Chapter 1

Biomarkers in Breast Cancer

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Abstract

Breast cancer is the most common cancer in women and its incidence experienced an important increase, thanks to the introduction of a systematic screening. The increased incidence of early breast cancer has led to debates on its over-treatment, which may cause unnecessary harm to patients with favorable prognosis. Therefore, modern research is in the quest of finding the perfect prognostic marker to avoid overtreatment in patients with a favorable prognosis. In this perspective, many molecular markers have been studied in the last decades in order to provide both a useful prognostic tool, able to determine whether the cancer is likely to be indolent or aggressive, and a possible therapeutic target. In this chapter, we review the current knowledge about the principal biomarkers, which are usually immunohistochemically tested on breast surgical specimens, including ER and PR, Mib1/Ki-67 and HER2/neu expression. Furthermore, we will analyze other possible prognostic markers which may have in the future a key role in breast cancer management, such as several multigene panels (OncotypeDX, Mammaprint, NanoString Prosigma). Finally, we will discuss the role of genetic tests for some know genetic mutations associated with higher breast cancer susceptibility (BRCA1 and 2 genes).

Keywords: breast cancer, biomolecular markers, biohumoral markers, therapy target, prognostic factors

1. Introduction

Breast cancer is the most common cancer in women, accounting for about one-third of cancer cases in women and more than 10% of all cancers worldwide [1], and its incidence experienced an important increase, thanks to the introduction at the beginning of this century of a systematic mammographic screening in the most developed countries, and the subsequent successful detection of an always greater number of early breast cancers [2–4]. The incidence of breast

cancer is also rapidly rising in developing countries, so that it will become in the next decades a major health burden in both developed and developing countries.

Improvement in the adjuvant chemotherapy and endocrine therapy decreased breast cancer mortality by approximately 50%. However, the increased incidence of early breast cancer has led to debates on its overtreatment, which not only increases social and family burden, but may also cause unnecessary harm to patients with a favorable prognosis [5, 6]. Therefore, the research is focusing on the development of new adjuvant therapies with a more precise target and fewer side effects. In this perspective, many molecular markers have been studied in the last decades in order to provide both a useful prognostic tool, able to determine whether the cancer is likely to be indolent or aggressive [7, 8], and a possible therapeutic target.

Breast cancer includes a heterogeneous group of tumors with a wide spectrum of morphologically and molecularly different subtypes, resulting in different biological behaviors, presentation, and prognosis. Along with the disease stage and the patient performance, the molecular pattern of the tumor is fundamental to identify patients who will particularly benefit from a given treatment. Among the molecular markers associated with breast cancer, the estrogen receptor (ER), the progesterone receptor (PR), the human epidermal growth factor receptor (HER2) and the Mib1/Ki-67 proliferation index are the most important ones and are firmly established in the standard care of all primary, recurrent, and metastatic breast cancer patients.

In this chapter, we review the clinical relevance of the principal biomarkers, which are usually immunohistochemically tested on breast surgical specimens. We discuss about their implication in the prognosis and treatment of breast cancer patients, and thus how this information is translated to treatment decision-making, the valid assays for these markers, and the guidelines for testing them. Furthermore, we analyze other possible prognostic markers which may have in the future a key role in breast cancer management, such as several multigene panels, which have been developed to predict the possibility of distant metastasis in the hormonal receptor-positive disease [9–11]. Finally, we discuss the role of genetic tests for some know genetic mutations associated with higher breast cancer susceptibility in the screening and follow-up of women at high risk.

2. Estrogen and progesterone receptor: Prognosis prediction and treatment planning

Human breast cancer usually depends on sexual hormones for its growth, as it arises from breast tissue that normally responds to endogenous hormones [12]. As in 1896 was firstly noticed that bilteral oophorectomy could induce a significant regression in breast cancer in the fertile age [13], endocrine therapy became quickly a standard of care in the treatment of breast cancer, but only one-third of patients responded.

Then, as in the early 1960s, radiolabeled estrogens were observed to concentrate on specific target organs, the existence of an estrogen receptor (ER) was hypothesized, which could be a predictive factor for the endocrine responsiveness of breast cancer to ovarian ablation [14, 15].

In fact, about 60% of ER-positive tumors, but only about 8% of ER-negative ones showed an objective response to endocrine therapy. The small proportion of patients who respond to hormone therapy with ER-negative disease may be mostly due to false-negative receptor assay results.

