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Pathogenesis of Triple-Negative Breast Cancer

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Abstract

Triple-negative breast cancer (TNBC) encompasses a heterogeneous group of fundamentally different diseases with different histologic, genomic, and immunologic profiles, which are aggregated under this term because of their lack of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 expression. Massively parallel sequencing and other omics technologies have demonstrated the level of heterogeneity in TNBCs and shed light into the pathogenesis of this therapeutically challenging entity in breast cancer. In this review, we discuss the histologic and molecular classifications of TNBC, the genomic alterations these different tumor types harbor, and the potential impact of these alterations on the pathogenesis of these tumors. We also explore the role of the tumor microenvironment in the biology of TNBCs and its potential impact on therapeutic response. Dissecting the biology and understanding the therapeutic dependencies of each TNBC subtype will be essential to delivering on the promise of precision medicine for patients with triple-negative disease.

Keywords

triple-negative; basal-like; subtypes; breast cancer; genomics; molecular pathology

INTRODUCTION

Breast cancer is a diverse disease in terms of histologic types, natural history, clinical behavior, and response to treatment. With the development of effective treatments for subsets of the disease that expressed hormone receptors, namely estrogen receptor (ER) and progesterone receptor (PR), as well as with the astounding success of anti–human epidermal growth factor receptor 2 (HER2) treatments during the 2000s, from a clinical management standpoint, breast cancers have been classified for the last 15 years according to their expression of ER, PR, and HER2. In the mid-2000s, the moniker triple-negative breast cancer (TNBC) was coined to refer to the subset of breast cancers lacking ER, PR, and HER2 (1, 2). TNBC was initially perceived as a clinical entity that would be closely

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related to the basal-like subtype discovered through the seminal gene expression microarray studies of the early 2000s (3); however, subsequent studies have supported the contention that TNBC is merely an operational term. On the basis of the currently available evidence, TNBC would be best considered as an umbrella term covering a variety of entities with marked genetic, transcriptional, histological, and clinical differences (2).

As a group, TNBCs display an aggressive clinical behavior. These tumors appear to be enriched in younger women and in women of African or Hispanic descent (2, 4). TNBCs constitute the most frequent type of invasive breast cancer developing in the context of patients harboring *BRCA1* germline mutations (5). In fact, >85% of breast cancers developing in the context of *BRCA1* germline pathogenic variant carriers display a triple-negative phenotype, and 11% to 19% of patients with triple-negative disease harbor *BRCA1* germline or somatic mutations (6, 7). Despite the more aggressive clinical behavior of TNBCs, several studies have now demonstrated that patients with these cancers more frequently evolve to pathologic complete response following neoadjuvant chemotherapy (8, 9). In the metastatic setting, however, TNBCs remain lethal despite the recent US Food and Drug Administration (FDA) approvals of different modalities of therapeutic agents for patients with this disease (10-12).

In this review, we focus first on the diversity of TNBCs at the histologic and molecular levels. Then we focus on the characteristics and pathogenesis of each subtype, followed by the recent advances in molecular classifications of TNBC and what we learned from these studies in terms of the pathogenesis of this heterogenous group of cancers. Finally, we seek to discuss recent findings on the pathogenesis and disease progression of this fascinating and heterogeneous group of tumors.

CLASSIFICATIONS OF TNBC

Histological Diversity

TNBCs comprise a panoply of histologic types of breast cancer (13, 14). Several studies have demonstrated that the vast majority of these tumors are high-grade invasive ductal carcinomas of no special type (IDC-NSTs) (14-16). Collectively, these high-grade IDC-NSTs harbor a constellation of histologic features that render them rather distinctive pattern, given that TNBCs more often harbor pushing borders, brisk lymphocytic infiltrates, and necrosis, not uncommonly in a geographic and central pattern (16, 17). In addition, the presence of medullary features (18), such as syncytial growth, and metaplastic elements (19), in particular in the form of squamous and spindle cells, are more frequently found in TNBCs than in breast cancers expressing hormone receptors and/or HER2 (20).

The comprehensive analyses of TNBCs, however, have revealed that there are some special histologic types of breast cancer that are preferentially of the triple-negative phenotype, including metaplastic breast cancers (17, 18, 20), carcinomas with medullary features (19), and carcinomas with apocrine features (8, 21, 22) (Figure 1). Collectively, these high-grade TNBCs, regardless of histologic type, are characterized by high levels of genetic instability, complex genomes, and recurrent *TP53* mutations (8, 14); however, there are some notable distinctions in the repertoire of somatic genetic alterations according to the tumor

type (2, 14). As compared with the common forms of TNBCs, metaplastic TNBCs more frequently harbor alterations affecting the phosphoinositide 3-kinase (PI3K) pathway genes and genes pertaining to the Wnt pathway (18, 20, 23, 24). Importantly, however, *CTNNB1* somatic mutations as well as nuclear expression of β-catenin are remarkably rare in these tumors (25). Carcinomas with apocrine differentiation have also been shown to display a distinctive repertoire of somatic alterations, pointing to activation of the PI3K pathway as one important molecular pathogenesis of the disease, including mutations in *PIK3CA*, *PIK3R1*, and *AKT1* (21). Other genes recently potentially implicated in pathogenesis of apocrine carcinomas are *NF1*, *DIS3, NCOA2*, and *PTPN11* (21).

