Comparison of multi-gene assays: Onco*type* DX and MammaPrint

Assay	**Onco*type* DX**	**MammaPrint**
Method	qPCR	Microarray
Genes tested	21	70
Material required	FFPE or fresh tissue	Fresh/frozen tissue
Processing limitations	May require microdissection	May impact routine surgical procedures
Current indication	Node-negative, ER+	Node-negative
Validated in retrospective studies	Yes	Yes
Prospective clinical trials in progress	TAILORx	MINDACT

Figure legends

Figure 1. Fluorescent in situ hybridisation (FISH). Within the cell the HER2 fluorescent DNA probe (red) hybridises to the Her2 gene and the Chromosome 17 centromeric enumeration DNA probe (CEP17, green) hybridises with Chromosome 17 DNA.

Figure 2. Fluorescent in situ hybridisation example of Her2 amplification (A) and an example of equivocal HER2 copy number (B). A case with clonal amplification (red circle - region of Her2 amplification, blue circle - region of diploid Her2 copy number) by Her2 immunohistochemistry (C) and HER2 chromogenic in situ hybridisation (D). Chromogenic in situ hybridisation showing Topoisomerase II alpha amplification (E) and Chromosome 17 “polysomy” (F). All images are at 1000X magnification.

Figure 3. Molecular methods for clinical diagnostics can assess changes in DNA, mRNA and protein levels and include examples such as HER2 FISH - PathVysion, qPCR - Onco*type* DX, Microarray - MammaPrint and Her2 IHC. Each method has specific tissue requirements and different levels of throughput.

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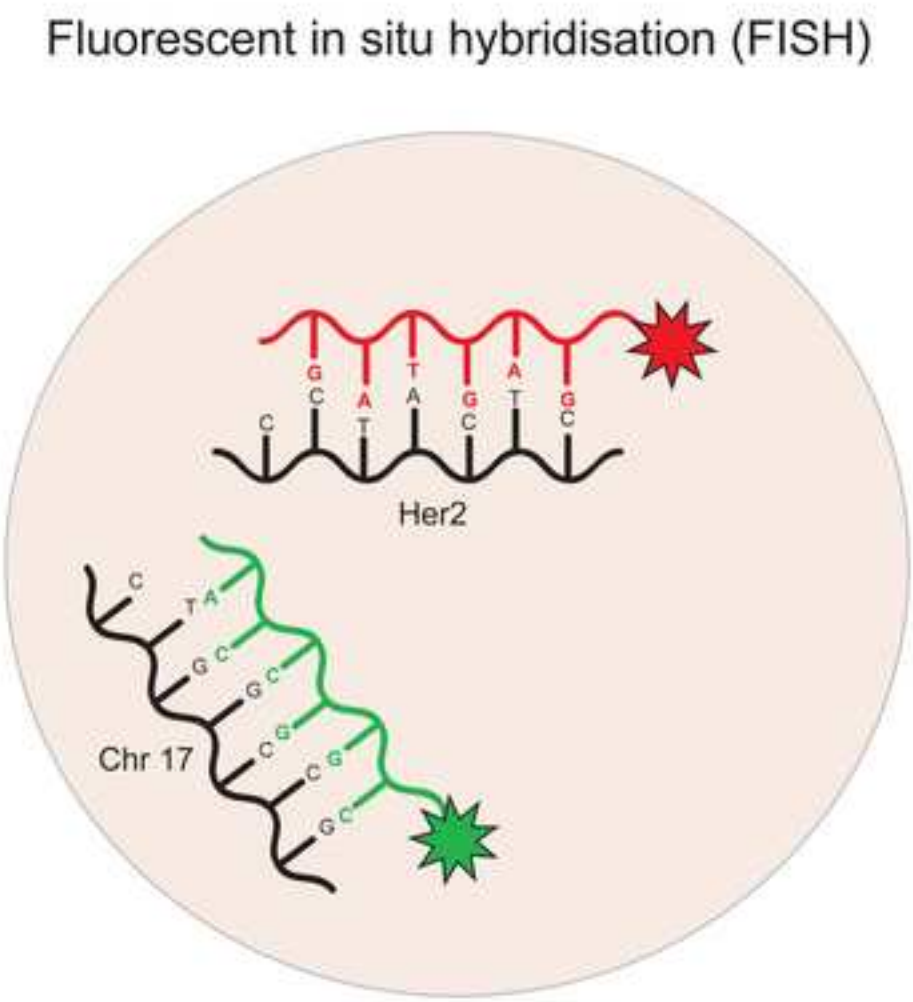