The identification of the estrogen receptor has not only proved to be a successful therapeutic target for the treatment and prevention of breast cancer, but has also represented a selective molecular model for all subsequent efforts to design oncological targeted therapies. Estrogen and progesterone receptor (PR), together with the HER2 status represent the most important molecular markers in the standard care of all primary, recurrent, and metastatic breast cancer patients, and the standardized assessment of the ER/PR/HER2 status is crucial in the evaluation of every newly diagnosed breast cancer.

Hormonal receptor-positive disease represents usually an indolent and slowly growing tumor with longer time to recurrence. The responsiveness of a tumor to hormone therapy is an important parameter in breast cancer management in both adjuvant and metastatic settings. The clinical aspects of anti-hormonal treatments are exposed in the following sections.

2.1. Biology of hormone receptors

The ER is a ligand-regulated, cytoplasmic receptor that belongs to the steroid nuclear receptor family, which in the ER-positive breast disease, promotes cell proliferation, survival, and invasion. The key components of ER are the DNA-binding domain, which binds with high affinity and specificity to estrogen response elements (ERE sequence) of DNA to regulate the transcription rates of target genes, and the ligand-binding domain, which binds estrogens [16]. The binding of estrogen to its receptor is essential for its translocation into the nucleus, where it functions as a transcription factor and transduces hormonal signals into a large variety of physiological responses in various target organs.

Two forms of ER, *ERα* and *ERβ*, are encoded by two separate genes that are differentially expressed in tissues. In the normal mammary gland, both *ERα* and *ERβ* bind estradiol to control cell proliferation and differentiation [17, 18]. *ERα* is also responsible for estrogen-induced mitogenic signaling in epithelial cells in breast, uterine, and ovarian tissues [19] and is prevalently expressed by breast cancer cells [20], whereas *ERβ* is usually associated with less aggressive tumors, as it inhibits both *ERα*-mediated transcription and estradiol-induced proliferation in various types of cancer cells [21]. The *ERα*/*ERβ* ratio may play a critical role in the regulation of estradiol activity in breast cancer cells [22].

Estradiol binding to ER activates the receptor through phosphorylation, which undergoes conformational changes and dissociates proteins which usually tightly wrap the DNA [23]. Thereafter, ER binds to the ERE sequence within the gene promoter, and dynamically and sequentially recruits various regulatory protein complexes that contribute to chromatin remodeling and enhance transcriptional activity [24].

ER-mediated transcription involves also other multiple coregulatory proteins, which coordinately act to influence gene transcription, cell cycle regulation, cell differentiation and apoptosis.

Nuclear receptors coactivators of ER include the ubiquitary general transcription factor P300/CBP, some methyltransferases such as CARM1 and PRMT1, some members of the p160 protein family such as the steroid receptor coactivators (SRC1, SRC2, and SRC3) [25, 26].

The regulation of ER and PR function can occur at three levels: differential translation of exons, splicing of their mRNA, and post-translational modifications. These lasts include phosphorylation, ubiquitylation, acetylation, and methylation. Among the multiple kinases that can phosphorylate *ERα* are p38 mitogen-activated protein kinase (MAPK), cyclin A-CDK2, CDK7, c-Src, pp90rsk1, extracellular regulated kinase (Erk) 1 and 2, protein kinase A (PKA) and B (Akt) [27–35]. The effects of this phosphorylation involve receptor turnover, cellular localization, and transcriptional activity and are complex and interdependent. The PR can be phosphorylated at different sites coordinately regulated by ligands or kinases [36].

Few mutations of the *ERα* gene have been reported in the literature, resulting in a receptor with hypersensitivity to the estrogen-mediated growth-promoting effects. These mutations in breast cancer correlate with older age, larger tumor size, nodal involvement, and poor prognosis [37–39].

Along with their classical genomic activity, ER and PR exhibit also a more rapid, nongenomic activity, which occurs within seconds to minutes independently of gene transcription, by mediating signaling cascades originating from the membrane or the cytoplasm through direct interaction with signal-transduction mediators [40, 41]. ER may establish a cross talk with other signal transduction pathways, such as that of growth factors, using its membrane and cytoplasmic receptors to transmit their signals through kinase cascades, triggering the phosphorylation and activation of the epidermal growth factor receptors (EGFR), insulin-like growth factor-1R (IGF-1R), transforming growth factor (TGF)α, Src kinase, Shc adaptor protein, and phosphatidylinositol 3-kinase (PI3K), and the inhibition of TGFβ and tyrosine phosphatases [20, 42–45]. Finally, ER may also use calcium, cyclic adenosine monophosphate (cAMP), and other second messengers for signal transduction.

