Review Article
Breast cancer molecular subtypes: from TNBC to QNBC

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Abstract: Treatment protocols for breast cancer depend predominantly on receptor status with respect to estrogen (estrogen receptor alpha), progesterone (progesterone receptor) and human epidermal growth factor [human epidermal growth factor receptor 2 (HER2)]. The presence of one or more of these receptors suggests that a treatment targeting these pathways might be effective, while the absence of, or in the case of HER2, lack of overexpression of, all of these receptors, termed triple negative breast cancer (TNBC), indicates a need for the more toxic chemotherapy. In an effort to develop targeted therapies for TNBC, it will be necessary to differentiate among specific TNBC subtypes. The subset of TNBC that expresses androgen receptor (AR) has been determined to express genes consistent with a luminal subtype and therefore may be amenable to therapies targeting either AR, itself, or other pathways typical of a luminal subtype. Recent investigations of the AR signal pathway within breast cancer lead to AR as a significant target for breast cancer therapy with several clinical trials currently in progress. The subclass of TNBC that lacks AR, which we have termed quadruple negative breast cancer (QNBC) currently lacks a defined targetable pathway. Unlike AR-positive TNBC, QNBC predominantly exhibits a basal-like molecular subtype. Several subtypes and related pathway proteins are preferentially expressed in QNBC that may serve as effective targets for treatment, such as ACSL4, SKP2 and EGFR. ACSL4 expression has been demonstrated to be inversely correlated with expression of hormone/growth factor receptors and may thus serve as a biomarker for QNBC as well as a target for therapy. In the following review we summarize some of the current efforts to develop alternatives to chemotherapy for TNBC and QNBC.

Keywords: QNBC, TNBC, quadruple negative, ACSL4, breast cancer

Introduction

Breast cancer is a major health concern in the United States, and is a leading cause of cancer death in women [1]. Breast cancer has long been recognized as a heterogeneous disease. While many factors have been investigated as a means to stratify patients by risk and treatment options (age, parity, family history, etc.), receptor status has proved to be the most useful in predicting prognosis and responsiveness to treatment [2-4]. Immunohistochemical (IHC) techniques are utilized to measure expression of estrogen receptor (ER), progesterone receptor (PR), and overexpression of human epidermal growth factor receptor 2 (HER2/neu). Breast cancers are then classified with respect

to the presence or absence of these receptors, with cancers lacking all three designated as triple negative breast cancers (TNBC). Those cancers that express ER, PR or Her2-neu are amenable to targeted therapies directed at these receptors; however TNBC patients are treated with traditional chemotherapeutic reagents. These biomarker designations are related to, although not identical with, more recent determinations of intrinsic molecular subtypes based on patterns of gene expression as determined by genetic microarray testing [5].

Intrinsic molecular subtypes

Expression array analysis has resulted in the classification of breast cancer according to

intrinsic molecular subtypes, and it is now widely accepted that there are four distinct intrinsic molecular subtypes: luminal A, luminal B, HER2-enriched, and basal-like breast cancer (BLBC) [5-7]. A fifth subtype, designated normal-like, is generally thought to arise from contamination of samples with normal mammary cells. Based on these findings, a 50-gene molecular signature (PAM50) has been devised for clinical use in prognosis and treatment decisions. These subtypes can be approximated clinically using IHC determination of receptor status as follows: luminal A approximates ER and/or PR positive, and HER2 negative, luminal B approximates ER and/or PR positive, and HER2 positive, HER2-enriched approximates ER and PR negative, and HER2 positive; and BLBC approximates TNBC. Although clinical designation by IHC can be used to approximate these subtypes, the subtypes do not always neatly fall into the IHC designation. An example of this is seen in a study by Parker, et al. [8] which showed that of the samples that tested positive for ER, 73% were luminal, 11% were HER2-enriched, 5% were basal-like, and 12% were normal-like. These subtypes have been associated with different prognoses, with patients with luminal A tumors having the best prognosis, and patients with BLBC having the worst prognosis. Furthermore, patients can be separated by treatment options based on their subtypes, as patients with luminal A and B, and HER2-enriched subtypes are sensitive to targeted treatments, while patients with BLBC currently have only chemotherapy as an option [4, 6-10].

