RESEARCH ARTICLE

Triple negative breast cancer – Understanding the clinical implications of heterogeneity

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Abstract

Triple negative breast cancers (TNBC) have long been associated with a worse prognosis in contrast to hormone receptor (HR) positive and human epidermal growth factor receptor 2 (HER2) positive breast tumours. However, advances in the classification and molecular categorization of TNBC have led to meaningful advances, highlighting substantial TNBC heterogeneity and distinct histomolecular subtypes. While TNBC has historically been treated with single or combined chemotherapeutic agents, novel targeted therapies are being developed and evaluated. A better understanding of oncogenic drivers and the pathogenicity of germline and somatic mutations has also paved the way to new targeted treatments. More recently, the neoadjuvant space has become increasingly popular to better understand the behaviour of TNBC subtypes, leading to the development of biomarker-driven trials to better select targeted treatments. This has ultimately led to a number of advances and new potential targets for treatment which remain under investigation and will undoubtedly lead to a better understanding and personalized approach for patients diagnosed with TNBC.

INTRODUCTION

Triple negative breast cancers (TNBC) account for 15% to 20% of all newly diagnosed breast cancers (1, 2). They have predominantly been associated with an earlier age of onset, a more aggressive clinical course with the early development of visceral and central nervous system metastases and tend to confer a worse overall prognosis (1-4). Further, gene expression analyses of breast tumours reveal a higher occurrence of brain and lung metastases in patients with basal-like and claudin-low tumours, the majority of which are in fact TNBC (5). Nearly 50% of patients with metastatic TNBC will go on to develop CNS relapses (6).

TNBC has traditionally been defined as the absence of the estrogen receptor (ER) and progesterone receptor (PgR) by immunohistochemistry (IHC) with less than or equal to 1% expression and the absence of the human epidermal growth factor receptor 2 (HER2) overexpression by IHC and/or fluorescence in situ hybridization (FISH) based on the most recent American Society of Clinical Oncology (ASCO) Guidelines (7-9). As patients with TNBC have not derived significant benefit from targeted treatments directed at these biomarkers (10-13), the mainstay of treatment remains chemotherapy either given as monotherapy or in combination. The increased risk of relapse coupled with the chemo sensitivity of TNBC and evidence for benefit, results in a lower threshold for a recommendation for chemotherapy in the adjuvant setting (14). While TNBC have historically been thought of as basal-like cancers, which tend to express genes characteristic of normal basal and myoepithelial cells, less aggressive forms of TNBC which do not harbour these same characteristics have been recognized (15). Surgery and radiation are important in early stage disease, particularly in subtypes which are less chemo sensitive. Although there may be a role for surgical debulking in advanced disease, the aggressive and widespread nature of TNBC usually demands systemic treatment. However, in cases where there are disparate responses, surgery may provide a basis for understanding the heterogeneity in an individual patient and may be of benefit in removing resistant disease if the malignancy is otherwise controlled. The pathological assessment may therefore provide a basis for a rational choice of targets.

Despite advances in the other clinically recognized breast cancer subtypes, the time, frequency and pattern of relapse for the heterogenous group of TNBC has not markedly changed over time, prompting more directed research efforts for this group (2, 11). This review addresses the heterogeneity of TNBC and highlights some of the distinct subgroups with a divergent clinical course of potential relevance for targeted treatment selection and benefit.

TNBC CLASSIFICATION: A MOVING TARGET

Breast cancer classification has significantly evolved over the last 20 years. While histopathological types were first used to describe the wide spectrum of breast cancer behaviour, the advent of IHC-defined subtypes quickly gained popularity in the early 2000s given their predictive value for

selected targeted treatments and their prognostic relevance (11, 16). However, the method of assessment for ER/PR positivity has evolved over time (17, 18). The process utilizing a ligand-based assay was abandoned in the late 1980s in favor of a well validated immunohistochemistry (IHC) method (17), which has been further perfected since its original implementation (18), although it remains subject to pre-analytical, analytical and post-analytical limitations (19). Many classifications have emerged over the years, although those with the most promise of clinical relevance are emphasized herein.