2.2. Clinical relevance of hormone receptors

Hormone receptors are expressed by about two-thirds of invasive breast cancers in women younger than 50 and approximately 80% of tumors in women older than 50 [46]. Measurement of hormone receptors has become a routine part of the evaluation of breast cancers, as they represent a predictive factor for hormone therapy responsiveness. Both ER and PR increasing levels directly correlate with better response, longer time to treatment failure, and longer survival [47, 48].

Hormone receptor expression represents also an important favorable prognostic factor, being an important marker of growth rate, rather than metastatic potential. In particular, patients with ER+/PR+ tumors have a better prognosis than patients with ER+/PR- tumors, who in turn have a better prognosis than patients with ER-/PR- tumors [49]. ER expression is significantly associated with some favorable prognostic indicators, such as older age, low grading, lower fraction of dividing cells, lower genetic mutation, but not with nodal involvement [46, 50–53].

Adjuvant hormone therapy can halve the recurrence rate of patients with ER-positive breast cancer [54] and, due to its quite limited side effects, it can be administered with success also in the elderly or in the presence of comorbidities, and responses can last for many years in some patients with metastatic disease. Patients with stage I ER-positive breast cancer, who receive no systemic therapy, have a 5–10% lower probability of recurrence at 5 years in comparison with ER-negative patients [55]. On the other hand, in ER-negative tumors are unlikely to respond to hormone therapy and respond better to cytotoxic chemotherapy.

The literature demonstrates that the benefit of 5 years of adjuvant tamoxifen treatment depends on the tumor ER and PR status [54, 56], and the efficacy of tamoxifen in reducing local, contralateral and distant relapse or death was strongly confirmed by more recent large prospective trials [57, 58]. A marginally significant relationship between ER level and time to recurrence was observed also in patients treated with aromatase inhibitors [59]. However, studies about adjuvant tamoxifen in early breast cancers did not show any benefit of PR expression among ER-positive patients, but only a benefit among ER-negative patients [54, 60, 61]. Definitely, ER+/PR+ tumors had a 15–30% lower risk of recurrence and death than ER+/PR- ones [49].

Hormone therapy may be an interesting option also in the advanced disease, as the level of ER expression is associated with good responses in the ER-positive disease, and provides good palliation, better quality of life, and improved survival [62]. In fact, approximately 30–40% of patients with ER-positive metastatic disease will respond to first-line hormone therapies, another 20% will experience disease stabilization, and despite a gradual efficacy decline, about 20–30% will respond to subsequent lines of hormone therapy [63]. ER status is also prognostic for the site of metastasis, metastasizing ER-positive tumors more frequently to the bone, soft tissue, or the reproductive and genital tracts, and ER-negative ones to visceral organs or the brain [64].

As the hormone receptor status of the metastases should be more predictive than that of the primary tumor, before making treatment decisions, the molecular markers of breast cancer should be retested in the metastatic lesions when possible, due to the risk of discordance between the hormone receptor status between the relapse/metastases and the primary tumor. In fact, a conversion rate of 20–30% has been reported from ER-positive to ER-negative status, related with a poorer prognosis, while less frequent conversion has been reported from ER-negative to ER-positive status [65–68]. The same happens for what concerns PR expression in metastatic lesions, which often converts from PR-positive to PR-negative [66, 67].

Beyond the probable technical causes of false-negative or false-positive results, possible explanations for the hormone status changes include the tumor dedifferentiation over time and the intratumoral heterogeneity, leading to clonal selection of hormone receptor negative and more resistant clones, as an adaptive mechanism to prior treatments [68]. Apart from the hormon status change, the resistance to endocrine therapy may be explained by the modulation of many cellular signaling networks, which usually provide alternative mitogenic and survival stimuli for the cells. Therefore, multigene predictive scores have been developed to predict tumors hormonal responsiveness, such as the Oncotype DX 21 gene assay, which includes several downstream ER-regulated genes and several proliferation genes in addition to ER mRNA, and will be discussed in another section [11].