Triple negative breast cancer

TNBC makes up 10-30% of all breast cancers. It is associated with younger age and higher stage at diagnosis, higher nuclear grade and mitotic activity, and poorer prognosis [2, 9, 11, 12]. Within the TNBC designation are heterogeneous characteristics. TNBC can be categorized by its morphological appearance: infiltrating ductal carcinoma, not otherwise specified (NOS), medullary carcinoma, adenoid cystic carcinoma, myoepithelial carcinoma, squamous carcinoma, metaplastic carcinoma, apocrine carcinoma, secretory carcinoma, or carcinoma arising in the background of microglandular adenosis. Despite TNBC having a more aggressive nature as a whole, there are sub-

types that are much more indolent. For example, adenoid cystic carcinomas are considered slow growing, with a very good prognosis status post surgical excision [13, 14]. Based on genetic expression profiling, TNBC has been categorized into six TNBC subtypes: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL) and luminal androgen receptor (LAR) [15]. The intrinsic molecular BLBC subtype has controversially been considered synonymous with TNBC. It is called basal-like for its expression of markers for basal type cells (CK5/6, CK14, CK17, EGFR), and is defined as ER/PR/HER2 negative, CK5/6 positive, and/or positive for EGFR. Although it has been argued that TNBC and BLBC are the same subtype, not all TNBC express basal cell markers characteristic of BLBC; positivity for basal markers is associated with poorer prognosis than TNBC overall, and may be seen across the different genetic subtypes of TNBC [11, 15-18]. BLBC typically has a worse prognosis, but when treated with adjuvant chemotherapy, patients with BLBC showed longer disease free survival when compared to patients with TNBC as a whole [11]. Despite better response rates of TNBC versus non-TNBC to chemotherapy, overall prognosis is still poor [19]. Furthermore, better response rates may be due to BLBC being grouped with TNBC, thus patients with non-BLBC TNBC fare the worst [11]. Claudin-low breast cancer, similar to BLBC is found mostly in TNBC, and represents 25-39% of all TNBC. In molecular cluster analysis, it is found in close proximity to BLBC, however claudin-low tumors do not consistently express basal keratins. Furthermore, it has characteristics of mesenchymal and MSL molecular subtypes, which are also seen by IHC, with positive vimentin and N-cadherin. It is called claudin-low because of its lack of expression of tight-junction components, claudin-3, claudin-4, and claudin-7. This type is associated with poor prognosis, as well as poorer sensitivity to chemotherapy than BLBC [20, 21].

More recently, Burstein, et al. revisited the grouping of TNBC, and redefined subtypes into four, rather than six, subtypes using RNA and DNA gene expression profiling. Subtype 1 tumors have AR, ER, prolactin and ErbB4 signaling despite being ER negative via IHC. This subtype highlights ER negative tumors that may still respond to ER antagonists. They cor-

relate with the tumors previously categorized as LAR subtype, and subsequently have been termed the LAR subtype. Subtype 2 highly express growth factors and genes oetherwise only seen with osteocytes and adipocytes and show pathways regulated in breast cancer. This subtype correlates with the mesenchymal stem-like subtype and claudin-low tumors, and have been termed the mesenchymal (MES) subtype. Subtype 3 exhibits downregulation of immune regulating pathways and cytokine pathways with basal-like expression. It has been termed the basal-like immunosuppressed (BLIS) subtype. Subtype 4 has basal-like expression, but has upregulation of immune-regulating pathways, and has been termed the basal-like immune activated (BLIA) subtype. The BLIA subtype has the best prognosis, while the BLIS subtype has the poorest prognosis. The prognostic implications of BLIA vs BLIS subtypes are of interest because of the observation that tumor-infiltrating lymphocytes (TILs) in TNBC are associated with better prognosis. The International TILs Working Group 2014, recently proposed a possible standardized method to be used to assess TILs in breast cancers by H&E evaluation. However, there are still no recommendations for clinically relevant TIL thresholds [23-26].

Currently, the only treatment for patients with TNBC is chemotherapy. In patients who receive neoadjuvant chemotherapy and show a pathological complete response on resection, prognosis is very good. However, in patients who do not show a pathological complete response, they have a worse prognosis with a higher incidence of recurrences. Thus there is an urgent need to find targeted therapies and stratify patients by treatment options [12, 19, 27].

