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CLINICAL TRIALS (PF PEDDI, SECTION EDITOR)

Updates on Molecular Classification of Triple Negative Breast Cancer

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Abstract

Purpose of Review Clinical management of triple negative breast cancer (TNBC) is challenging as patients have heterogeneous responses to systemic therapy, and there is no established therapeutic target. Gene expression profiling and genomic sequencing analysis are among the first steps in understanding the biology of TNBC. In this paper, we review the molecular classification of TNBC and discuss the implications for systemic therapy.

Recent Findings Clonal and mutational spectrum analyses of TNBC show that it is highly heterogeneous with a diverse mutational pattern and can be clustered into different subtypes including basal-like, luminal androgen receptor, and mesenchymal, based on gene expression profiling. Although knowledge of these subtypes is not used in routine clinical practice, studies have shown that patient outcomes differ according to subtype, with higher pathological complete response rates to chemotherapy reported in basal-like subtypes. Clinical trials with targeted agents are now starting to incorporate molecular subtypes into eligibility criteria.

Summary TNBC is molecular heterogeneous, and therefore, a wide spectrum of patients' clinical outcomes exists. Incorporating molecular subtypes into treatment algorithms may offer clinicians greater precision in managing TNBC patients.

Keywords Triple negative breast cancer · Molecular subtypes · Therapeutic implications · Clinical trials · Heterogeneity

Introduction

Breast cancer is the most commonly diagnosed cancer in women with an estimated incidence of over 250,000 new cases in the USA in 2017 [1]. It is the second most common cause of cancer death in women, after lung cancer, and accounts for approximately 6.8% of all cancer deaths in women [1]. The clinical classification of breast cancer is discernable by the presence of certain markers, including the estrogen receptor (ER), the progesterone receptor (PR), and overexpression and/or amplification of the human epidermal growth factor receptor 2 (HER2). The knowledge of expression of these three markers guides management, allowing rational and tailored management for breast cancer patients. Attempts to provide further insights into breast cancer heterogeneity based on gene expression patterns has resulted in the development of the molecular classification of breast cancer. The description of the so-called intrinsic subtypes based on gene cluster analysis led to the classification into luminal A, luminal B, Her2-enriched, basal-like, and normal subtypes [2]. Since then, other studies have also described additional breast cancer molecular subtypes [3, 4].

The subset of breast cancer that lacks expression of ER, PR, and HER2 is termed "triple negative breast cancer" (TNBC). Therefore, TNBC is a diagnosis of exclusion. It accounts for approximately 10–20% of all breast cancers [5, 6] and is more commonly diagnosed in younger women, under the age of 50 years [7]. Racial disparities have also been noted in the incidence of TNBC; women of African-American descent have a higher attributable risk than Caucasian women (odds ratio [OR] 2.41, 95% CI 1.81–3.21) [7]. TNBC is also more common in individuals with a germline *BRCA* mutation, especially *BRCA1* [8, 9]. As such, the *National Comprehensive Cancer Network* (NCCN) recommends genetic risk evaluation for individuals diagnosed with TNBC before the age of 60 years [10]. TNBC is also associated with more biologically aggressive disease at presentation than ER positive breast cancer [11]. As TNBC lacks therapeutic targets, the standard approach for systemic treatment is cytotoxic chemotherapy. Although a subset

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of TNBC is chemosensitive and carries a good prognosis, resistance to chemotherapy is common and is associated with a much higher risk of early disease recurrence compared to other breast cancer subtypes [11]. Although generally an aggressive subtype, there have been descriptions of low-grade TNBC variants, including adenoid cystic and secretory carcinomas [12].

Clonal and mutational spectrum analyses of TNBC suggest that it has a higher mutational frequency than other breast cancer subtypes [13–15]. The only frequently recurrent somatic mutation identified is in *TP53*, present in over 80% of patients [13–16]. Mutations seen in this gene are commonly frameshift or nonsense mutations versus missense mutations seen in TP53 in patients with luminal breast cancers [17]. The Cancer Genome Atlas study lists *PIK3CA* as the second most common somatic mutated gene (9%) [17]. Although aberrations in these genes are clonally dominant compared with others, the clonal frequencies in some TNBC patients are not consistent with a founder status, also supporting the observed mutational heterogeneity [13]. In this review, we present two clinical cases to highlight differences in TNBC outcome and discuss molecular classifications of TNBC.