The results of adjuvant chemotherapy trials support that ER-negative tumors derive more benefit from chemotherapy than ER-positive ones, as well as luminal A (ER/PR+/HER2-) tumors [54, 69]. A study comparing adjuvant TAC (docetaxel-adriamycin-cyclophosphamide) with FAC (fluorouracil-adriamycin-cyclophosphamide) showed a benefit of adding taxanes regardless of ER status, as they exhibit endocrine effects by inducing amenorrhea in premenopausal women [70]. On the other hand, as expected, the ovarian ablative effects of chemotherapy are not observed in postmenopausal patients. Neoadjuvant chemotherapy trials have also shown the effect of ER status in pathologic complete response (pCR) rates, which result significantly higher in the ER-positive group than in ER-negative one [71].

2.3. Methods for measuring hormone receptors

Various assay methods have been used to measure ER expression in breast cancer specimens, which is fundamental for the therapeutic planning. The dextran-coated charcoal/ligand-binding assay (DCC/LBA) was the first available standard inked immunosorbent assay (ELISA). Thereafter, since 1990s, immunohistochemistry (IHC) of formalin-fixed paraffin-embedded specimens began to replace the DCC assay because it needs smaller tissue amounts, does not require fresh/frozen tissue, correlates staining with histology, and allows the storage and retrieval of archived slides for later testing [72].

The last guidelines for hormone receptor testing, reported by the Society of Clinical Oncology (ASCO)/Collage of American Pathologists (CAP) in 2010, establish mandatory proficiency testing and inspection criteria to improve the accuracy of these tests [73]. Breast resection specimens should be fixed as quickly as possible (within 1 h from reesection) in an adequate volume of fixative (optimally 10-fold greater than the volume of the specimen). After being received in the pathology laboratory, specimens should be oriented and carefully inked for surgical margin assessment, sectioned at 5 mm intervals, and placed in 10% neutral (phosphate) buffered formalin for no less than 6 h and for not more than 72 h before processing.

After treatment for antigen retrieval, the tissue sections are incubated with a primary antibody directed against the ER or PR, and subsequently with a secondary detection systems that are conjugated to an enzyme to amplify the chromogenic signal, and finally microscopically evaluated. External and internal controls can be used to ensure the proper performance of IHC test. The percentage of cells with nuclear staining is reported by either estimation or quantitation, which may be performed either manually or by image analysis. Both the average intensity (weak, moderate, strong) and extent of staining (as a percentage) are reported. ER or PR expression is considered positive or negative in case of immunoreaction in respectively ≥1 or <1% of tumor cell nuclei [73].

3. HER2/neu testing: Prognosis prediction and targeted therapies

3.1. Biology of HER2/neu

The human epidermal growth factor receptor 2 (HER2/neu) gene, localized on chromosome 17, encodes a a 185 kDa, transmembrane member of the tyrosine kinase epidermal growth factor

receptors, which are normally expressed at low levels in all epithelial cells in normal fetal and adult tissues, but are also essential for cancer proliferation and survival [74]. HER2 gene amplification has been associated with increased levels of expression of HER2 mRNA and protein product, which lead to oncogenic signaling and resultant self-sufficiency in growth signals, uncontrolled proliferation, sustained angiogenesis, survival, enhanced invasion, and metastasis processes, which are drivers of carcinogenesis [75–77]. The HER2/neu gene results amplified in a variable percentage of breast [77, 78], ovarian [77], bladder, endometrial [79], salivary gland [80], and gastric cancer [81].

The human epidermal growth factor receptor (HER) family consists of four members: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. The structure of these receptors consists of a ligand-binding extracellular domain, a transmembrane domain, and a cytoplasmic catalytic kinase domain that drives downstream signaling pathways, such as the PI3K/Akt/mTOR and RAS/RAF/MEK/ERK ones [82]. HER heterodimers are more potent in signal transduction than are homodimers. HER2 is the preferred partner for dimerization, triggering with its overexpression breast cancer progression with a poor prognosis, and the HER2-HER3 heterodimer is known to be the most potent oncogenic combination in breast cancer.

HER2 overexpression ultimately activates ligand-independent HER2/HER3/PI3K complex formation and kinase activity in tumor cells, so that the resistance to trastuzumab can be circumvented through PI3K inhibition, as well as gain-of-function mutations of PI3K and the loss of PTEN. Furthermore, the upregulation of insulin-like growth factor receptor 1 (IGF-1R) results in sustained activation of the PI3K/Akt pathway, thereby leading to resistance to antihormonal and HER2-targeted therapies.

3.2. Clinical relevance of HER2/neu

Having a look at the currrent literature, HER2 results amplified in approximately 15–30% of breast cancers [75, 83]. HER2 overexpression, in the absence of adjuvant treatment, correlates with a poor prognosis in terms of both overall and disease-free survival, independent of tumor size, grade and hormone receptor status [84]. However, HER2 is also an important predictive marker for responsiveness to HER2-targeted therapies, in both metastatic and adjuvant settings [85, 86].