Further work describing intrinsic subtypes and patterns defined by gene expression profiling (15, 20, 21) and structural alterations by next generation sequencing dramatically changed the classification of breast cancer by providing a more in depth knowledge of breast tumour heterogeneity and the underlying molecular complexity (22-24). While the groups defined as claudin-low, basal-like, HER-2 related, luminal B, luminal A and normal-like were more or less thought to correspond to the histopathological subtypes by IHC classification, there was no seamless overlap. Amongst the tumours pathologically defined as TNBC, 6 distinct molecular subtypes lumped into 3 subgroups were defined, described as basal-like (BL1, BL2, IM), mesenchymal-like (IM, MSL) and luminal-like (LAR) (1,25). This also heralded subsequent work by Lehmann et al. which further refined TNBC molecular subtypes into 4, comprising BL1, BL2, M and LAR, whereas the immunomodulatory (IM) was thought to be a reflection of tumor infiltrating lymphocytes (TILs) and the mesenchymal stem-like (MSL) subtype, a reflection of tumor-associated stromal cells (26).

The work of both Lehmann et al. and Perou et al. is mostly concordant in suggesting 4 TNBC subtypes, of which the majority, comprising 70 to 80% of tumours, can be grouped in the basal subtype and 20-30% in the luminal subtype. Those aligning with a basal subtype could then be further divided into either claudin-low/mesenchymal tumours with low proliferation and chemosensitivity as opposed to the well described basal-like tumours with high proliferation and chemosensitivity. Within the luminal group encompassing the remaining 20-30% of tumours with androgen receptor (AR) expression, distinct classifications were also defined, a luminal group with minimal chemosensitivity and low proliferation and a HER-2 enriched highly proliferative and chemosensitive group (1,26,27).

With the introduction of the TCGA and enhanced knowledge of tumour biology, it's become evident that breast cancers encompass a range of entities which extend beyond the groups defined by IHC, genome and transcriptome data, highlighting the need for an integrative classification beyond histomolecular subtypes (28). In fact, a comparison of the TCGA TNBC datasets revealed that 86% corresponded to the basal-like intrinsic subtype defined by PAM50, whereas 6% were HER2-enriched, 5% luminal A, 1% luminal B and 2% normal-like (29). Further, the distribution of intrinsic subtypes from TCGA and METRABRIC TNBC datasets revealed that

a large proportion (86% and 78%, respectively), but not all TNBC tumours were basal-like (29). It also emphasized the fact that not at basal-like cancers identified in TCGA and METABRIC datasets were clinically classified as TNBC by IHC (78% and 75%, respectively) (29). The interplay of molecular alterations in combination with histological features and a better understanding of tumour microenvironment and immunogenicity emphasises the complexity of TNBC classification (29). It remains to be seen whether novel and more comprehensive histomolecular phenotypes can be predictive of responses to tailored treatment, as has been proposed by other groups (30). Clinical trials which clearly define outcomes in specific molecular groups are needed. Further, the pragmatic utilization and incorporation of multi-omics in clinical practice remains a key limitation that requires further consideration (31), due to the availability, reliability, turnaround time and cost, amongst others.

NEOADJUVANT OUTCOMES HIGHLIGHT TNBC HETEROGENEITY

The use of pathologic complete response (pCR) as a surrogate endpoint for long-term survival outcomes has been well demonstrated (32) and remains an important tool in refining our understanding of biological subgroups, their natural history and their response to systemic treatment (33,34). Studies using immunohistochemical markers to infer molecular subtype demonstrated that basal-like tumours are more chemosensitive, but are ultimately associated with a worse prognosis due to a higher likelihood of relapse, deemed the TNBC paradox (12). More recently, the concept of adapting treatment for patients based on the presence of residual disease has shown promise, with TNBC patients obtaining a significant DFS and OS advantage when treated with 8 cycles of capecitabine if residual disease was identified following neoadjuvant chemotherapy (35). Efforts to establish a consistent framework to define residual disease have therefore been undertaken, which has resulted in the validation of a new model, the residual cancer burden (36). In an institutional validation study, an independent association of the residual cancer index score to prognosis was demonstrated (36). Understanding the relationship between pCR and survival outcomes can therefore inform the design of pre-operative trials by identifying agents or combinations that result in less residual disease (37). In turn, this highlights the importance of understanding the heterogenous biological subgroups and the mechanisms by which rates of pCR and/or lower RCB scores can be enhanced.