Androgen receptors (AR) in breast cancer

AR, similar to ER and PR, is a steroid receptor that is expressed in the normal breast as well as 60-90% of all breast cancers. This is of interest, particularly in the subset of TNBC that express AR, referred to as LAR subtype by Lehmann, et al. [15], as AR may be a target for therapy. Androgen deprivation therapy has long been used to treat castration resistant prostate cancer, and more recently targeted anti-androgen therapies have been developed. These same targeted therapies could be used in AR positive TNBC along with chemotherapy,

as well as in ER positive breast cancers that become resistant to anti-estrogen therapy. Although the biological role of androgens in the breast is not fully understood, it has been suggested that they have multiple roles within breast cancer. In breast cancers that express ER, PR, and AR, treatments with androgens elicits an inhibitory growth response, while in ER/PR negative, AR positive breast cancers, androgens induce a proliferative effect [28-30]. Furthermore, patients showing an AR/ER ratio of greater than or equal to 2 may have increased risk of resistance to tamoxifen [31].

Expression of AR can be evaluated by IHC. In fact, by using IHC measurements, AR is the most commonly expressed biomarker in breast cancers, overall [28]. After binding with its ligand, testosterone, AR translocates to the nucleus, where it proceeds to regulate gene transcription. Thus, nuclear staining by IHC is indicative of active AR receptors. Although there is currently no standard scoring method for IHC testing of AR, CAP/ASCO guidelines for ER and PR staining have been used due to similarity to AR. However, it should be noted that the prevalence of positive biomarkers may differ within studies, depending on the threshold used, as CAP/ASCO guidelines have changed in the past few years [28, 32-34]. While some studies use a cut-off value of 1% staining [33, 35], many studies, including a recent clinical trial in the metastatic setting showing benefit of anti-AR therapy, use a cut-off value of 10% [34, 36, 37]. Depending on the thresholds of positivity used, AR is expressed in 10-43% of TNBC, and positivity of only AR is far more common in patients than positivity in only ER, PR or HER2 [33, 34, 38]. Furthermore, in AR positive staining TNBC, AR expression is preserved in lymph node metastases and tumor recurrence. In the cases where there is loss of AR, patients showed increased tumor aggression [28, 34, 39].

There is AR expression within certain TNBC molecular subtypes. The molecular subtype with the most AR expression is LAR, with as much as ten times higher AR protein expression than non-LAR TNBC. Conversely, the basal-like TNBCs have the least expression of AR. AR positivity is associated with a lower Ki-67 index [32, 36, 38]. The lower Ki-67 index of AR positive TNBC may be an indication of why non-basal-like TNBC responds less well than basal-

like TNBC to chemotherapy [32, 35, 36]. Bicalutamide is a FDA approved therapy, in combination with a luteinizing hormone-releasing hormone analog, for treatment of Stage 2 metastatic prostate carcinoma. It is a non-steroidal anti-androgen that competitively inhibits androgen activity by binding AR in the cytosol, thus inhibiting gene transcription. Considering its action on AR, bicalutamide has been considered a possible therapeutic drug for AR positive breast cancers. There have been many investigations on Bicalutamide as a therapeutic option for TNBC. In AR positive TNBC, inhibition of AR results in decreased proliferation and increased apoptosis [38]. A recent Phase II clinical trial for non-steroidal anti-androgen therapy (bicalutamide) for ER-/PR-/AR+ breast cancer showed a 19% clinical benefit rate [37], which suggests that evaluating AR status may be useful for assessing treatment options in the metastatic setting.

While the LAR subtype of TNBC is the most sensitive to AR antagonists, owing to its high expression of AR, there is some AR expression in non-LAR subtypes. A second generation AR antagonist, enzalutamide, is currently FDA approved for metastatic castration resistant prostate cancer, and some studies have found that some patients who did not respond to bicalutamide, showed response to enzalutamide. Similar to these prostate cancer patients, some non-LAR patients who did not show a response to bicalutamide, showed sensitivity to enzalutamide. Enzalutamide targets more than one point in the AR signal pathway, including blocking localization of AR to the nucleus. Due to the complexity of AR expression, AR antagonists may not be reserved for LAR subtypes of TNBC alone [31, 32, 38].