Trastuzumab, the most famous humanized monoclonal antibody against HER2, significantly improves response rates, time to progression and survival when used alone or added to chemotherapy in both early stage and metastatic breast cancer [87]. Other HER2-targeted drugs, including the tyrosine kinase inhibitor lapatinib, the antibody pertuzumab, and the antibody drug conjugate adotrastuzumab emtansine (T-DM1), improve outcomes in HER2-positive metastatic breast cancer [88–90].

A controversial association exists between HER2 positivity and resistance to hormone therapies, but randomized trials in either adjuvant or metastatic settings failed to provide supporting evidence. [91]. This probably happens due to a physiological cross talk between the HER2 and ER signal transduction pathways, but other mechanisms of hormone independent endocrine resistance of HER2-expressing cells have been described, such as phosphorylation of the ER, ligand-independent ER activation, and regulation of hormone receptor

expression [92]. Moreover, some data suggest that endocrine resistance may be specific to selective estrogen modulator (SERM) therapy such as tamoxifen and perhaps not to estrogen depletion therapies such as aromatase inhibitors [59, 93]. Furthermore, the response to ligand-depleting therapies such as ovarian ablation or aromatase inhibitors is not affected by HER2 overexpression.

HER2 may be associated with either sensitivity or resistance to some chemotherapeutic agents. For example, HER2 positivity is associated with better outcomes in response to adjuvant anthracycline containing regimens in most studies, probably due to the coamplification of HER2 with topoisomerase II, which is the direct target of anthracyclines [94]. Anyway, the combination of trastuzumab and anthracycline has cardiotoxicity concerns, so that an accurate determination of HER2 alterations in breast carcinomas is mandatory. On the other hand, data about the possibile correlation of HER2 positivity with responsiveness to paclitaxel containing chemotherapy are still contradictory [95].

3.3. Methods for measuring HER2/neu

HER2 gene amplification is directly correlated with its mRNA expression and protein levels, and HER2 status can potentially be evaluated at any of these levels. A great number of commercially available testing kits are approved from FDA for the assessment of patients suitable for the treatment whit trastuzumab (humanized mouse 4D5 monoclonal antibody) may be a suitable treatment. Overexpression of the HER2 protein product may be evaluated by Western blotting, ELISA or IHC; overexpression of its mRNA by Northern blotting or RT-PCR, and its gene amplification by fluorescence (FISH), chromogenic (CISH) or silver-enhanced in situ hybridization (SISH) [96].

FISH is more accurate, reproducible, and robust than IHC [97], but IHC has been more widely used as the primary test for HER2 status because it results quicker, is viewed using a conventional bright-field microscope, permits parallel viewing of tumor morphological features, and stained tissues do not degrade over time [98]. Moreover, automated IHC techniques may enable more rapid testing.

Recommendations for tissue handling as well as preanalytic, analytic, and postanalytic factors in ER/PR testing are also suitable for HER2 testing. Laboratories performing these tests should follow all accreditation requirements, which conform to the 2010 ASCO/CAP recommendations for ER/PR testing, one of which is the initial testing validation [83]. Laboratories are responsible for ensuring the reliability and accuracy of their testing results and should review and document external and internal controls with each test and each batch of tests.

The final IHC result is classified as 3+ in the case of a complete circumferential membrane staining in >10% of neoplastic cells, 2+ in the presence of moderate circumferential membrane staining of >10% of neoplastic cells, 1+ or 0 if there is incomplete membrane staining or no staining in >10% of neoplastic cells. A positive result includes the 3+ and the 2+ in the presence of a ISH confirmation [83].

4. Mib1/Ki-67: Prognosis prediction and treatment planning

Numerous measures of tumor cell proliferation have been studied over time, including thymidine labeling index, flow cytometry and S-phase fraction, thymidine kinase, cyclins D and E and their inhibitors p27 and p21, topoisomerase *IIα*, p53, bax, bcl-2, and Ki67, but methodological shortcomings precluded attribution of prognostic or predictive significance to any of these potential markers [99].

The mitotic index, which is one of the three components of the tumor grading assessment, results the strongest prognostic discriminant in node-negative breast cancer, being the most significant predictor of survival, and rendering less significant the other two elements of tumor grading evaluation, pleomorphism and tubular formation [100]. In particular, patients with mitotic index ≥10 should be considered at high risk and be offered adjuvant therapy.