With an increased awareness of TNBC molecular subtypes and differential responses to neoadjuvant chemotherapy, rates of pCR relative to TNBC type have been explored in retrospective datasets (38). Utilizing the refined classification proposed by Lehmann et al., 5 gene expression datasets were retrospectively evaluated using PAM50 and TNBCtype-4 (26). Ultimately, this study highlighted the implications of TNBC subtype on rates of pCR. It also confirmed the differing responses observed with chemotherapy, in which the greatest responses achieved were

in TNBC tumours classified as BL1, in contrast to lower rates of pCR for patients with LAR and BL2 tumours (26). Another similar study supported these findings, with BL1 tumors demonstrating the highest proliferation indices and achieving the highest rates of pCR (39). It also reported that the patients with LAR tumors had lower rates of proliferation, low rates of pCR and ultimately were comprised of non-basal-like intrinsic subtypes, namely HER2-enriched and luminal A (39). The ability to assess treatment efficacy in vivo by use of a surrogate for responses has therefore proven itself to be an attractive means to test novel therapeutics in vivo (40). While some neoadjuvant studies looked at the use of selected agents amongst all TNBC (41, 42), other studies have sought to evaluate the predictive value of TNBC subtype by Lehmann's refined classification (43). Interestingly, subtypes defined by this classification remained predictive of responses to neoadjuvant treatment in multi-variate analyses which included traditionally recognized clinical and pathological factors. It also confirmed that patients with BL1 tumours were associated with a younger age at diagnosis and higher proliferation index compared to the other subtypes (43).

One of the most recent examples of the neoadjuvant setting illustrating the heterogeneity of TNBC and interplay with the tumour immune status using clinical was recently published by Prado-Vazquez et al. Gene expression data from nearly 500 TNBC tumours was analyzed and tumours were divided according to their immune activity, either high or low (52% vs 48%), which identified a significantly better prognosis for highly immune active-cells (44, 45). Studies like these continue to challenge our understanding of tumour behaviour but may ultimately inform clinical trials directed by a greater understanding of tumour biology. This may in turn predict differential responses to immune-targeted treatments. Examples of neoadjuvant studies and the utilization of biomarker informed trials are discussed further in the subsequent sections of this review.

ACTIONABLE MOLECULAR TARGETS

The identification of actionable molecular targets in TNBC remains an important topic of research and interest (46, 47) and has been the subject of many reviews (25, 48). Within the TCGA analysis, 65 of the basal-like breast cancers were found to be triple negative, with a high frequency of TP53, RB1 and BRCA loss and high activation of the PI3K pathway, amongst others (28). It is now well recognized that TNBC is associated with a higher risk of germline deleterious mutation in cohorts unselected for family history, particularly in patients with an earlier age of diagnosis (49-52).

Amongst the most common and recognized oncogenic drivers, the breast-related cancer antigen 1 (BRCA1) and breast-related cancer antigen 2 (BRCA2) genes have been identified as potential oncogenic targets. Firstly, patients with deleterious BRCA mutations are known to have damaged double-strand DNA repair mechanisms, making them increasingly vulnerable to agents that induce DNA damage such as anthracyclines and DNA cross-linking agents such as platinum