The systematic study of breast cancers with a triple-negative phenotype has also revealed the existence of a subset of low-grade tumors, often with an indolent clinical behavior. Albeit vanishingly rare in the breast (2, 14, 23), these low-grade types of TNBC have counterparts in the salivary glands and are known in the field of breast pathology as salivary gland–like tumors of the breast (Figure 1) (26, 27). Collectively, these low-grade forms of TNBC harbor simple genomes often characterized by a few highly recurrent, if not pathognomonic, genetic alterations (14, 26) which are also present in their salivary gland counterparts, and include secretory carcinomas (28), adenoid cystic carcinomas (AdCCs) (29-32), and mucoepidermoid carcinomas (33, 34). In a way akin to their salivary gland counterparts, secretory carcinomas harbor the *ETV6-NTRK3* fusion gene in >95% of cases, and this gene constitutes a biomarker for the use of FDA-approved tyrosine receptor kinase inhibitors in the context of recurrent/metastatic disease (35-37). AdCCs have been shown to be driven by activation of the MYB pathway, through the oncogenic *MYB-NFIB* fusion gene, *MYBL1* rearrangements, or *MYB* gene amplification (14, 26, 29-31). Mucoepidermoid carcinomas have been recently reported to harbor *MAML2* rearrangements (34). In addition, polymorphous adenocarcinomas of the salivary glands have been shown to be underpinned by a highly recurrent *PRKD1* E710D somatic mutation (38) or by rearrangements affecting *PRKD1, PRKD2*, or *PRKD3* (39, 40) and may also be diagnosed in the breast. ER-negative adenomyoepitheliomas of the breast, which bear remarkable similarities to epithelial-myoepithelial tumors of the salivary glands (41, 42), are underpinned by *HRAS* Q61 hotspot mutations in conjunction with PIK3CA hotspot mutations or PIK3R1 loss-of-function mutations, which are causative of the cardinal histologic and phenotypic features of these tumors (41). The exception in this group is the acinic cell carcinoma of the breast (43-46); although these tumors are often of low histologic grade, they are often found in association with or can progress toward high-grade TNBCs (45, 46). These tumors do not bear a histologic resemblance to their salivary gland counterparts and, in fact, lack the known genetic alterations found in acinic cell carcinomas of the salivary glands (43). Instead, acinic cell carcinomas of the breast harbor *TP53* mutations, display complex genomes, and are remarkably similar, at the genetic level, to microglandular adenosis of the breast, a precursor of high-grade triple-negative disease (26, 44, 45, 47).

Some rare special types of breast cancer that are often but not exclusively of the triple-negative phenotype may also harbor rather specific constellations of genetic alterations. For instance, tall cell carcinomas with reversed polarity, a rare type of TNBC, have been shown to be driven by the combination of *IDH2* R172 hotspot mutations or, less frequently, *TET2*

loss-of-function mutations in conjunction with mutations affecting the PI3K pathway (48, 49); together, when expressed in 3D models of nonmalignant breast epithelial cells, these alterations result in the acquisition of the characteristics of reverse polarization (48, 49).

These findings highlight the plethora of distinct entities under the banner of TNBC and also illustrate the importance of TNBC histologic subtyping, given that the high- and low-grade forms of TNBC have fundamentally different genetic features, clinical behavior, and response to therapy.

Molecular Classification and Clues to the Pathogenesis of TNBC

The seminal work by Perou and colleagues (50, 51) resulted in the microarray-based classification of breast cancers into five intrinsic subtypes, namely luminal A, luminal B, HER2-enriched, normal-like, and basal-like breast cancers (51, 52). This classification has matured into the development of a system for the identification of these subtypes, known as intrinsic gene or PAM50 subtypes (53, 54). Although TNBCs were once considered to be synonymous with basal-like breast cancer, it is currently accepted that TNBCs display a remarkable diversity at the gene expression level as well. While most TNBCs fall into the basal-like intrinsic subtype on the PAM50 subtyping assay, the overlap between TNBC immunohistochemistry subtype and the basal-like molecular subtype is not complete (55). Further refinement of the initial classification identified a claudin-low subset within the basal-like subtype (56). Although grouping TNBC into basal and nonbasal subtypes was an oversimplification of the TNBC molecular heterogeneity, these studies brought up interesting questions in terms of the pathogenesis of TNBCs at the cell of origin level (see below for further discussion and the spectrum of TNBC heterogeneity (55).

The heterogeneity of TNBC has been further explored by Lehmann et al. (57), with evolving transcriptomic studies initially demonstrating the existence of six distinct subtypes within this group, namely basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR). In a follow-up study, the same research team refined their classification to four tumor-specific subtypes (BL1, BL2, M, and LAR), as they recognized that the initial transcriptomic features of the IM and MSL subtypes were derived from tumor-infiltrating lymphocytes (TILs) and tumor-associated stromal cells, respectively (58). These four TNBC subtypes were characterized by distinct expression patterns, whereas the immune-modulatory infiltrates vary within each of the subtypes. The BL1 subtype displayed enrichment of the genes involved in cell-cycle and cell-division and DNA damage response (DDR) pathways (57). The BL2 subtype displayed unique genetic profile that involved growth factor signaling, glycolysis and gluconeogenesis, and myoepithelial marker expression. The M subtype was enriched in gene expression for cell motility (57, 59). The LAR subtype was characterized by androgen receptor signaling with the gene ontologies being heavily enriched in hormonally regulated pathways, including steroid synthesis, porphyrin metabolism, and androgen-estrogen metabolism, despite being ER negative (57). Consistent with the notion that four robust subtypes of TNBC can be detected at the transcriptomic level, Burstein et al. (60) also identified four discrete subtypes: LAR, mesenchymal (which they refer to as MES), basal-like immune suppressed (BLIS), and basal-like immune

activated (BLIA). Of note, the BLIA and BLIS subtypes showed, respectively, the best and the worst disease-free survival.

