



Molecular and Cellular Analyses of Breast Cancers in Real Life

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Introduction

Breast cancer is the most common women's malignancy in the world and in South American countries, including Peru, and it is the leading cause of cancer-related death in women. Different reports indicate that race and a country's income can influence prognosis in breast cancer. Part of these disparities is because women from low-income countries have a delay in cancer management and are diagnosed with more advanced stages. However, different publications also describe that rates of aggressive tumor features have a higher prevalence in some races (Castaneda et al. 2018).

Biomarkers in Breast Cancer

As we are entering the era of personalized medicine, much attention has been paid to identify biomarkers of prognosis and response to therapies targeting a tumor's genetic background (André et al. 2019).

One of the most immediate challenges after diagnosis is to identify who should receive adjuvant treatment and to select the most suitable therapy in early stages and how to choose the most effective and least toxic therapy in

advanced disease. A substantial amount of research has been invested in the development and validation of prognostic factors and predictive biomarkers.

A good biomarker should be analytically valid, reproducible, and respond to a relevant clinical question. It must also be affordable and accessible to pathologists and laboratory scientists in both academic and community practice centers worldwide to be incorporated into daily practice (Fig. 1) (Salgado et al. 2019).

Biomarkers in Cancer Cells

The most recognized prognostic tumor factors in early breast cancer are the two macroscopic pathological features, regional lymph node metastases and tumor size, and the microscopic feature tumor grade. Histology grade is widely informed based on the Nottingham system. This system utilizes three microscopic features: nuclear pleomorphism, gland or tubule formation, and the number of dividing cells.

Detection of estrogen receptor (ER), progesterone receptor (PgR), HER2, and the widely used Ki67 index has become a requirement for choosing the treatment through the worldwide cancer centers (Rebaza et al. 2018). Although the last one has been described as having a poor interlaboratory precision and lack of a validated cutoff point, the International Ki67 in the Breast

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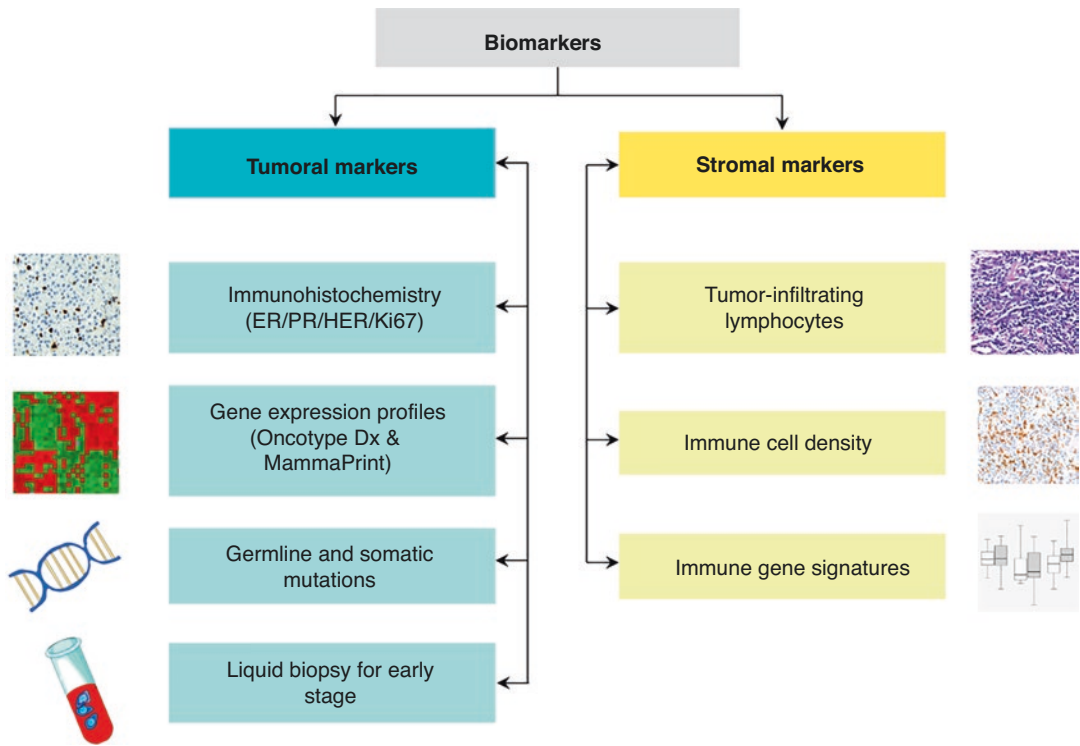


Fig. 1 Biomarkers in breast cancer

Cancer Working Group makes technical recommendations for improving assay results and indicates that <5% and >30% Ki67 counts have better interlaboratory precision (Nielsen et al. 2021).