derivatives, ultimately leading to synthetic lethality (53, 54). Supporting the benefit of platinum drugs, the phase III Triple Negative Breast Cancer Trial (TNT) demonstrated a doubling of the objective response rate (ORR) for patients with BRCA1 or BRCA2 mutations (68% vs 33%; p=0.03) and a PFS advantage (6.8 months vs 3.1 months; p=0.03) when treated with carboplatin as opposed to docetaxel, in contrast to the non-basal like tumours who fared better with docetaxel (55). Further, poly(ADPribose) polymerase 1-2 (PARP) inhibitors have also been associated with benefit in the advanced setting in contrast to unselected TNBC populations which did not derive notable benefit (56). In a study of just over 200 patients, median PFS was prolonged by nearly 3 months and ORR doubled (29% vs 60%; p<0.001) for patients treated with olaparib 300mg twice daily in contrast to those treated with standard single-agent chemotherapy drugs (57). A recent study of talazoparib, another PARP inhibitor, demonstrated an RCB-0 rate of 53% and RCB-0/I of 63% when used neoadjuvantly once daily for 6 months in patients with a germline BRCA pathogenic variant (58). A larger neoadjuvant trial utilizing this approach is ongoing (ClinicalTrials.gov identifier: NCT03499353).

Targeting the DNA repair pathway beyond BRCA mutations has also lead to the notion of BRCA-ness, which is associated with high grade tumours with triple negative IHC and frequent TP53 gene mutations with sensitivity to DNA damage (59). Within this spectrum of BRCAness, the low expression of BRCA1/2 due to promoter hypermethylation or BRCA1/2 somatic gene mutations have been proposed as mechanisms associated with the BRCAness phenotype (59). Additionally, a number of genes involved in HR repair may theoretically confer sensitivity to DNA-damaging agents. In a study of over 1800 unselected patients for family history, deleterious germline mutations in genes other than BRCA1/2 were found in 3.7% of patients, the majority involving homologous recombination, including PALB2, BRAD1, RAD51D, RAD51C and BRIP1 (51). While studies in the TNBC setting have not been definitive (60,61), some evidence of benefit from platinum compounds and PARP inhibitors has been described in advanced breast cancer with HRD signatures (53) and in non-BRCA mutant HRD phenotypes in ovarian cancer (62), respectively. Ongoing studies of PARP inhibitors, namely talazoparib, in patients with mutations in non-BRCA homologous recombination pathway genes are ongoing (NCT02401347). The homologous recombination deficiency (HRD) score has also been associated with sensitivity to DNA damaging agents irrespective of pathogenic germline mutational status, deemed BRCA-like tumors in BRCA 1/2 non-mutated tumours. In a pooled analysis of six phase II trials conducted across TNBC cohorts, rates of RCB 0/I and/or pCR were significantly higher for those with a high HRD score treated with DNA-damaging agents compared to those with a low score (pCR 33.0% vs 11.0% with platinum compounds) (63). However, in the TNT trial described previously, patients with BRCA1 methylation or a high score with Myriad

HRD assay did not appear to derive the same benefit in contrast to those with a germline BRCA mutation (55). It remains to be seen whether other genes associated with DNA repair and genomic instability will be associated with similar benefit from agents targeting DNA repair in personalized medicine or tumor agnostic trials.

Beyond the most well described susceptibility gene mutations associated with a high risk of TNBC, more extensive panel testing has been surmised to provide a greater understanding of tumour biology and oncogenic drivers. Other genes enriched in TNBC cohorts such as BARD1, BRIP1, PALB2 and RAD51C relative to other breast cancer subtypes, have not yet demonstrated clear clinical utility, particularly as it relates to treatment selection and sensitivity (64). In a study of 8753 TNBC, germline pathogenic variants across 21 genes of interest were detected in 12.0% of patients, although only 3.7% were non-BRCA1 and 2 (65). The genes identified beyond BRCA1 and 2 associated with high OR of breast cancer (PALB2, BARD1, RAD51D) remain part of the cluster of genes which have not yet been predictive of targeted treatment benefit (65). However, their involvement in the homologous recombination pathway makes them interesting for future study.