Taken together, the studies reported above and subsequent studies have demonstrated that the most parsimonious number of subtypes of TNBC at the gene expression level is likely four. BLIA represents the majority of TNBCs with a complex genomic profile, having *TP53* mutations in more than 90% of cases and a high frequency of a homologous recombination (HR) DNA repair deficiency (HRD)–related signature (see below for discussion (60). BLIS also shows a high mutation rate in *TP53,* complex genomic profiles, and an HRD-associated signature but is associated with significantly lower TILs. BLIA and BLIS combined represent many of the basal-like tumors with germline and/or somatic *BRCA1* mutations (60-63). The mesenchymal subtype is characterized by lower genomic complexity and activation of the PI3K pathway (60, 62). LAR represents TNBC tumors with the lowest genomic complexity, with mutations in *PIK3CA, AKT1, NF1, GATA3*, and *CDH1* (63, 64). The LAR subtype displays mutations similar to those detected in luminal B cancers and its microenvironment is described as “cold,” with low TILs, in comparison with the “desert” microenvironment in the mesenchymal subtype (65) and “hot” microenvironment in BLIA (Figure 2). It should be noted, however, that in these gene expression classification systems, the vast majority of TNBCs analyzed were of high grade; hence, it remains unclear as to how the low-grade forms described above would fit into this taxonomy or if these low-grade forms would constitute completely different entities at the transcriptomic level.

SOMATIC GENOMIC ALTERATIONS

The genomic landscape of TNBC has been studied by means of whole-exome, whole-genome, and targeted sequencing analyses, which have shown that, as a group, these tumors display remarkably complex genomes, with a plethora of gene copy number gains and losses and a relatively high mutation burden as compared with other forms of breast cancer (21, 61, 66, 67). Interestingly, however, TNBCs display a limited number of highly recurrent mutated genes. In fact, of the known breast cancer drivers, *TP53* is the most frequently mutated driver gene (>80%), followed by *PIK3CA*. Other notable genetic alterations detected at low (<5%) frequency include *PTEN*, *KMT2C*, and *RB1* (61, 68).

The copy number alterations (CNAs) in TNBCs, as a group, are complex, with multiple gains and losses across all chromosomes and a few amplifications. Gains of 1q, 8q, and 10q; losses of 5q and 8p; amplifications of *EGFR* and *FGFR2*; and *PTEN* loss are found frequently in TNBCs (69-72). Importantly, the concurrent 1q gains and 16q losses, which are typical for ER-positive breast cancers, are not found in TNBCs (15).

On the basis of the integration of CNAs and gene expression profile, Curtis et al. (72) brought up 10 integrative clusters (IntClust-1 to −10), in which IntClust-10 encompasses mostly the poorly differentiated TNBCs with a high rate of *TP53* mutations and intermediate genomic instability. This TNBC-enriched subgroup had characteristic chromosome 5q deletion, associated with alterations in key cell-cycle-related and DNA damage repair genes (72). Some TNBCs (~25%) are classified as belonging to the IntClust-4 subtype, characterized by low levels of genomic instability and the absence of CNAs (72). This

CNA-devoid subgroup showed a strong inflammation signature and nearly twice as many deletions at the T cell receptor (TCR) loci on chromosomes 7 (TRG) and 14 (TRA), when compared with other nonbasal enriched clusters. Deletion of TCR loci in these tumor cells was related to severe lymphocytic infiltration (72), suggesting an interplay between genetic alterations in tumor cells and their microenvironment (see below). The role played by the disproportionately high percentage of inflammatory cells in the CNA profiles of these tumors, however, remains to be fully elucidated.

Notably, some CNAs appear to be significantly associated with distinct TNBC subtypes (64, 73). For example, the BL1 subtype, which displays the highest CNAs among all TNBC subtypes, harbors gains/amplifications in *MYC, PIK3CA, CDK6, AKT2, KRAS, FGFR1, IGF1R, CCNE1*, and *CDKN2A*/*B* and deletions in *BRCA2, PTEN, MDM2*, and *RB1*, whereas the LAR subtype displays recurrent gains/amplification of *EGFR* and *AKT1* as well as a high frequency of deletions affecting *CCND3, AKT2, ESR1, CDKN2A/B, SMAD4, NF1, NCOR1*, and *TP53*. The M subtype, on the other hand, was found to harbor recurrent gains/amplification of *DNMT3A* and *TP53* with high-frequency deletions of *PDGFRA, RB1*, and *MAP3K1* (64).

Jiang et al. (74) defined six clusters in TNBCs, on the basis of CNAs, and studied their association to different molecular subtypes of TNBCs in a large multiomic profiling study. Despite the lack of a specific one-to-one correspondence between most of the transcriptomic subtypes (62, 74) and the proposed CNA clusters, this endeavor demonstrated that most of the LARs (85%) were grouped in 8p21 loss or in low chromosomal instability clusters. Moreover, *CDKN2A* losses/deletions were noted to be enriched in the LAR subtype (65% of LARs versus 36% of other subtypes), emphasizing the role of cell-cycle signaling in the pathogenesis of this subtype of TNBC (74). Most TNBCs with 9p23 amplification (61%) and 12p13 amplification (74%) were of the BLIS subtype.

Mutational Signatures in TNBC

Seminal studies by Alexandrov et al. (75-77) and Nik-Zainal's group (78, 79) have demonstrated that the repertoire of somatic genetic alterations in a cancer, including the passenger genetic alterations, can provide important information about the biological phenomena that shaped the genome of a cancer. In fact, mutational signature analyses have demonstrated that in breast cancer, the most frequent mutational processes are aging or clock-like [Catalogue of Somatic Mutations in Cancer (COSMIC) signatures 1 and 5]; apolipoprotein b mRNA editing enzyme, catalytic polypeptide-like (APOBEC) mutagenesis (COSMIC signatures 2 and 13), likely stemming from the activity of the APOBEC cytidine deaminases, which function as a viral protecting agent as well as in RNA editing; and HR DNA repair defects (COSMIC signatures 3 and 8) (67, 74-76). The mutational processes captured by the mutational signatures appear to vary according to ER and HER2 status (67, 78, 79); whereas the most frequent mutational signatures in ER-positive disease are aging and APOBEC, in triple-negative disease, an enrichment for the HRD signature is observed. These mutational signatures, however, appear not to be specific to any PAM50-classified breast cancer subtype (67, 74, 80).