The American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) periodically reviews the guidelines for cataloging ER and PgR, and the last actualization was performed in 2020. They categorize positive status for ER or PgR when 1–100% of tumor nuclei positive are found. The low ER-positive status is demonstrated when 1%–10% of tumor cell nuclei are immunoreactive (Allison et al. 2020).

Similarly, a periodical review of the guidelines for HER2 status is performed and the last was done in 2018. It classifies the status as positive when the IHC result is 3+ and negative if the IHC result is 0 or 1+. If the IHC result is 2+ (weak to moderate complete membrane staining observed in <10% of tumor cells), a dual ISH or FISH is recommended to be performed. Combination between HER2/CEP17 ratio and

mean HER2 copy number can fit in one of five scenarios. The first (HER2/CEP17 ratio ≥ 2.0 and average HER2 copy number ≥ 4.0) and the third scenarios (HER2/CEP17 ratio < 2.0 and HER2 copy number of ≥ 6) are classified as positive (Wolff et al. 2018).

There have been described other immunohistochemistry biomarkers like androgen receptor, and their analysis has been described by my group at the Peruvian Cancer Institute in a local series of 95 triple-negative breast cancer (TNBC) samples (Castaneda et al. 2019).

Additionally, platforms of tumor gene expression have demonstrated their prognostic value and some of them a predictive role for adjuvant treatment (Sparano et al. 2018; Cardoso et al. 2020; Müller et al. 2021; Gnant et al. 2015). Oncotype Dx is the most widely used multigene signature for predicting outcome in breast cancer, and the tool is available in the different continents. It tests 21 genes at the mRNA level by using RT-PCR, including 16 linked to cancer, and provides quantification of gene expression for

ER, PgR, and HER2. It generates a recurrence score (RS) as a continuous variable that divides patients into prognostic groups as well as benefit from adjuvant chemotherapy. The prospective trial TAILORx with more than 10,000 node-negative breast cancer cases demonstrated that there was a low risk of recurrence after endocrine therapy alone in patients with RS = 0–10. The endocrine therapy alone was noninferior to adjuvant chemotherapy plus endocrine therapy in the overall population with RS = 11–25 and a high likelihood of benefit from chemotherapy in patients with RS = 26–100. A chemotherapy benefit was noted in patients ≤ 50 years old with an RS of 16–25 (Sparano et al. 2018). A new tool (RSclin) that integrates RS with tumor grade, tumor size, and age has demonstrated its value to predict chemotherapy benefit (Sparano et al. 2021). Recent analyses find that race influences the value of the test, and Black women had worse clinical outcomes despite similar RS (Hoskins et al. 2021). Finally, the recently presented RxPONDER trial enrolled 5015 stage II/stage III breast cancer patients and found that postmenopausal women with ER-positive, HER2-negative breast cancer with 1–3 positive nodes and RS ≤ 25 derived no benefit from adding chemotherapy to endocrine therapy. On the other side, premenopausal women experience a 46% reduction in recurrence risk with the addition of chemotherapy (Müller et al. 2021).

MammaPrint assay is another platform that is a microarray-based technique that evaluates a 70-gene signature related to proliferation, invasion, and angiogenesis. The MINDACT trial is a phase III trial evaluating 6693 node-negative and 1–3 node-positive early breast cancer patients that demonstrated to have better prognostic value than the evaluation using standard clinicopathological features. A recent long-term analysis confirmed the prognostic value of the tool in women >50 years (Cardoso et al. 2020).