Clinical Case Studies

Patient 1 A healthy 56-year-old postmenopausal woman discovered a palpable lump in the upper outer quadrant of her left breast. Physical examination was remarkable for a 3.5-cm firm mass at the 2 o'clock position of the left breast, with multiple bulky left axillary lymph nodes. Diagnostic breast imaging revealed a 3-cm hyperdense irregular mass at the 2 o'clock position in the left breast, with several markedly enlarged left axillary lymph nodes suspicious for metastatic disease. Her staging CT of the chest, abdomen, and pelvis, and bone scan did not show distant metastatic disease. After initial breast and lymph node biopsies confirmed high-grade TNBC with nodal involvement, she underwent neoadjuvant carboplatin and docetaxel chemotherapy on a clinical trial, followed by mastectomy and axillary node dissection. Pathology review indicated a residual focus of invasive ductal cancer measuring 1.5 cm, with one of three nodes involved with a 3-mm focus of carcinoma. All surgical margins were negative. Genetic testing did not reveal a deleterious mutation in *BRCA1* or *BRCA2*. She received adjuvant post-mastectomy radiation therapy. Six months after therapy completion, she developed abdominal pain and was found to have widespread recurrence in the liver, lungs, lymph nodes, and bones. A liver biopsy confirmed recurrent TNBC. She received palliative treatment with eribulin and pembrolizumab on a clinical trial but died from liver failure due to disease progression within 2 months.

Patient 2 A healthy 54-year-old postmenopausal woman discovered a palpable lump in the upper outer quadrant of her right breast. Physical examination revealed a 4-cm firm mass in the upper right breast, with no palpable right axillary lymph nodes. Diagnostic breast imaging revealed a 4.2-cm upper outer quadrant right breast mass, with a 7-mm satellite mass located 1.8 cm anterior to the dominant mass. Her staging CT showed two indeterminate non-calcified subcentimeter left upper lobe lung nodules. After initial breast biopsy confirmed high-grade TNBC, she also underwent neoadjuvant carboplatin and docetaxel chemotherapy on a clinical trial, followed by lumpectomy and sentinel lymph node biopsy. Pathology review indicated residual invasive ductal cancer measuring 2.4 cm, with one of three nodes involved with macro-metastatic carcinoma. All margins were negative. Genetic testing did not reveal a deleterious mutation in *BRCA1* or *BRCA2*. She received adjuvant post-lumpectomy radiation therapy. She remains disease-free 4 years after therapy completion.

Both patients were managed by the same multidisciplinary team and received the same chemotherapy regimen. The disease course experienced by each patient highlights the diversity in clinical outcomes seen in TNBC, even in individuals who present at similar stages, and are managed in similar ways. This suggests that other unmeasured factors such as tumor biology influence long-term clinical outcomes.

Molecular Heterogeneity of TNBC

In order to deepen our understanding of TNBC biology, several attempts have been made to subclassify TNBC. Studies by Lehmann et al. identified six TNBC subtypes ("TNBCtype") using gene expression profiles of 587 TNBC cases: (i) basal-like 1 (BL1) subtype enriched in genes involved in cell cycle and proliferation; (ii) basal-like 2 (BL2) involving growth factor signaling; (iii) immunomodulatory (IM) associated with immune cell and cytokine signal transduction pathway; (iv) mesenchymal like (M) genes involved in cell motility, growth, and differentiation; (v) mesenchymal stem-like (MSL), similar to M subtype, however, with low level of genes associated with proliferation; and (vi) luminal androgen receptor (LAR) enriched with genes involved in steroid synthesis metabolism [18, 19]. This subclassification was recently refined as "TNBCtype-4" (BL1, BL2, M, and LAR) based on studies that showed that the gene expression patterns for the previously defined IM and MSL subtypes were heavily influenced by tumor associated stromal cells and infiltrating lymphocytes [20]. Similarly, Burstein et al. identified four distinct TNBC subtypes using mRNA and DNA profiling: (i) basal-like immunosuppressed (BLIS) characterized by downregulation of immune cell and cytokines pathways; (ii) basal-like immune activated (BLIA) with upregulation of genes associated with B, T, and NK cell functions; (iii) mesenchymal (MES) enriched in pathways associated with cell cycle, mismatch repair, and growth factor; and

(iv) LAR exhibiting androgen, ER, and ErbB4 signaling, with negative ER staining on IHC [21]. Another group defined three distinct subtypes of TNBC using analysis of microarray gene-expression profiles of 107 TNBC patients: (i) basal-like with low immune response and high M2-like macrophages, (ii) basal enriched with high immune response and low M2-like macrophages, and (iii) LAR [22]. Subsequently, Liu et al. proposed a new classification system by integrating gene expression profiles of mRNAs and lncRNAs of TNBC into four subtypes (i) BLIS, (ii) IM, (iii) LAR, and (iv) MES [23].