The ability to capture and analyze a comprehensive repertoire of genomic alterations—encompassing single-base substitutions, double-base substitutions, insertions and deletions (indels), and structural variants in cancer genomes—to call mutational signatures brought a new insight into the causative factors driving many cancers (67, 75, 76, 78), including TNBCs (67, 76). In addition to the single-nucleotide-based signatures, two indel signatures and six rearrangement signatures (RS-1 to −6) have been identified in the context of breast cancers (67). RS-1 and RS-3 (cumulatively accounting for 27% of all the rearrangements) are associated with tandem duplications (TDs) (see below for further discussion). The TDs associated with RS-1 were relatively large (>100 kb) and mostly found in *TP53*-mutated TNBCs without *BRCA1* mutation or promoter hypermethylation. By contrast, more than 90% of *BRCA1*-mutated or hypermethylated TNBCs had small (<10 kb) TDs associated with RS-3 and HRD (67, 79, 80).

Of the structural rearrangement signatures, the tandem duplicator phenotype (TDP) merits additional consideration in the context of triple-negative disease. The TDP is a pro-oncogenic, genome-wide configuration that is significantly enriched in these tumors, with up to 52.8% of high-grade TNBCs displaying this genomic pattern, which likely stems from the combination of the genomic instability and replicative drive found in TNBCs (81).

The TDP can be scored for each tumor using the number of TDs and their chromosomal distribution and can be classified on the basis of the span size of their TDs, small (<10 kb) and relatively large (>100 kb) TDs (67). TNBC was shown to be a TDP-rich cancer, with almost half of all TNBCs showing TDs of different classes. The relatively small (~10 kb) TDs, named group 1 TDP, were found in 29% of all TNBCs and specifically enriched by *BRCA1*-associated, but not *BRCA2*-inactivated, TNBCs (81). Other classes of TDs were also detected in TNBCs; midsize class 2 TDs (~50–600 kb) are highly enriched in tumors exhibiting CCNE1 pathway activation, whereas *CDK12* mutations were found to be enriched in tumors that harbor numerous larger class 3 TDs (~2 Mb) (81, 82). Further studies are warranted to understand the target genes and functional importance of the TDP in different TNBC subtypes.

The role of the TDP in the pathogenesis of TNBC and the therapeutic dependencies that specific subsets of these tumors display merit further exploration. In fact, some known tumor suppressor genes and oncogenes map to the breakpoint hotspot regions associated with different classes of TDs. For example, among the tumor suppressor genes, *PTEN* and *RB1* are affected in 16% and 15% of TNBCs with class 1 TDs, respectively (82). *MYC* is the frequently (21%) duplicated oncogene in TNBCs with class 2 TDs. Therefore, small TDs have the potential to disrupt several driver tumor suppressors (e.g., *PTEN* and *RB1* in TNBC) simultaneously (82). As a result, one plausible scenario in which small TDs may play a role in the pathogenesis of TNBCs could be when multiple driver genes are influenced by the TDP; then, their functional contribution to cancer cell fitness may simply be the result of multiple haploinsufficiencies (83).

In addition to the genomic signatures described above, another phenomenon identified in the context of TNBC is whole-genome duplication (WGD), which stems from tetraploidization and is believed to buffer the deleterious effects of higher mutational burden, contributing

to the biology of a subset of TNBCs (84, 85). In this way, cancer can be envisioned as analogous to asexual evolution, where polyploidy is the mechanism to mitigate Muller's ratchet (i.e., the accumulation of deleterious somatic mutations due to the absence of recombination) (85). Breast cancers, in general, are reported to have high rates of WGD, with approximately 45% showing at least one iteration of WGD (66, 86, 87), which is frequently associated with an increased rate of other types of somatic CNAs (86), as well as tumor mutation burden (84, 85). In TNBC, WGD appears to be a frequent event at least in some subtypes of TNBCs, such as BLIS; however, further studies are required to address the frequency and causality of this event in tumor evolution (74). Interestingly, WGD in breast cancer is reported to precede the acquisition of the driver genetic alterations and play a role in the branching of the evolving clones (88).

Homologous Recombination DNA Repair Deficiency in TNBCs

Defects in double-stranded DNA repair, due to either germline or somatic mutations in *BRCA1* and other genes involved in HR, have been known to play an important role in the pathogenesis of a subset of TNBCs. Several proteins with different roles are involved in DDR and HR. These can be categorized as sensors (e.g., *ATM*), mediators (e.g., *BRCA1* and *CHK2*), effectors (e.g., *BRCA2* and *RAD51*), or facilitators of the HR pathway (e.g., *PALB2* and *BRIP1*) (89).

Germline mutations in *BRCA1* occur in up to 19% of unselected TNBCs, and *BRCA1* mutations are mostly of the basal-like subtype (61, 90, 91). In the absence of *BRCA1*/*2* germline mutations, a substantial proportion of TNBCs still harbor the BRCA-like HRD phenotype (67) [also defined as BRCAness (91)]. Thus, in addition to alterations in *BRCA* genes, other alterations that could induce features of BRCAness have been investigated. Alterations in other specific HR genes have been shown to result in increased risk of breast cancer development, namely *PALB2, CHEK2, ATM*, and *NBN* (92). Multiple studies used gene panels to estimate the frequency and distribution of other genes implicated in HRD in TNBC, estimating a high frequency of alterations in genes other than *BRCA1* (in more than 50% of HRD-related TNBCs), including *PALB2* (~9%) (92, 93). In fact, 14.6% of all TNBCs were reported to harbor deleterious germline mutations in one of the HRD-related genes (94); deleterious germline mutations were identified in *BRCA1* (8.5%), *BRCA2* (2.7%), and other HRD genes (3.7%), including *PALB2* (1.2%) and *BARD1, RAD51D, RAD51C,* and *BRIP1* (0.3% to 0.5%) (94). These findings imply that these specific genes within the HR pathway can be related to BRCAness.