Mib1/Ki-67 is a proliferation index used as both a prognostic and predictive marker, although its widespread use is limited by the lack of standardization of the assay and its interpretation [99, 101]. This marker of proliferation results an independent prognostic factor for DFS, is significantly predictive for responsiveness to both adjuvant chemotherapy and endocrine therapy, and is predictive for pathological complete response in the neoadjuvant setting [102, 103]. In fact, the Mib1/Ki-67 decrease in the post-treatment samples of women who underwent neoadjuvant therapies is a strong independent predictor of better clinical outcomes [104].

5. Genomic markers, prognosis, and personalized treatment

In the past, breast cancers were simply treated based on some clinicopathological features, such as tumor size, lymph node status, patients age and menopausal status, and tumor biomarkers such as ER, PR, and HER2/neu. Then, systemic chemotherapy was applied nearly universally to locally advanced breast cancers regardless of their biomolecular profile, and to about 60% of early breast cancers, but often without any significant effect on women prognosis [105]. As a consequence, a great debate has emerged about quality-of-life issues, acute and long-term side effects of systemic therapies, and the cost of unnecessary treatments [54, 106]. Therefore, in the last decades, quantitative approaches for prognosis prediction and treatment individualization have been developed, and genomic and molecular technologies are routinely applied to prevent overtreatments.

Recently, thanks to the increased level of knowledge regarding the molecular pathways and underlying genetic changes in breast cancer, the molecular signatures of gene expression have been correlated with breast cancer recurrence risk [7, 107, 108]. Anyway, their current clinical application is still limited due to reproducibility questions and the need for fresh or frozen tissue.

In this section, we discuss about susceptibility genes, the carriers of which results to have an increased breast cancer risk and consequently deserve a more frequent and specific screening,

and about some signatures, which are usually used to predict breast cancer responsiveness to adjuvant and neoadjuvant therapies.

5.1. BRCA1 and BRCA2

Inherited susceptibility to breast cancer has been hypothesized due to the discovery and characterization of a number of high-risk, relatively uncommon genes responsible for the clustering of breast cancer in certain families, which thereafter had a significantly increased risk in comparison with the general population [109, 110]. Many studies suggest that breast cancer susceptibility is transmitted in an autosomal dominant mendelian way [111], but the actual risk of developing breast cancer in a mutation carrier is based on the penetrance of the gene, which consists in the likelihood that the effect (phenotype) of a mutation (genotype) will become clinically apparent.

The BRCA1 gene was firstly identified in 1994 [112] and is localized on the 17th chromosome, whereas the BRCA2 gene was found some years after and is localized on the 13th chromosome [113]. The big size of these genes is important in the context of genetic testing because of the increased probability of mutations and the consequent technically demanding and costly mutations testing, but fortunately the use of modern next generation DNA sequencing is already overcoming these technical and cost issues. Moreover, the BRCA1 gene contains a large number of repetitive elements that facilitate the generation of large deletions and duplications.

BRCA1 is a nuclear protein with two important regions of sequence similarity with known functional motifs: a 42–amino-acid RING (Really Interesting New Gene) domain at the beginning of BRCA1 which binds zinc and is essential in cell growth and differentiation, and the BRCT (breast cancer-1 terminus) motif at the carboxyl terminus, which acts as a phosphoprotein docking motif and a transcriptional activation domain [114, 115]. BRCA2 is also a nuclear protein composed of the following major structural motifs: the eight tandem BRC repeats in the central portion of the protein, which mediates the critical interaction of BRCA2 and RAD51, the TR2 at the carboxyl terminus, which binds RAD51 exists, a single-strand and double-strand DNA binding domain in the C-terminus [113, 116].

Both BRCA1 and BRCA2 genes encode large proteins with multiple functions, which act mainly as tumor suppressor gene products, affecting transcription, cell cycle regulation, genome stability maintenance, and repair of doublestranded DNA breaks for protection of the genome during replication [117, 118]. In particular, BRCA1 prevents replication of damaged DNA by altering chromatin structure and nucleosome organization at the local site of damage, facilitates access by repair complexes, and promotes the use of the error-free repair pathway of homologous recombination-mediated repair rather than the error-prone process of nonhomologous end joining [118]. BRCA2 affects the choice between the two homologous recombination pathways in favor of the error-free one, by interacting with RAD51 [118]. When the wild-type BRCA1 or BRCA2 allele is lost, mutated, or silenced, a high degree of chromosome instability is observed and defective DNA repair may occur, with the consequent accumulation of additional mutations during replication and promotion of carcinogenesis [119].