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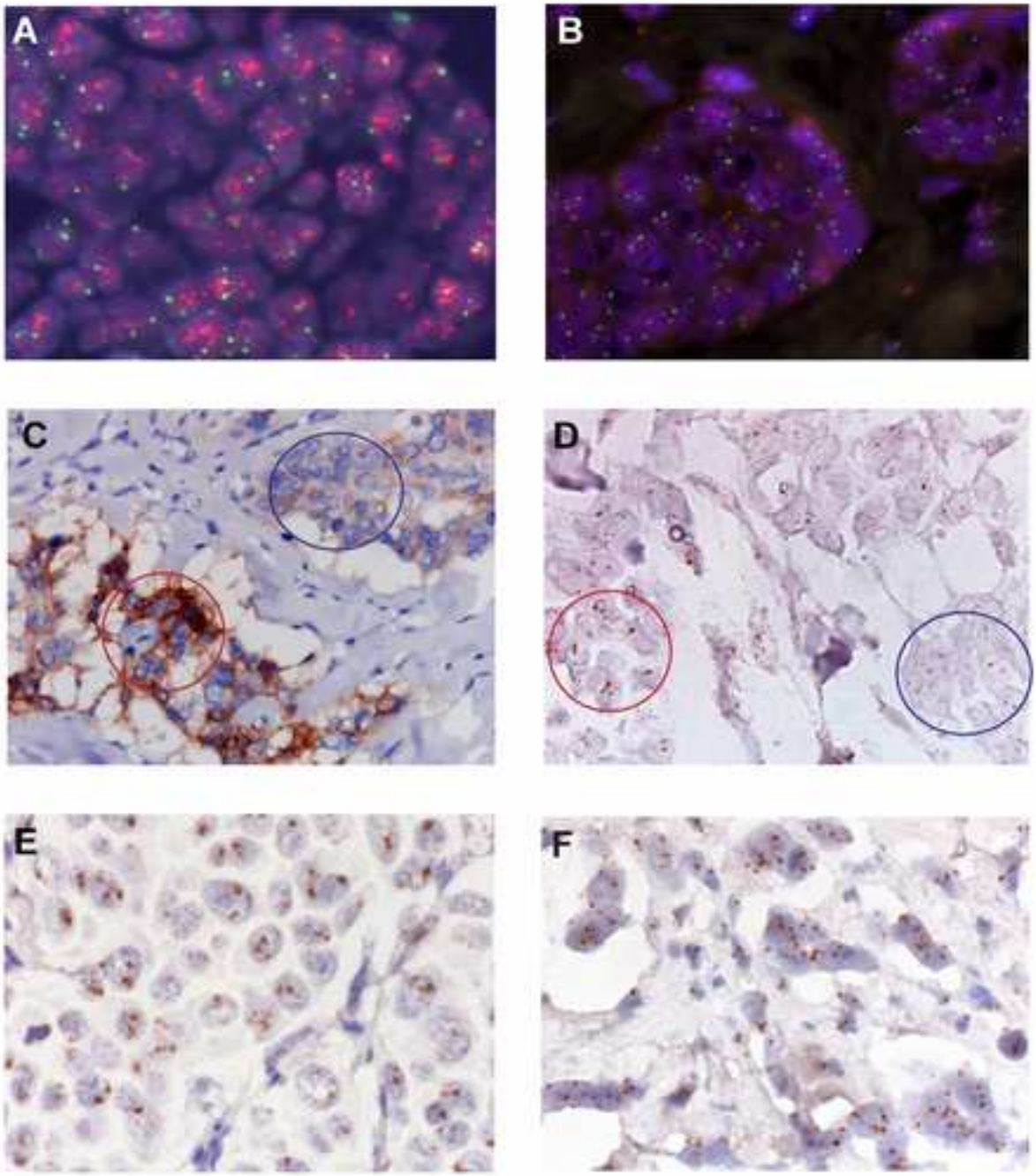