Importantly, only biallelic, and not monoallelic, inactivation of *BRCA1* and other HR genes appears to be associated with genomic features of HRD (95, 96). This finding has been shown not only in breast cancer but also in a pan-cancer analysis of The Cancer Genome Atlas (TCGA) data, demonstrating that only biallelic inactivation of HR-related genes results in features consistent with HRD and that these biallelic alterations are mutually exclusive of one another (95). Polak et al. (96) analyzed 995 TCGA breast cancer cases, using signature 3 (as described above) to identify the cases with BRCAness, and found that 247 cases were in the top quartile of signature 3 (HRD) activity. Of those, 88 (35%) showed biallelic loss in *BRCA1* or *BRCA2*, whereas the remaining 159

(64%) cases were classified as having BRCAness. Alterations of *RAD51C*, *PALB2*, and *BARD1* (but not *ATM* or *CHEK2*) were shown to account for 13% of cases associated with signature 3 (Ref. 96). Consistent with these observations, subsequent studies have demonstrated a lack of mutational signature 3 in *ATM*- and *CHEK2*-altered breast cancers (97, 98). Furthermore, epigenetic silencing of *RAD51C* and *BRCA1* has been shown to be associated with BRCAness (96). These findings are in line with another recent study, using HRDetect (see below) , showing that almost 39% of TNBCs have an HRD phenotype via germline/somatic mutations of *BRCA1/BRCA2, BRCA1* promoter hypermethylation, *RAD51C* hypermethylation, or biallelic loss of *PALB2* (99).

The studies by Riaz et al. (95) and Polak et al. (96) demonstrated that mutational signature 3 and other genomic features of HRD can be employed in the identification of tumors harboring *BRCA1/2* germline mutations as well as BRCAness. However, the majority of factors causing this phenotype remained unresolved. HRD, however, has been shown not only to affect the patterns and sizes of indels (resulting in signatures 3 and 8) but also to cause indels with microhomology, which is due to activation of the alternative error-prone end-joining mechanism for resolving double-strand breaks by aligning short homologous sequences (i.e., microhomology) to the broken ends (79, 100). The formation of TDPs has been mechanistically linked to this process, combining microhomology-mediated end joining with break-induced replication. Small TD formation was found to be a specific feature of *BRCA1*-inactivated TNBCs, developed in the context of the *TP53*-null genotype (100a), implying that *BRCA1* and *TP53* deficiencies, together, may lead to genomic instability and replicative drive, resulting in the formation of short TDs (43). The *BRCA1* gene is among the most significantly downregulated genes in TDP TNBCs in comparison with non-TDP TNBCs (100b). While the difference between the function of *BRCA1* and other HR-related genes is yet to be completely explored (100c), an intriguing study on primary mammalian cells showed that the loss of *BRCA1*, but not *BRCA2*, causes an aberrant replication restart mechanism at stalled forks, which contributes to TD formation (100d). Also, as described above, HRD phenotype signatures 3 and 8 could be linked to loss of only a few of the proteins implicated in HR, including BRCA1, RAD51C, PALB2, and BARD1, but not others such as ATM or CHEK2 (95, 96).

Error-prone repair of double-strand breaks in cancer cells has other structural genomic consequences (100), including CNAs and loss of heterozygosity (LOH), which can be detected and quantified using various measures, such as the fraction of LOH in the genome, telomeric allelic imbalance (TAI) (the number of allelic imbalance extending to the telomere), large-scale transitions (LSTs) (the number of chromosomal breaks occurring between regions of at least 10 Mb), or the number of intermediate-sized LOH events (exceeding 15 Mb but shorter than the length of a complete chromosome) (100).

Since identifying the cases with HRD affects therapeutic decision-making, multiple assays based on genomic alterations, described above, have been developed. While there are several HRD surrogates available, most capture only part of the phenotype. A combined score (i.e., HRD score) is defined as the arithmetic mean of genomic instability scores, including LSTs and TAI (100e, 100f). The HRD phenotype is defined when the HRD score is >42 (96, 100f). Whether the HRD score constitutes a predictive marker for response to

treatment remains to be determined. Clinical trials using different scores to predict HRD in the absence of germline *BRCA1* mutations have been conducted. In phase 3 of the Triple Negative Breast Cancer Trial (TNT), which compared carboplatin with docetaxel in unselected advanced TNBCs, patients with BRCA-mutated tumors achieved higher overall response rates after carboplatin treatment than after docetaxel treatment (68.0% versus 33.3%). No significant differences in the overall response rate of the patients having a high HRD score (detected byMyriad HRD assay) to carboplatin and docetaxel treatment were detected (101). Analyses of TNT outcomes using HRDetect and other signature profiles are under development. Multiple other trials, beyond the scope of this review, have been developed to assess the predictive value of these assays (see 102).