Determination of germline mutations including BRCA1, BRCA2, and other genes related to DNA repair mechanisms has been largely associated with hereditary breast cancer, and somatic mutations in similar genes have similarly been described in tumor lesions. Their presence has

been described as predictive for response to platinum chemotherapy and enzyme-poly-ADP-ribose-polymerase inhibitor compounds. The methodology for their detection has also been recently implemented in South American countries, and the experience in their interpretation is increasingly required in daily practice (Castaneda et al. 2018; Oliver et al. 2019; Oh et al. 2021).

Circulating Biomarkers

The analysis of compounds in blood such as CA15-3 and CEA has all been shown to predict poor outcome in patients with breast cancer (Molina et al. 2010).

Several studies have shown that somatic mutations identified in ctDNA are widely representative of the tumor genome and can provide an alternative noninvasive method that overcomes many difficulties related to tissue biopsy. The detection of mutation in the ligand-binding domain of ESR1 in ctDNA that confers constitutive activity of ER is an emerging predictor of endocrine therapy resistance in the metastatic setting. ctDNA levels also closely reflect changes in tumor burden and can predict the progressive disease several months before the standard imaging. Levels of ctDNA may also be an important indicator of prognosis; however, prospective studies in larger cohorts of patients are still needed to validate their prognostic role. CTCs are cancer cells that have been shed or actively migrate into the vasculature from the primary tumor or metastatic lesions and circulate in the bloodstream. They can give rise to metastases (seeding hypothesis) in distant organs (Stanton et al. 2016; Denkert et al. 2018). CTC enumeration has demonstrated to have a prognostic value in the metastatic setting and to predict early and late recurrences as well as shorter overall survival (OS) in early breast cancer. Beyond enumeration, there is interest in genotypic and phenotypic characterization of CTCs that may help in revealing the underlying mechanism of tumorigenesis and metastases. In contrast to CTCs, a cutoff of ctDNA that correlates with a worse prognosis has not been identified yet (Rossi et al. 2018).

During the last 4 years, we have been working in the detection of ctDNA through digital PCR equipment in plasma samples from 183 breast cancer patients. We found a PIK3CA mutation in ctDNA in 35% cases (most with E545K), and it was associated with lower levels of tumor-infiltrating lymphocytes (TILs) ($p = 0.04$). PIK3CA in ctDNA tended to be associated with advanced stages ($p = 0.09$) in whole series and with higher recurrence rates ($p = 0.053$) in the nonmetastatic setting. Patients with presence of PIK3CA mutations in their ctDNA tend to have shorter OS ($p = 0.083$) (Galvez-Nino et al. 2020).

Biomarkers in Stromal Cells

Malignant cell transformation alters the structure of cell membrane proteins and induces antitumor responses against tumor antigens which eliminate the developing tumor cells. Dendritic cells can take antigens and migrate to lymphoid organs, where they present their antigens to adaptive immune cells.

Effector T-cells include various subsets: T helper cells, T helper 1 (TH1), TH2 cells, TH17 cells, regulatory T (Treg) cells, T follicular helper cells, and cytotoxic T lymphocytes (CTLs).

TH1 cells produce cytokines, such as IFN-gamma and IL-2, which play important roles in activating and regulating the CTL responses. Meanwhile, TH2 cells secrete cytokines that stimulate humoral immune responses as well as IL-4 and IL-10 which downregulate the pro-inflammatory state and inhibit the synthesis of TH1 cytokines.

CTLs confer cytolytic activity by releasing perforin and other cytotoxins that induce apoptosis. The antitumor activation of T-cells relies on T-cell receptor (TCR) recognition of antigenic peptides presented by major histocompatibility complex molecules on the neoplastic cells.

However, tumors have developed some mechanisms inhibiting T-cell responses. Upregulation of CTLA-4 in CD8+ T-cells produces an inhibitory effect over the stimulatory activity of the CD28 receptor after TCR engagement with antigens. Another inhibitory receptor in T-cells is

PD-1 that is activated by their ligand PD-L1 which can be upregulated by tumor cells. In addition, there are inhibitory cells present in the tumor microenvironment, including M2-polarized tumor-associated macrophages (TAMs) and Tregs. Tregs are identified as a population of CD4+ FOXP3+ T-cells that express CD25, a subunit of the receptor for the T-cell-stimulating cytokine IL-2, and also constitutively express CTLA-4. TAMs represent a highly heterogeneous immune cell population that express CD68 marker and have two polarized phenotypes, M1 and M2. The former is traditionally associated with antitumor effects and expresses CD80 and CD86, and the latter is typically showing protumorigenic characteristics and expresses CD163, CD204, and CD206.