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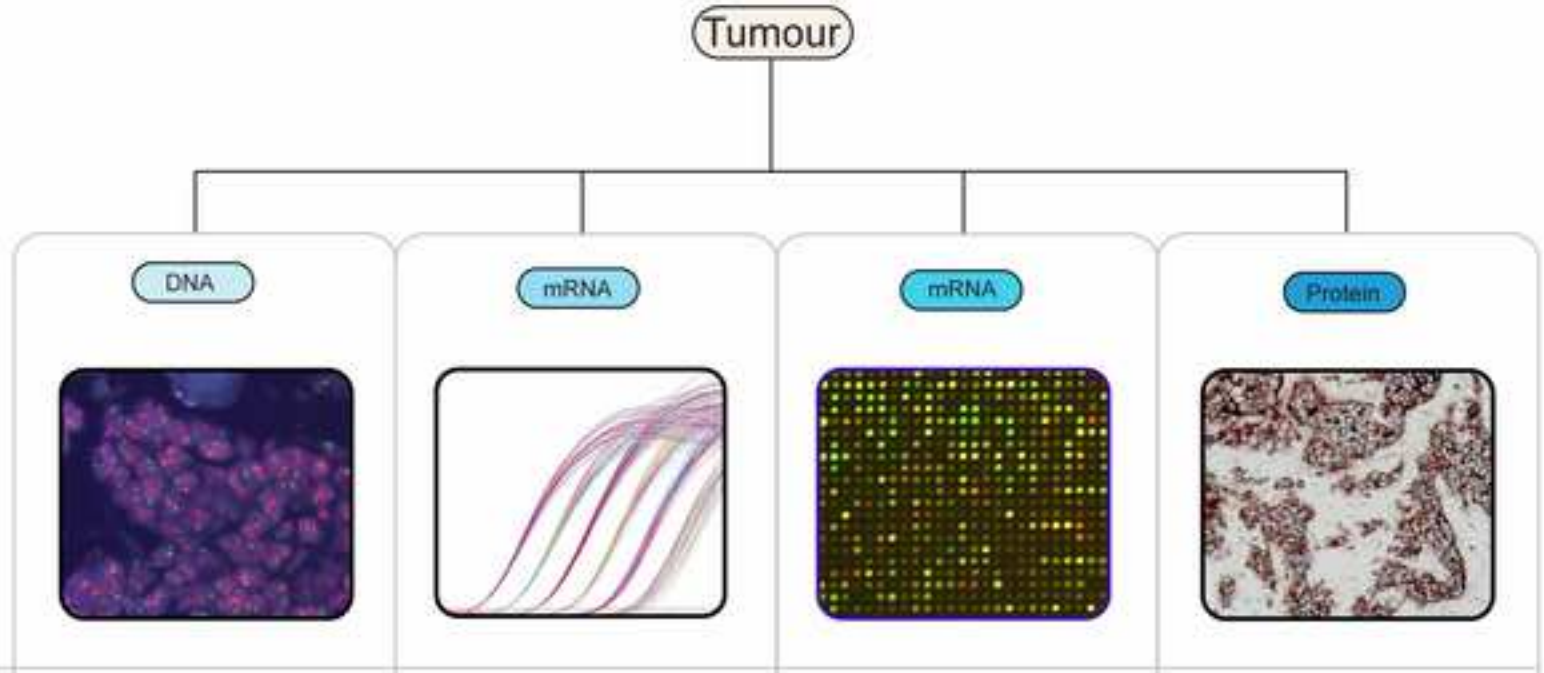

Method	FISH	qPCR	Microarray	IHC
Molecular assessment	Translocations, amplifications and deletions	Gene expression	Gene expression	Protein expression
Clinical example	Her2 PathVysion (Abbott Molecular)	Oncotype DX (Genomic Health)	MammaPrint (Agendia)	Her2 IHC
Tissue required	FFPE tissue slide	FFPE tissue	Fresh/frozen tissue	FFPE tissue slide
Level of throughput	Low	Medium	High	Low