HRDetect, a promising HRD assessment method, is a composite approach that integrates the information from various previously described signatures reflective of HR dysfunction, including microhomology-associated small deletions; mutations signatures 3 and 8; RS-3 and RS-5; and an HRD index that is an arithmetic sum of LOH, TAI, and LST scores (44). HRDetect classifies TNBCs into three groups, namely HRDetect-high, HRDetect-intermediate, and HRDetect-low. The HRDetect-high TNBC subgroup comprises cases in which germline/somatic alterations in *BRCA1*/*2* and *PALB2* or epigenetic changes in *BRCA1* and *RAD51C* could (67%) and could not (33%) be detected. The tumors with high HRDetect scores are associated with better prognosis and sensitivity to standard adjuvant chemotherapy compared with the HRDetect-low subgroup, regardless of whether a genetic/epigenetic cause was identified (99). The HRDetect-intermediate subgroup showed enrichment of *CCNE1* amplifications, and the HRDetect-low subgroup was enriched for PIK3CA/AKT1 pathway abnormalities. The findings advocate for whole-genome sequencing of TNBCs as a single assay to improve therapeutic decision-making. Not only because whole-genome sequencing may identify many poor responders to current standard-of-care that cannot be detected by other methods; but also, because other limited sequencing modalities miss many HRD-high tumors that do not have genetic/epigenetic drivers but are predicted to have good outcomes.

Although genomics methods to detect HRD can identify the lack of HR during the development and progression of a cancer, currently they cannot determine whether HR would be defective at a given time in the evolution of a cancer. Hence, functional assays such as *RAD51* foci formation after therapeutic intervention (103) or by means of ex vivo ionizing radiation (104) have been tested and shown to correlate with response to chemotherapy and genomics features of HRD, respectively. This approach may help with identifying HRD-related cancers in a real-time manner; however, logistical challenges in the implementation of such assays remain a barrier for their testing in the context of prospective clinical trials.

Intratumor Genetic Heterogeneity and Clonal Evolution of TNBC

The spectrum of the mutational and clonal evolution of TNBCs is wide, both within the tumor (105, 106) and between the tumors from different patients (68, 106). Intratumor genetic heterogeneity has been well documented in breast cancers (107), and even driver genetic alterations, such as *HER2* gene amplification, can be heterogeneously present in

primary breast cancers at diagnosis. In most TNBCs, *TP53* and *PIK3CA* mutations have the highest clonal frequencies, implying their roles in early tumorigenesis (68). In a small subset of TNBCs, however, *TP53* and *PIK3CA* mutations can be subclonal (68), supporting the contention that in a subset of these cancers, these alterations may not constitute a founder driver event. Importantly, hundreds of subclonal mutations can be found in a TNBC tumor mass, highlighting how prevalent intratumor genetic heterogeneity is in triple-negative disease (107, 108). The heterogeneity of TNBCs has also been demonstrated by single-cell sequencing approaches (105, 108, 109). Although CNAs have been shown to be acquired in punctuated bursts of evolution, whereas mutations are acquired in a gradualistic manner, at diagnosis, TNBCs have now been shown to be constituted by multiple subclonal subpopulations both at the CNA and mutational levels.

TUMOR MICROENVIRONMENT AND TUMOR IMMUNE MICROENVIRONMENT IN TNBC

The tumor microenvironment (TME) is a combination of such multiple cell types, including fibroblasts, TILs, and lymphatic vascular channels. The active interaction between tumor cells and the microenvironment affects the pathogenesis and evolution of the tumor. Initial studies indicate that high levels of TILs (which are specifically found in the IM TNBC subtype) were associated with better prognosis and response to chemotherapy in both the neoadjuvant and adjuvant contexts (110, 111).

Despite the numerous molecular studies on TNBC, the only biomarker of outcome for patients with TNBC treated with the mainstay chemotherapy regimens supported by level IB evidence is the quantification of TILs (112). This includes histologic evaluation of the tumor and quantification of stromal TILs (sTILs) within the borders of the invasive tumor, as outlined in recommendations by an International TILs Working Group (111-113). Several ring studies were designed to enhance the standardization of sTIL assessment by Working Group pathologists and to tackle the challenges in intra- and interobserver reproducibility (113).

Quantification of sTILs on hematoxylin-and-eosin stained slides, however, is only the beginning of the efforts to understand the contribution of the TME as a biomarker for patients with TNBC. Pooled analysis of five large clinical trials of anthracycline chemotherapy in TNBC showed a 13% relative risk reduction for every 10% increment in sTILs (hazard ratio, 0.87; 95% CI, 0.83–0.91) (114). A recent retrospective study showed that sTILs can identify a subset of stage I TNBC patients (with >30% sTILs) in early stage disease who have an excellent prognosis [5-year overall survival rate of 98% (95% CI, 95% to 100%)] in the absence of chemotherapy, paving the way for trials on chemotherapy de-escalation in early TNBC (115).

The presence of TILs in a subset of TNBCs raised hopes for immunotherapies targeting the programmed death receptor 1 (PD-1) pathway, which is frequently co-opted by tumors to evade an immune response. In fact, a clinical trial [Phase II KEYNOTE-086 trial (116)] on pembrolizumab (a monoclonal IgG4-K antibody against PD-1) monotherapy in patients with metastatic TNBC showed that TIL levels were independent predictors of response (116).

The KEYNOTE-173 trial showed that the presence of sTILs is significantly associated with pathologic complete response to the therapeutic regimen, which included pembrolizumab added to NAC156 (117).