In the non-germline setting, distinctive mutational patterns are seen between the basal and non-basal like tumours. Whereas TP53 mutations are often identified, they are mostly within basal-like tumours while other mutations such as PIK3CA, NF1 and PTEN loss are more frequently identified in the luminal subtypes (28,46,66), although meaningful benefit from targeted treatment for this pathway has yet to be established. Additionally, basal-like tumours also have high rates of RB1 deficiency, with concurrent TP53 and RB1 alterations in nearly 40% of basal-like breast cancers (67). Although the basal subtype is associated with more structural alterations by NGS (23), few of these have led to targeted treatments with meaningful clinical benefit. Further, while much of the prior research has focused on targeting key signalling pathways associated with proliferation, angiogenesis and survival, little progress has been made relative to historical outcomes. In addition to the genomic findings discussed, additional work is being done on non-coding microRNAs (miRNA), namely miR-363, which can effectively regulate gene function at a post-transcriptional level (68). Selected treatments based on transcriptomics have also been undertaken in novel trial designs, although beyond HRD gene signatures discussed earlier in this review and immune signatures discussed later in this review, the predictive benefit of transcriptomics data remains uncertain (69).

Another potential targetable alteration which has been most commonly identified in tumours within the LAR group is the androgen receptor activation and its downstream effects. While this subgroup generally appears to benefit from a better overall prognosis attributed to their low proliferation index (27), they tend to be less responsive to traditional chemotherapeutic agents. Thus, anti-androgen treatments such as bicalutamide, enzalutamide and abiraterone have been explored, revealing some clinical benefit, albeit short-lived, with

a median PFS of 3 months, 2.9 months and 2.8 months, respectively (70-72). Studies of agents targeting the androgen receptor in combination with PI3K/mTor inhibition and cell cycle inhibition are ongoing given the frequent co-amplification of PIK3CA in AR positive tumors (73) and their greater dependence on CDK4/6 phosphorylation (74), respectively.

NOVEL TARGETS OF INTEREST

In recent years, immunotherapeutics has become an increasingly attractive treatment strategy extending well beyond targeted treatments specific to DNA, RNA or signatures. The interplay between the immune system has long been purported as an important therapeutic target for breast tumors, with studies supporting a significant correlation between regulatory T cells and risk of relapse (75). It was subsequently shown that stromal tumour-infiltrating lymphocytes were associated with significantly better outcomes in primary TNBC based on two neoadjuvant phase III trials (76). In node positive samples from the BIG 02-98 trial, the increase in intratumoural and stromal lymphocytic infiltration by 10% increments resulted in a significantly lower risk of death in TNBC patients, regardless of chemotherapy received (27% and 17% reductions, respectively) (77). In a recent pooled analysis of over 2000 TNBC patients, TILs located in the stroma were lower in older individuals, those with larger tumors and those with nodal involvement (78). Further, node negative patients with stromally located TILs >=30% had excellent outcomes, with a 3-year invasive DFS (iDFS) rate of 92% and a median OS of 99%, adding further support to the important role of TILs as a prognostic indicator (78), which appears to be an independent prognostic factor for DFS outcomes (79).

Relating this back to the efforts to subtype TNBC, Lehmann et al. underscored the impact of the tumour microenvironment and immune milieu of the tumour on gene expression profiles (26). It was demonstrated that the highest percentage of lymphocytes was identified in the tumours classified as IM, in contrast to the other defined subtypes, despite TILs being present in all TNBC molecular subtypes (26). RNA sequencing revealed a significantly higher expression of PD-1, PD-L1 and CTLA-4 in those classified as the IM subtype (1, 26). In a similar observation, those with the M subtype had an inverse correlation to IM, suggesting an unfavourable microenvironment for lymphocytes (26). These observations, amongst others, suggest that the immunogenicity of the tumour can in fact have prognostic value, but perhaps more importantly, may be predictive of the benefit of checkpoint inhibitors and immunomodulatory treatment approaches (80).

PD-L1, which is not expressed in normal breast tissue, seems to be enriched in patients with basal-like tumours (81) and can be found in 20-30% of TNBC patients and associated with infiltration of lymphocytes (80,81). In a phase IB single-arm study, a PD-1 inhibitor, pembrolizumab, had activity in heavily pre-treated patients with TNBC with an ORR of 18.5% (82) and studies of the PDL-1 agents, atezolizumab (83), and avelumab (84), suggested benefit

in similar populations, although the method of PDL-1 expression was assessed using different IHC antibodies and staining patterns. Given the activity in heavily pre-treated populations in the advanced setting, combinations with additional checkpoint inhibitors, chemotherapy and targeted agents were undertaken.