Mutations in BRCA1 and BRCA2 genes are the most frequent hereditary genetic aberrations in breast cancer and account for approximately half of all hereditary breast cancers. Initial estimates found BRCA1 mutations to be responsible for 45–90% of breast cancer cases in families with apparent autosomal dominant transmission of breast cancer, and this percentage rises if the median age at onset of breast cancer is younger than 45 years [120, 121]. Estimates of BRCA1 and BRCA2 mutation prevalence in unselected patients with breast cancer are in the range of 2–3% [122].

Among BRCA1 mutation carriers, the estimated breast cancer risk is about 65%, the estimated risk of contralateral breast cancer occurrence results 60%, the cumulative risk of ovarian cancer varies between 27 and 45%, and there is also a significantly increased risk of fallopian tube, uterine and cervical cancer, as well as of male breast cancer, stomach, pancreatic, colon and testicular cancer [123–126].

Among BRCA2 mutation carriers, the estimated lifetime breast cancer risk ranges between 45 and 84%, and that of ovarian cancer between 10 and 20% [123, 127]. There is an increased male breast cancer risk of about 6%, as well as an increased risk of prostate, pancreatic, stomach, gallbladder and bile duct cancers, and malignant melanoma [122, 128].

More than 500 coding region sequence variations have been detected in BRCA1 and 250 in BRCA2. Most unequivocally confirmed mutations reported to date are truncating mutations, adding little in the way of clues for defining functional regions. Although few mutations have been identified in either gene in sporadic breast cancers, a phenotype termed "BRCAness" exist, in which the BRCA1 and BRCA2 proteins act may be somehow disrupted also in sporadic cancer [129]. Finally, there are also some syndromes characterized by an increased breast cancer susceptibility, which are discussed in the following section.

Although germline mutations in BRCA1 and BRCA2 confer a high risk of breast cancer, a great deal of variability has been observed in cancer risk among individuals, both between and within families, as many environmental or genetic factors can modify the penetrance of BRCA1 and BRCA2 mutations. The Consortium of Investigators of Modifiers of BRCA1 and BRCA2 (CIMBA), analyzing DNA and clinical data from approximately 10,000 BRCA1 and 5000 BRCA2 mutation carriers, demonstrated the role of a number of gene variants in affecting the penetrance in mutation carriers [130].

The most important prognostic modifiers of BRCA1 and BRCA2 mutation carriers are prophylactic oophorectomy and the use of tamoxifen for chemoprevention, which approximately halve the breast cancer risk and decrease the risk of ovarian cancer by about 95% [131, 132].

In comparison with sporadic breast cancers, those related with high-penetrance susceptibility genes correlate with younger age at diagnosis, more aggressive tumor biological behavior, bilaterality, and the eventual coexistence of other cancers in the same individual or in other individuals of the same family, including ovarian, colon, prostate, pancreatic, and endometrial cancers, as well as sarcomas and male breast cancer [133].

Breast cancers arising in BRCA1 mutation carriers are frequently, although not exclusively, basal-like and ER-negative, probably because BRCA1 plays a crucial role in the transcriptional

regulation of ER [129, 134]. On the other hand, breast cancer originated in BRCA2 mutation carriers are typically much more similar to sporadic cases, despite with higher grading, higher frequency of ER-positivity and lower frequency of HER2/neu overexpression [135].

Breast cancer prognosis in BRCA1 and BRCA2 mutation carriers is still controversial, and if some authors describe a worse prognosis even in classically low-risk node-negative disease [136], other exclude any significant difference if compared to the general breast cancer population [137]. Furthermore, BRCA1 and BRCA2 mutation carriers result to have an increased risk of contralateral breast cancer occurrence [138].

New drugs have been purposed as a promising therapeutic strategy in BRCA defective tumor cells, such as the inhibitors of the poly(adenosine diphosphate-ribose) polymerase-1 (PARP1), which is an enzyme involved in the single-stranded DNA repair that use base excision repair.