How AR relates to the prognosis of patients is still controversial. In patients with ER+ tumors, expression of AR has been correlated with a more favorable prognosis [31, 34]. There have been studies showing TNBC patients with positive AR expression having poor overall survival [33, 40]. Other studies have shown no correlation between AR expression and prognosis [13, 29, 35, 36]. A recent meta-analysis on AR receptor expression and survival outcomes by Kim Y et al. showed an association of improved survival outcomes and AR expression in patients with TNBC [41].

A recent advance in AR biology in prostate cancer is the identification of splicing variants of AR (AR-Vs) with 15 different AR-Vs described to date [42, 43]. It is currently hypothesized that these variants may be derived via two mechanisms, genomic rearrangement and/or alternative pre-mRNA splicing. Genomic rearrangements have been demonstrated in castration resistant prostate cancer cell lines that consistently express AR-Vs. The most commonly identified AR-Vs are AR-V7 (also known as AR3) and ARv567es (also known as AR-V12). AR-V7 is associated with resistance to androgen ablation therapy in prostate cancer [33-36], and very recently, AR-V7 expression has been associated with poor prognosis [44]. Only limited data on AR-Vs in breast cancer exists [36]. Considering current clinical trials, and possible future treatment of targeting AR in AR positive breast cancers, AR-Vs in breast cancer are of considerable interest. The complexity of the AR signaling pathway may be a reason for the different findings in prognosis and AR expression [35]. Considering the possible targeted treatment, it has been proposed that TNBC should be classified as either positive for AR or negative for AR [13, 14, 32].

Quadruple negative breast cancer (QNBC)

The presence of AR in TNBC is associated with a luminal subtype as determined by gene expression microarray data, as mentioned above, while the majority of AR negative TNBC exhibit a basal-like molecular subtype. We have recently suggested that AR negative TNBC be considered as a separate molecular subtype from AR positive TNBC and be referred to as quadruple negative breast cancer (QNBC). A number of genes are differentially expressed as a function of TNBC or QNBC status (Jinhua and Gang's unpublished data), suggesting that it might be advantageous to redefine TNBC as AR positive and QNBC as AR negative subsets of ER, PR and HER2 negative tumors [45]. Currently, use of a QNBC category is not in clinical practice. While TNBC have the potential of a targetable AR, QNBC have been shown to express unique proteins that may be amenable to use in the development of targeted therapies. One such protein is the fatty acid activating enzyme, long chain fatty acyl-CoA synthetase 4 (ACSL4).

Fatty acyl-CoA synthetase 4 (ACSL4) as a biomarker and target in QNBC

ACSL4 is an enzyme that catalyzes the activation of long chain fatty acids subsequent to their utilization in distal metabolic events. It is over expressed in a number of cancers, including liver, colon and aggressive forms of breast cancer [45-49]. In breast cancer cells, it is associated with increased tumor growth, migration, and invasion [45, 48-50]. Expression of ACSL4 has been demonstrated to be inversely correlated with expression of ER, PR, AR and HER2 in both cell lines and tissue samples, suggesting that ACSL4 might function as a single biomarker for QNBC status [45, 49]. Wu et al. found that ACSL4 status can predict hormone receptor status in breast cancer cell lines with a sensitivity of 78% and specificity of 86%. The biomarker function of ACSL4 in tissue samples remains to be validated. In addition to this negative correlation, forced expression of ACSL4 in cell lines that express ER, PR and AR, such as MCF7 cells results in a reduction in receptor expression for all three hormones [50]. Furthermore, co-expression of ACSL4 with ER results in decreased sensitivity to receptor-targeted treatments, suggesting ACSL4 might also function as a biomarker for hormone resistance in cells.

When ACSL4 expression is evaluated as a function of intrinsic molecular subtypes in breast cancer cell lines, the greatest level of expression is seen in the claudin-low and basal-like subtype [45]. There is a subset of basal-like breast cancers that do not express ACSL4, but the significance of this has not yet been determined. ACSL4 expression is lowest in luminal and HER2-enriched breast cancer. The same results apply to mRNA expression as a function of molecular subtype in breast tumor samples; however these results have not yet been validated for protein expression in tumor samples.