In the recent randomized double-blind IMPASSION-130 study, patients with a higher PDL-1 status, defined as PD-L1 IC ≥ 1%, derived a significant DFS and OS advantage form nab-paclitaxel in combination with atezolizumab compared to nab-paclitaxel and placebo. However, in contrast to studies in other tumour sites, PD-L1 staining was done on the immune cells in contrast to tumour cells (85), reflecting the importance of stromally located TILs. In a post-hoc analysis of PD-L1 staining for patients enrolled on the IMPASSION 130 study, 3 commercially available tests including SP142, SP263 and 22C3 were compared to assess analytical concordance and their predictive capabilities. This study ultimately revealed that the overall percentage agreement ranged between 63-69%, although SP142 PDL1+ populations seem to derive the greatest benefit, which highlights the importance of using the appropriate complementary diagnostic immunohistochemistry assay for the specified agent (86), especially given that other studies of a combination of chemo-immunotherapeutics did not identify PDL1 as a predictive marker of response (87). In the neoadjuvant setting, the KEYNOTE 522 study looked at the addition of Pembrolizumab concurrently during the chemotherapy portion and subsequently continued in the adjuvant treatment setting for an additional 6 months, revealing an improvement in pCR (88). Interestingly, while the absolute numbers of patients who achieved pCR was greater for those with PDL1 positive tumours, with a 14% benefit with the addition of a PD-1 inhibitor (69% vs 55%), incremental gains in pCR were seen amongst those with PDL-1 negative tumours (45% vs 30%) (88).

These studies reinforce the fact that interactions between immune cells, tumour cells and the microenvironment remain complex, highlighting the heterogeneity of TNBC even within tumours which may share phenotypic traits. In fact, pre-clinical models revealed that despite adaptive immune cell infiltration in claudin-low tumours, immune checkpoint inhibitors were ineffective in controlling tumour growth in those subtyped as claudin-low tumours. In mouse models, a large proportion of the TILs in claudin-low tumours were T regulatory cells which appear to suppress the effector T cell responses, suggesting that Treg depletion potentiates checkpoint inhibition in claudin-low breast cancer (89). Understanding the heterogeneity of this group may therefore aid in the selection of personalized treatment approaches and support the premise of designing clinical trials based on multi-omics analysis, while considering the epigenome and immune microenvironment (29). Therefore, many groups are advocating for biomarker directed trials to improve patient selection and allow for more in depth correlative work and translational studies. In turn, the hope is that this would aid in the identification of patients who may benefit from

immunotherapeutics and novel drug combinations (90). Finally, a number of other targets are being explored. New drug conjugates are being developed and may provide further options for therapy (91). The issues of tumour heterogeneity and the subtypes that will benefit from these therapies require further investigation for us to understand their potential.

CONCLUSION

In summary, the clinical relevance of tumour biology for patients newly diagnosed with TNBC is at the forefront of research initiatives to better select informed treatment and refine prognosis. With the discovery of potential therapeutically relevant aberrations and predictive biomarkers of response to targeted treatment, the landscape of therapeutic options for TNBC has finally started to move beyond chemotherapeutics. Biomarker driven trials designed with pre-screened populations and enriched for specific biologic subgroups will most likely be utilized, as the significant heterogeneity of TNBC is recognized. However, while this review addresses the primary issue of inter-tumour heterogeneity, a greater understanding of intra-tumour heterogeneity is needed. The complex interplay between multi-omics, selective pressures, immunogenicity and tumour microenvironment undoubtedly has a large yet poorly understood effect on clonal mutational evolution and the development of resistance which warrants further study alongside the spectrum of TNBC subtypes. Hopefully in the near future we can abandon the term ‘triple negative’ and replace it with more meaningful and clinically relevant labels for this heterogeneous group of breast cancers.

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