Other breast cancer susceptibility genes have been described, which can be divided into three categories in terms of mutation risk and the frequency of mutation. Along with BRCA1 and BRCA2 gene mutation, the first category includes PTEN and TP53 gene mutations, which are classified as high-penetrance, low-frequency predisposition genes, and the occurrence of even one of these mutations can increase the risk of breast cancer to 25% [139, 140]. The second category includes the CHEK2, ATM, PALB and BRIP1 2 genes, which are moderate-penetrance, low-frequency predisposition genes, and lead to an increased risk of cancer of twofold to fourfold [110]. Finally, the third category consists of the FGFR2, MAP3K1,and TGFB1 gene mutations, which are low-penetrance, high-frequency predisposition genes [141].

5.2. Multigene signatures

In the last decades, many genomic and molecular classification have been described with a prognostic intent. The most famous divides breast cancers into the following subtypes: luminal A, luminal B, HER2-enriched, basal-like, and normal-like [142]. Luminal subtypes express high levels of ER, they usually have an indolent clinical course, with a low distant recurrence rate, which anyway persists even up to 15 years after the diagnosis. Luminal B subtypes express fewer ER-related genes, have a higher proliferation rate and may overexpress HER2/neu, so that they usually require to be treated with both hormonal therapy and chemotherapy. HER2-enriched subtype exhibits HER2/neu gene amplification but does not express ER-related genes, they have an aggressive natural clinical course but fortunately respond very well to HER2-targeted therapy. The basal-like or triple-negative subtype does not express ER, PR, and HER2/neu but expresses basal cytokeratins 5/6 and 17, they have a poor prognosis and a high recurrence rate.

The first molecular signature of breast cancer was determined in 2000 by the expression of a set of genes within the tumor, which were able to predict the clinical outcome [143]. The main limitations of gene signature profiling include difficulties in reproducing the specific gene sets, testing expense, and reporting standardization. However, gene signature cannot be substituted by IHC surrogates which have significant discordances with genetic profiling [144].

The three multigene tests for breast cancer which are commercially available and currently used in the clinical practice are the Oncotype DX test (Genomic Health, Redwood, CA, USA), the MammaPrint test (Netherlands Cancer Institute™ and Agendia™, Netherland), and the Prosigma one (NanoString Technologies, Seattle, WA, USA). The Oncotype DX test is the most widely used molecular test in the therapeutic decision-making, is strongly predictive for endocrine responsiveness in hormone receptor-positive breast cancers with 0–3 positive nodes, and is recommended by both the National Comprehensive Cancer Network (NCCN) and the St. Gallen Consensus [7, 145, 146].

The Oncotype DX is a real-time reverse transcriptase chain reaction (RT-PCR) assay, which measures the expression of a panel of 21 genes in formalin-fixed paraffin-embedded samples, including 16 cancer-related genes (ER, PR, Bcl2, SCUBE2, HER2, GRB7, Ki-67, STK15, survivin, cyclin B1, MYBL2, stromelysin 3, cathepsin L2, GSTM1, CD68, and BAG1) and 5 housekeeping control genes (beta-actin, GAPDH, RPLPO, GUS, and TFRC), to generate a recurrence score to stratify breast cancer patients into three risk groups. The low risk group (score < 18), the intermediate (score 18–30), and the high risk one (score ≥ 31) have a 10-year distant recurrence rate of respectively 6.8, 14.3, and 30.5% [144].

The MammaPrint assay is the second most commonly ordered molecular test approved by the US-FDA and measure the expression of 70 genes involved in the cell cycle, invasion, proliferation, angiogenesis, metastasis, and signal transduction, none of which is tested by the Oncotype DX assay. The MammaPrint customized microarray contains a reduced set of 1900 probes suitable for high-throughput processing, and allows the use of less RNA and a short processing time of 5 days. This assay can be applied in both node-positive and node-negative and both hormone-positive and hormone-negative cancers, it is predictive for responsiveness to chemotherapy and prognostic for early distant recurrence within the first 10 years after diagnosis, which results 13 and 56% respectively in the low- and high-risk group [147–149].

The Prosigna test is an assay approved by the US-FDA which measures the expression of 50 target genes and 5 constitutively expressed normalization genes, using a proprietary technology called the "nCounter Dx Analysis System." The assay is highly sensitive and precise and uses 250 ng of RNA from formalin-fixed paraffine-embadded tumor tissue, and generates a risk of recurrence score, which assesses the 10-year risk of distant recurrence for hormone receptor-positive stage I–III breast cancers to be treated with adjuvant endocrine therapy, and correlates to one of the five molecular subtypes previously described [143, 150]. The Prosigna assay results superior to the Oncotype DX test in predicting late distant recurrence after 5–10 years [9, 151].

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