Despite the interest in TILs, the biomarker utilized for the approval of PD-1 immune checkpoint blockade in breast cancer is immunohistochemistry utilizing specific antibodies for programmed death-ligand 1 (PD-L1). The IMpassion130 trial evaluated the addition of atezolizumab (a monoclonal antibody that blocks the interaction of PD-L1 with PD-1) to nab-paclitaxel as a first-line treatment in patients with locally advanced or metastatic TNBC (11). Atezolizumab plus nab-paclitaxel prolonged progression-free survival among patients with metastatic TNBC in the PD-L1–positive subgroup (11). In this study, PD-L1 expression was defined as >1% of tumor area with expression of PD-L1 as defined by the SP-142 antibody. In the KEYNOTE-355 trial, the addition of pembrolizumab to chemotherapy (nab-paclitaxel, paclitaxel, or gemcitabine plus carboplatin) was compared with placebo plus chemotherapy. This study also confirmed a significant increase in progression-free survival in the subset of TNBCs displaying expression of PD-L1; however, the significant differences were observed in the population of TNBCs that displayed a combined positive score >10, as defined by the anti-PD-L1 antibody 22C3 (12). Although atezolizumab and pembrolizumab have now been approved for use in the context of PD-L1–positive metastatic TNBC patients, the methods for the identification of this patient population vary according to the drug, and the overlap between the populations identified by the SP-142 and 22C3 antibodies is far from complete (118-120). Further studies to define the optimal approach for the delivery of biomarkers for the use of immune checkpoint blockade in TNBC patients constitute an important unmet medical need at present.

The tumor immune microenvironment (TIME) is not composed of TILs alone; other immune cells also play pivotal roles. Tumor-infiltrating neutrophils and macrophages may be involved in TNBC pathogenies and response to treatment. Neutrophil-enriched subtypes were shown to be resistant to immune checkpoint blockade, due to the immunosuppressive effects of immature neutrophils (121). Evidence also suggests that their localization and distribution may vary according to the TNBC subtype. When the presence and differential localization of $CD8^+$ T cells is considered, TNBCs can be divided into different groups (65). The CD8-high group may be fully inflamed with involvement of both stroma and epithelial cells or have stroma-restricted $CD8^+$ T cell accumulation, excluding the epithelial compartment. The CD8-low group has very low numbers of $CD8^+$ T cells in the core of the tumor, namely an immune desert TIME, and if $CD8^+$ T cells do exist, they are margin restricted. Interestingly, TIME classification was associated with expression patterns described in TME. For example, SR tumors were mainly associated with a metabolism-related pathway activation pattern (73).

The TME role in the pathogenesis of TNBC is a multilayered process. The complexity of studying TME effects on TNBC pathogenesis is due not only to the multiple components of the microenvironment but also to the dynamic nature of it. Evidence suggests that different TNBC molecular subtypes show distinct TME patterns. These distinct TME patterns can be captured on the basis of which different biological processes are observed, including immune response (64, 73), vascularization (64), stroma compartment (122), and metabolic

process (64, 73). Bareche et al. (122) have shown that the LAR and M subtypes were of high stroma and metabolic expression levels. The abundant stromal signature places emphasis on the role of carcinoma-associated fibroblasts and stromal cells in pathogenesis and cancer progression in these specific subtypes. Carcinoma-associated fibroblasts are shown to promote cancer progression by providing a survival niche for cancer stem cells in the breast (122). The IM subtype was shown to be predominantly associated with an immune response signature; basal-like tumors were enriched with a metabolic signature, and MSL was mainly associated with high levels of lymph angiogenesis (64). Importantly, however, the cancer cell–intrinsic mechanisms, which can dictate the substantial heterogeneity within the TME, remain largely unresolved. Recent studies have shown a link between the genetic makeup of the cell (e.g., loss of *TP53* in cancer cells), intercellular signaling (e.g., cancer cell autonomous Wnt ligand secretion), and systemic neutrophilia that potentiates metastatic progression in breast cancer (123). Further studies are necessary to clarify the interplay between tumor cells of distinct types of TNBC and their microenvironment.

CELL OF ORIGIN

With the identification of the different cell lineages of breast epithelial cells, numerous models and potential cells of origin have been proposed for the different subtypes of breast cancer in general and even for distinct subtypes of TNBC. Historically, from a breast cancer molecular taxonomy perspective, it was postulated that each of the five molecular breast cancer subtypes (i.e., basal-like, HER2-enriched, normal breast-like, luminal A, and luminal B) would originate from different types of stem/progenitor cells within the different lineages of breast epithelial cells (Figure 3). According to this hypothesis, if a transforming event affected an ER-negative mammary stem cell, it would give rise to ER-negative basal-like or HER2-enriched breast cancer. Conversely, a transforming event affecting a more differentiated progenitor would give rise to an ER-positive luminal breast cancer (124, 125). Within luminal tumors, luminal A cancers were hypothesized as originating from more differentiated ER-positive lineage cells, whereas luminal B tumors would stem from more primitive ER-positive progenitors (124). These hypotheses were called into question by the seminal study by Lim et al. (126), which demonstrated that breast cancers developing in the context of a *BRCA1* germline mutation and basal-like breast cancers likely originate from ER-negative luminal progenitor cells rather than from basal cells.

Much has been learned in the last decade about the different cell types of breast epithelial cells and the origins of TNBC. Albeit TNBCs were originally perceived as stemming from basal stem cells (127, 128), given the transcriptomic similarities between the subset of basal-like TNBCs and the gene expression profiles of basal and myoepithelial cells of the breast, there are now multiple lines of evidence to support the contention that the likeliest cell of origin for the majority of TNBCs appears to be contained within the ER-negative luminal-progenitor cell compartment (126, 129, 130).