All classification systems and the significant overlap between them not only support the remarkable degree of heterogeneity in TNBC tumors but also emphasize the need for a more uniform and standardized classification system for the eventual translation to patient care (Table 1). The overlap suggests at least four distinct subtypes (basal-like, immuno modulatory, mesenchymal, and LAR) with potential clinical implications.

Basal-like

This subtype has the most controversy in its description by the classification systems. It was initially first coined by the "intrinsic subtype" model to describe a subset of breast cancers lacking ER and *ErbB2* expression but associated with a unique gene expression profile similar to that expressed by the basal epithelial cells. This group was initially thought to involve all TNBC tumors; however, research has shown that not all TNBC tumors are basal-like, with reported concordance of 70% [24]. Lehman et al. described two basal-like groups (BL1 and BL2) in their original and refined classification system [18]. BL1 is enriched in genes that were associated with cell cycle and cell division (*AURK*, *MYC*, *NRAS*, *PLK1*, *BIRC5*), and genes associated with DNA damage repair (*RAD5*, *FANC*, *MSH2*, *MDC1*). It is also associated with mutations in DNA damage repair genes (*BRCA1*, *ATR*) [18, 20–22]. Breast cancers in patients with germline *BRCA1* mutation have been described as basal-like and have a TNBC phenotype. Interestingly, although most basal-like TNBC patients do not have germline *BRCA1* mutation, a high degree of *BRCA1* dysfunction and low levels of *BRCA1* mRNA expression have been reported in this subtype [25]. The proposed mechanisms of decreased *BRCA1* expression include loss of 17q21 (the *BRCA1* locus), increased expression of ID4, regulating *BRCA1* transcription, and *BRCA1* promoter hypermethylation [26, 27]. These provide the basis of the *BRCAness* of basal-like breast cancer, and the rationale for the use of PARP inhibitors in this subtype. BL1 is also associated with a high Ki-67 mRNA expression supporting its proliferative nature and the observed chemosensitivity [28]. Masuda et al. described varying rates of pathological complete response (pCR) to neoadjuvant chemotherapy among the different subtypes, with BL1 having the highest pCR rate of 52% (M 31%, IM 30%, MSL 23%, LAR 10%, and BL2 0%) [29]. BL2 is associated with genes involved in growth factor signaling (*EGFR*, *MET*, *Wnt/β-catenin*) [18].

Other groups have proposed a substratification of the basal-like subtype based on the immune signature and tumor niche. Two separate groups proposed two basal-like subtypes, BLIS and BLIA (with low and high immune response), while Liu et al. described BLIS as the basal-like subtype [21–23]. BLIS (basal-like immunosuppressed) represent tumors with basal-like gene expression profile but exhibit downregulation of B cells, T cells, NK cells, cytokine, and complement pathways, but with the unique expression of several SOX transcription factors. BLIA (basal-like immune activated) as its name implies shows upregulation of genes associated with B, T, and NK cell functions and high expression of STAT family transcription factors [21–23]. Jézéquel reported that the subtype associated with downregulated immune response was associated with high M2 macrophages implicated with tumor invasion and metastases and associated with a poorer prognosis [22].

LAR

Androgen receptors (AR) belong to the steroid hormone group of nuclear receptor family, which is encoded on the long arm of X chromosome (Xq12) [30]. This intracellular binding of testosterone or 5-α dihydrotestosterone results in translocation of this complex into the nucleus, with binding to promoter regions of target genes associated with cell growth and survival [30]. AR is more commonly expressed in ER positive breast cancer (~ 80%); however, 10–35% of TNBC have expression of AR. Those tumors are described as LAR given similarity of gene expression profiles to those ER positive breast cancers [31–34]. Lehman et al. described that in the LAR subtype, AR mRNA was overexpressed, on average at 9-fold greater than the other TNBC subtypes [18]. LAR subtype displays a unique gene expression profile enriched in genes associated with hormone regulation and steroid synthesis. It involves expression of downstream androgen receptor target genes (*APOD*, *FASN*, *SPDEF*, *CLDN8*), estrogen receptor (*ESR1*), and genes associated with estrogen signaling pathways (*FOXA1*, *XBP1*, *GATA-3*) [18]. This demonstrates that although the LAR subtype is immunohistochemically negative for ER, it exhibits increased molecular activity of estrogen-mediated pathways. Taken together, these suggest the possibility of therapeutic benefit with anti-estrogens and anti-androgens.