With respect to the mechanism by which ACSL4 expression induces a basal-like phenotype in breast cancer cells, data suggest a role for enhanced arachidonic acid metabolism *via* PTGS2 and/or ALOX5, with subsequent involvement of the AKT/mTOR pathways [46-50]. The mTOR signaling pathway is a major regulator of cellular processes, and dysregulation has been implicated as a critical step in breast cancer growth [51-53]. In addition, forced expression of ACSL4 in ACSL4 negative cell lines is associated with significantly reduced expression of the transcription factor, AUTS2 mRNA, suggesting a possible role for this protein in breast cancer biology [44].

In addition to its function as a biomarker of QNBC and hormone resistance, data suggests that ACSL4 might also be a potential target for treatment [49, 50]. Down regulation of ACSL4 expression has been reported to induce ER expression in ACSL4 positive MDA-MB-231 cells (reference 45) as well as to increase AR expression in LNCaP-AI cells (Wu et al, Oncotarget, in press), suggesting that targeting ACSL4 might restore sensitivity to receptor-based treatments in hormone-resistant cancers. And simultaneous inhibition of ACSL4 with rosiglitazone has been shown to augment the effects of PTGS2 and ALOX5 inhibition [49] as well as of mTOR inhibition on growth of breast cancer cells [51].

S-phase kinase associated protein 2 (SKP2) in QNBC

The ubiquitin pathway is essential for cellular turnover and normal cell homeostasis. SKP2 is a subunit of the ubiquitin protein ligase complex, SCF-SKP2, which is involved in ubiquitin degradation, mainly p21, p27 and p57. SKP2 is a mediator of DNA replication, and has increased expression during the S phase of the cell cycle, but little expression during the G0/G1 phase. SKP2 expression is significantly higher in the tumors of patients with invasive breast cancer [54]. Expression of SKP2 has been inversely correlated with prognosis in invasive breast cancer. This is seen in both patient samples as well as in cell lines [55]. There is also an inverse correlation between expression of SKP2 with ER, and SKP2 with HER2 [56]. Dihydrotestosterone (DHT) is an androgen that is produced by the prostate, testes, hair follicles and adrenal glands. It has been demonstrated to bind to SKP2, which was correlated with increased degradation of p27 [57]. This supports a relationship between SKP2 and sex hormones, but it is not clear if there is any relationship with androgen receptor status. However, these data suggests SKP2 as a possible treatment target. There is currently a small molecule inhibitor of SKP2 in development that inhibits the SKP2-p26 interaction, which thereby reduces p27 degradation [58].

MicroRNA (miRNA) signatures

MicroRnas are short 18-22 nucleotide long non-coding RNAs that may alter gene expression by binding messenger RNAs (mRNAs), and may affect cell proliferation, differentiation, and cell death. Cancer associated miRNAs may be oncogenic, or tumor suppressive, with oncogenic miRNAs promoting tumor growth, and tumor suppressive miRNAs being suppressed in cancer. They may be a potential biomarker, as they are released by all cell types via exosomes and may be detected at any tumor stage. In light of the connection of miRNAs in TNBC, either individual or signatures of miRNA has been described. Functional studies in mice have investigated the possibility of targeting miRNAs in TNBC [59-61]. It is of great interest and importance to define miRNAs in QNBC in future.

Conclusion

In summary, breast cancer is a highly heterogeneous disease, of which QNBC is a distinct subtype distinguished from TNBC by the absence of AR. In considering new approaches for QNBC, multiple proteins and signal pathways need to be explored for use as biomarkers and/or targets for therapy. Current research has uncovered ACSL4, SKP2, EGFR and CD151 as potential candidates. ACSL4 and SKP2 may be both biomarkers and targets for therapy. Inhibition of ACSL4, which may be accomplished with Rosiglitazone, shows inhibited tumor growth. SKP2 may be bound by DHT and development of SKP2 inhibitors is currently underway. Future studies will further elucidate these signal pathways and networks in QNBC for therapeutic targets and prognostic biomarkers.

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