Stromal tumor-infiltrating lymphocyte (TIL) level has been extensively described to be higher in triple-negative breast cancer (TNBC) and HER2+ than luminal phenotype (Stanton et al. 2016; Denkert et al. 2018). In addition, TIL levels are lower in metastatic and in heavily treated diseases, while levels are lower in tumor lesions located in the liver (Luen et al. 2017).

TILs have been strongly associated with prognosis in early-stage TNBC and HER2-positive breast cancer. Additionally, Denkert and colleagues found that TILs were independent predictors for pCR in an initial cohort ($n = 218$) and the validation set ($n = 840$). TIL-positive tumors achieved 40% and 42% of pCR, whereas the TIL-negative tumors achieved only 3%–7% pCR in the discovery and the validation cohort, respectively (Denkert et al. 2010).

A recent meta-analysis found that higher TIL levels predict pCR (OR 2.14, 95% CI 1.43–3.19) and longer OS (HR 0.9, CI 0.97–0.93) and DFS (disease-specific survival) (HR 0.66, 0.57–0.76) in the TNBC subset (Denkert et al. 2018; Gao et al. 2020; Loi et al. 2019).

Finally, an international collaboration network where we participated evaluated the role of TIL in residual lesions of TNBC. In a final series of 375 residual TNBC samples, TIL levels were significantly lower with increasing post-NAC tumor size, nodal stage but did not differ by residual cancer burden (RCB) class. Higher TIL in resid-

ual disease was associated with improved RFS ($p < 0.001$) and OS ($p < 0.001$). Greater magnitude of positive effect was observed for RCB class II than class III (Luen et al. 2019).

Therefore, TILs reached level 1b evidence as prognostic marker in early TNBC in the 16th St Gallen International Breast Cancer Consensus Conference. WHO (World Health Organization) and ESMO (European Society for Medical Oncology) also recommended their incorporation in the routine pathology report of early TNBC samples. However, TIL was not recommended for guiding systemic treatment selection (Burststein et al. 2019).

A large series of studies evaluated the prognostic significance of CD8+ T-cells in over 1300 breast cancer patients who underwent mastectomy or lumpectomy with radiation. The number of CD8+ T-cells correlated with a higher grade and inversely correlated with ER expression. In a multivariate model that included tumor size, stage, grade, vascular invasion, HER2 and ER status, age, and adjuvant treatment, the number of CD8+ T-cells was independently associated with improved disease-specific survival (DFS) ($p = 0.001$). This association keeps among the ER tumors but not in ER+ tumors (Mahmoud et al. 2011). A further geographic analysis of CD8 cell distribution inside the tumor lesions describes that fully infiltrated and stroma-restricted CD8+ infiltration had the most favorable prognosis (Gruosso et al. 2019). Other series of studies describe that CD8 expression can also have a negative effect over survival as is strongly correlated with FOXP3 expression (Bottai et al. 2016).

García-Martínez et al. evaluated the role of CD3, CD4, CD8, CD20, CD68, and FOXP3 immune cells in pre- and post-neoadjuvant tumor samples in a series of 121 breast cancer patients in predicting response to neoadjuvant chemotherapy and survival. They found that higher pre-NAC infiltration by CD3, CD4, and CD20 was associated with pCR, and the predictive response role of CD4 was confirmed in six public genomic datasets. Higher CD68 density in residual post-NAC samples was associated with shorter OS. Analysis of the immune infiltrate in post-chemotherapy residual tumors identified a highly

CD3 and CD8 infiltrated profile with a worse DFS (García-Martínez et al. 2014).

A recent meta-analysis found that the CD4 TIL subgroup (high vs. low) showed a better OS (HR 0.49, 95% CI 0.32–0.76) and DFS (HR 0.54, 95% CI 0.36–0.8), and the CD8 TIL subgroup showed a better DFS (HR 0.55, 95% CI 0.38–0.81). FOXP3 TIL subgroup was associated with better DFS (HR 0.5, 95% CI 0.33–0.75) (Gao et al. 2020).