There is also increased frequency of *PIK3CA* activating mutations in the LAR subtype, suggesting a potential for therapeutic targeting with *PI3K/AKT/mTOR* inhibition [18, 21].

Table 1 Comparison of the different proposed classification systems

Author	Year	Classification model	Cohort size	Sample	Analysis	Proposed subtypes
Lehman et al.	2011	TNBCType	587	Gene expression	K-means	BL1 BL2 M MSL IM LAR
Lehman et al.	2016	TNBCType-4	587	Gene expression		BL1 BL2 M LAR
Burstein et al.	2015		198	RNA and DNA profiling	DEDS	BLIS BLIA M LAR
Jezequel et al.	2015		194	RNA profiling	Fuzzy clustering	Basal like with low immune response Basal like with high immune response LAR
Liu et al.	2016	FUSCC	165	mRNA and lncRNA	K-means ECDF	BLIS IM M LAR

Mesenchymal

This subtype has a gene expression profile that exhibits increased expression of pathways associated with cell motility and cellular differentiation. Several groups have described increased expression of genes associated with *TGF-β*, *mTOR*, *Wnt/β-catenin*, and *ALK* signaling pathways [18]. The initial proposed classification by Lehman et al. described two different subtypes including M and the MSL, with similar gene expression profiles. The major differences between both are increased growth factor signaling (*EGFR*, *PDGFR*), and low levels of proliferation genes in MSL compared to the M subtype [20]. The refined classification system proposed by Lehman et al. "TNBCtype-4" excludes the MSL subtype as the gene expression profile suggests an interaction between tumor cells and the microenvironment. However, subtypes proposed by others groups describe a mesenchymal type with gene expression profile enriched in growth factor signaling (*PGDGFR*, *VEGF*, *IGF*) and low levels of genes associated with cell proliferation, similar to the previously proposed MSL subtype. This suggests possible opportunities for therapeutic targeting with growth factor inhibitors.

Immunomodulatory

It remains unclear if this can be truly identified as a subtype. This was first proposed in the initial classification system proposed by Lehman et al. as enriched in genes involved in immune cell signaling [18]. The refined TNBCtype-4 classification system proposed that this subtype was associated with low tumor cellularity, with gene expression profile driven by the tumor infiltrating lymphocytes (TILs) [20]. The IM subtype has the highest amount of associated lymphocytes when compared to other subtypes, and high levels of immune checkpoint regulatory genes encoding for PD-L1 and PD1 [20], making this subtype a potential target for immune checkpoint inhibition. This subtype has been corroborated by the classification schema proposed by Liu et al. [23]; however, there seems overlap between this group and the BLIA/basal-like with high immune response.

Potential Therapeutic Applications

Although chemotherapy remains the mainstay of systemic therapy for TNBC patients, the molecular classification systems may allow for personalized and targeted therapy with the goal of improving clinical outcomes in TNBC.

Dysregulation of *BRCA1* in basal-like tumors leads to impaired homologous recombinant-dependent DNA repair pathways which are a mechanism of TNBC tumorigenesis [26, 27]. The *BRCAness* of basal-like breast cancer is the basis of the use of drugs that engage DNA-repair mechanisms. PARP1 inhibitors are approved for treatment of breast cancer patients with germline *BRCA1* and *BRCA2 mutations.* In vitro studies demonstrate that the combination of PARP1 inhibitors and

chemotherapeutic agents that induce DNA damage augment the cytotoxic and anti-proliferative effects of chemotherapy in TNBC cell lines [35, 36]. Clinical trials with PARP1 inhibitors in patients with *BRCA*-associated advanced breast cancer show improved clinical outcomes [37, 38]. Currently, there are several ongoing clinical trials investigating PARP1 inhibitors in TNBC, alone or in combination with cytotoxic chemotherapy (NCT00516724, NCT03205761, NCT01445418).

EGFR overexpression has been demonstrated in basal-like tumors and is reported as a predictor of worse survival independent of tumor stage [39, 40]. Interestingly, basal-like breast cancer cell lines are more sensitive to *EGFR* inhibition compared to luminal cell lines [39]. A phase II study of cetuximab, an *EGFR* inhibitor, and cisplatin doubled the overall response rate in patients with advanced TNBC [41]. Larger studies are needed to define patient subtypes with *EGFR* activating mutations that may benefit from *EGFR* targeted therapies.

TNBC cell lines with AR overexpression demonstrate increased proliferation in response to androgens and estrogens, but no response to treatment with estrogen antagonists, suggesting an AR-dependent, ER-independent growth response [42]. Anti-androgens have been investigated alone and in combination with chemotherapy in several clinical trials in TNBC with varying outcomes. A phase II trial of bicalutamide in AR-positive ER-negative breast cancer showed a 6-month clinical benefit rate (CBR) of 19% and a median progression-free survival (PFS) of 12 weeks [43]. A recently published phase II trial with enzalutamide in 118 AR-positive TNBC patients showed a 16-week CBR of 25%, a median PFS of 2.9 months, and a median overall survival of 12.7 months all in the intent to treat population [44]. Enzalutamide was very well tolerated with fatigue being the only treatment-related grade 3 or higher adverse event. In vitro studies have also demonstrated that the LAR subtype is much more sensitive to CDK4/6 inhibition compared to basal-like subtypes [45]. CDK4/6 inhibitors are approved in combination with aromatase inhibitors and fulvestrant for advanced ER positive breast cancer. Clinical trials evaluating the combination of anti-androgens and CDK4/6 inhibitors for AR-positive TNBC patients are ongoing (NCT02605486, NCT03090165). The increased frequency of activating *PIK3CA* mutations in TNBC also suggests a possible role for therapeutic targeting with *PI3k/mTOR* pathway inhibition [17, 18]. Taselisib, an oral *PI3K* inhibitor, is being studied in combination with enzalutamide for AR-positive TNBC patients (NCT02457910). Table 2 lists clinical trials with targeted agents in TNBC.

Table 2 Active and completed studies with targeted therapies in TNBC

PARP inhibition		
NCT02482311	Phase I	Olaparib
NCT02595905	Phase II	Veliparib + cisplatin
NCT01623349	Phase I	Olaparib + alpelisib
NCT03150576	Phase II/III	Olaparib + carboplatin/paclitaxel
NCT01173497	Phase II	Iniparib + irinotecan
NCT02401347	Phase II	Talazoparib
NCT01445418	Phase I	Olaparib + carboplatin
NCT00813956	Phase II	Iniparib + gemcitabine/carboplatin
NCT00892736	Phase I/II	Veliparib
EGFR inhibition		
NCT00463788	Phase II	Cetuximab + cisplatin
Androgen receptor inhibition		
NCT02929576	Phase III	Enzalutamide ± paclitaxel
NCT03055312	Phase III	Bicalutamide
NCT02971761	Phase II	Enobosarm + Pembrolizumab
NCT02750358	Phase II	Enzalutamide
NCT02689427	Phase II	Enzalutamide
NCT01889238	Phase II	Enzalutamide + paclitaxel
NCT02130700	Phase II	VT-464
NCT03383679	Phase II	Darolutamide
CDK4/6 inhibition		
NCT02605486	Phase I/II	Bicalutamide + palbociclib
NCT03090165	Phase I/II	Bicalutamide + ribociclib
PI3K inhibition		
NCT02457910	Phase I/II	Enzalutamide + taselisib

Role of Immunotherapy

TILs are more prevalent in TNBC compared to non TNBC, and their presence is a prognostic factor for disease-free survival, overall survival, and chemotherapy response in TNBC [46–48]. PD-L1 expression in TNBC ranges from 20 to 26% [49, 50]. Furthermore, the higher mutational frequency in TNBC compared to other breast cancer subtypes suggests immunogenicity in TNBC. These findings suggest a potential role of immunotherapy in TNBC. Pembrolizumab, an anti-PD1 monoclonal antibody, has been investigated in heavily pretreated PD-L1 positive metastatic TNBC with varying response rates of 5–18.5% (NCT01848834, NCT02447003).

Currently, there are several ongoing clinical trials using anti-PD1 or anti-PD-L1 monoclonal antibodies as monotherapy or in combination with other systemic therapies in the treatment of metastatic TNBC.

Conclusion

Various attempts to classify TNBC demonstrate its marked heterogeneity. There is no standard uniformly accepted molecular classification system. Several clinical studies investigating targeted therapies in TNBC patients have had varying results, which can be attributed to lack of proper patient selection. Although TNBC has a higher mutational frequency compared to other breast cancer subtypes, there is no approved targeted therapy for TNBC. To help address this gap, research should focus on the standardization of TNBC classification, as this may enable proper patient selection in future clinical trials with targeted therapies.

Compliance with Ethical Standards

Conflict of Interest Cynthia X. Ma has served on the advisory board for Merck, Pfizer, Novartis, Eli Lily, and AstraZeneca and has received a grant from Eisai.
Nkiruka Ezenwajiaku and Foluso O. Ademuyiwa declare that they have no competing interests.

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