The predictive value of TIL over response to targeted therapy has been demonstrated for HER2 therapy and anti-PD1 checkpoint inhibitors. Loi and colleagues have evaluated the predictive value of TIL in 935 patients randomized between chemotherapies along with or without trastuzumab. They found that trastuzumab was not associated with decreased risk of relapse in patients without lymphocyte infiltration (HR, 1.0; 95% CI, 0.55–1.75; $p = 0.99$). The three-year DFS rate was 96% in patients with high TIL tumors treated with chemotherapy and trastuzumab (Loi et al. 2012).

Higher TIL levels were also found to predict longer DFS and reduction in recurrence rates in the ShortHER trial that compared 1 year or 9 weeks of trastuzumab duration in 866 cases. They also found that cases with TIL < 20% obtained significant benefit from the longer but not from shorter trastuzumab schedule (Dieci et al. 2019).

TILs were associated with DFS in the whole population from APHINITY phase III trial who received adjuvant pertuzumab or placebo added to standard chemotherapy/trastuzumab after resection in early HER2+ breast cancer (Krop et al. 2019).

Furthermore, TILs also correlate with outcome after immune checkpoint blockade in metastatic TNBC (Loi et al. 2017; Emens et al. 2019; Voorwerk et al. 2019).

The IMpassion130 trial evaluated the addition of atezolizumab to first-line chemotherapy with nab-paclitaxel in 902 metastatic TNBC patients, showing a significant PFS benefit in both the ITT population and the cohort with the presence of at least 1% staining of PD-L1 on immune cells. In addition, although OS was not significantly improved in the ITT, an increase in OS was

observed among the PD-L1+ subgroups in the immunotherapy-containing arm (Schmid et al. 2018). Intratumoral CD8 were well correlated with PD-L1 and predicted progression-free survival (PFS) and OS; TILs had poor correlation with PD-L1 and were also associated with PFS but not OS (Emens et al. 2018). The KEYNOTE-355 trial evaluated the addition of pembrolizumab to three standard chemotherapeutic schedules in the first-line treatment of advanced TNBC. A significant PFS benefit was found with the addition of immunotherapy to first-line chemotherapy in the PD-L1+ population, defined by a combined positive score $\geq 10\%$ (Cortes et al. 2020).

There are two phase III randomized clinical trials that addressed the role of immune checkpoint inhibitors in the neoadjuvant setting of locally advanced TNBC. KEYNOTE-522 and IMpassion031 evaluated adding pembrolizumab or atezolizumab to standard neoadjuvant chemotherapy including anthracyclines. Although they demonstrated that the addition of antiPD1 therapy increased rates of pathologic complete response, they did not find that TIL levels or PD-L1 status predict the response (Schmid et al. 2020a,b; Loibl et al. 2019; Mittendorf et al. 2017, 2020).

After reviewing published information and analyzing our lab strengths and experience at the institute, a research team at the institute under collaboration with international research partners focused on evaluating the role of TIL levels and immune cell density in breast tumor samples before and after chemotherapy. After regulatory research issues, we evaluated 98 TNBC cases and found that higher TIL in pre-NST was associated with pathologic complete response and outcome. Post-NAC area with pCR had similar TIL levels than those without pCR ($p = 0.6331$). NAC produced a TIL decrease in full-face sections ($p < 0.0001$). Higher counts of CD3, CD4, CD8, and FOXP3 in pre-NAC samples had longer DFS. Higher counts of CD3 in pre-NAC samples had longer OS. Higher ratio of CD8/CD4 counts in pre-NAC was associated with pCR. Higher ratio of CD4/FOXP3 counts in pre-NAC was associated with longer DFS. Higher counts of CD4 in post-NAC area were associated with pCR (Castaneda et al. 2016).

Thereafter, we evaluated the role of TILs in 435 pre-NAC samples and found that they are associated with grade III, no luminal A subtype, RE negative, HER2 positive, and pCR (Galvez et al. 2018).

Conclusions

We can conclude that breast cancer is one of the most frequent malignancies, and biomarkers are allowing to improve treatment results. Technologies and procedures for evaluating biomarkers related to tumor cell behavior and their interaction with the stroma have been incorporated in the daily routine.

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