Conditional mouse model studies have now demonstrated that the targeted conditional deletion of *Brca1* in the luminal compartment (targeted by the use of Blg-Cre) or in the basal compartment (targeted by the use of Krt140Cre) in a *Trp53* heterozygous background mouse resulted in the development of TNBC of a basal-like subtype (129, 131). Importantly, these

studies have demonstrated that the conditional deletion of *Brca1* in the mammary luminal cell lineage of *Tp53*-null mice resulted in the development of basal-like tumors (129, 130). These findings not only demonstrate the role of luminal progenitor cells as the cell of origin but also point to the role of *BRCA1* in the differentiation of luminal cells and in the maintenance of the luminal cell fate (132, 133). Studies on transgenic mice with depleted *Brca2*, *Pten* and *Trp53* in either basal mammary epithelial cells or luminal ER-negative cells (134) revealed that basal cells gave rise to tumors of similar histologic traits regardless of the gene deleted, whereas luminal ER-negative cells gave rise to tumors of diverse phenotypic characteristics depending on the gene deleted, ranging from ER-positive to triple-negative and from luminal B to basal-like and claudin-low subtypes. Taken together, these findings suggest that basal-like breast cancers can originate from distinct cell lineages depending on the original genetic hit (e.g., *BRCA1* loss of function) and that multiple breast cancer subtypes, including both ER-positive and ER-negative cancers, may originate from a single epithelial cell type. On the basis of these observations, it is plausible not only that distinct cell types can constitute the substrate from which TNBCs develop but also that breast cancer phenotypes depend on the interplay between the cell of origin and the driver genetic alterations.

Single-cell RNA-sequencing analysis of TNBCs revealed enrichment of gene expression signatures related to basal, luminal progenitor, and mature luminal cells (135). The majority of malignant cells in studied primary TNBCs were basal-like and were associated with a luminal progenitor signature, supporting the luminal progenitor cell role as the cell of origin. Intriguingly, the LAR subtype was found to be more concordant with a mature luminal cell signature (135). While most malignant cells in this study showed prevalent luminal progenitor signatures, the tumors also contained malignant cells with more differentiated and mature luminal cell signatures (135). These findings suggest that TNBCs may be composed of distinct cell populations recapitulating different stages of epithelial cell differentiation. Further studies are warranted to investigate the prevalence of this phenomenon as well as to define its clinical and biological impact.

TUMOR EVOLUTION/PROGRESSION

The pathogenesis of breast cancer includes several pathways related to tumor evolution, starting from the precursors of the disease, evolving into the invasive tumor, and progressing into metastatic disease in other organs. Multiple models have been proposed for the initiation, transformation, and progression of breast cancer. Here, we focus on the proposed precursors of TNBCs and the characteristics and pathogenesis of metastatic TNBC.

Precursors

For ER-positive tumors, the linear model of breast cancer initiation proposed flat epithelial atypia, lobular neoplasia, atypical ductal hyperplasia, and ER-positive ductal carcinoma in situ (DCIS) as the nonobligate precursors. These preinvasive precursors and low-grade invasive lesions (invasive tubular, lobular, and low-grade IDC-NSTs) can subsequently progress to high-grade lesions (136). Considering all the histological and genetic evidence,

the term low-grade breast neoplasia family has been proposed to group all the low-grade ER-positive nonobligate precursors and low-grade invasive carcinomas (136).

In the context of TNBCs, high-grade ER-negative DCIS has been shown to be a precursor of invasive high-grade TNBCs (Figure 1). Approximately 7% of all DCIS lesions have a basal-like phenotype (137). The prevalence of *BRCA1* germline mutation among cases of DCIS is similar to that of invasive carcinoma (138). DCIS is a genetically advanced lesion, with marked intratumor genetic heterogeneity and with genetic alterations like those present in its synchronous IDC-NST (109, 139). Intralesion genetic heterogeneity has been documented in triple-negative DCIS (139); in this context, multiple clones within triple-negative DCIS have been shown to have the potential of becoming invasive cancer (139).

The less advanced precursors of TNBC have not been as well characterized to date. Genomic studies have provided circumstantial evidence to suggest that low-grade lesions such as microglandular adenosis (MGA) and atypical microglandular adenosis (AMGA) may constitute the substrate from which high-grade TNBCs develop. These lesions have been shown to harbor nearly identical patterns of CNAs and somatic genetic alterations to those observed in high-grade forms of TNBCs (140). Histological studies recognize the linear progression pattern in MGA, distinguishing the spectrum of the disease, including pure MGA without atypia, AMGA, and MGA associated with invasive carcinoma (141). The pattern of genomic alterations in TNBC, harboring recurrent mutations in *TP53* (~80%) and/or other common TNBC-related cancer genes, added another level of evidence to consider MGA as a nonobligate precursor of TNBCs, given that TNBCs synchronously diagnosed with MGA/AMGA were found to harbor identical *TP53* mutations and similar patterns of gene CNAs as those found in the associated MGA/AMGA (141). Despite the analysis of paired samples of MGA, AMGA, and invasive high-grade TNBCs, no somatic genetic alterations accounting for the distinct histologic features and behavior have been identified so far (15, 44, 45, 141). Interestingly, the so-called acinic cell carcinoma, a low-grade histologic type of TNBC that lacks the genomic features of its salivary gland counterpart, has been found to display similar histologic features as well as patterns of genomic alterations when compared with MGA and AMGA (44, 45). Importantly, these lesions have been shown to have a substantial proclivity to progress to high-grade triple-negative disease, given their frequent co-occurrence with high-grade TNBCs (44, 141). These observations have resulted in the development of the concept of a low-grade triple-negative breast neoplasia family, which can be perceived as a set of nonobligate precursors to high-grade TNBCs (44).

Salivary gland–like tumors of the breast, however, have simple genomes, lacking the CNA patterns associated with common forms of TNBCs and *TP53* somatic mutations. TNBCs arising in salivary gland–like tumors of the breast, including AdCCs or basaloid AdCCs, have been reported (142, 143); this phenomenon, however, appears to be remarkably rare, given that only a vanishingly rare subset of high-grade TNBCs harbors the genomic features present in breast salivary gland–like cancers (e.g., the *MYB-NFIB* fusion gene) (142).

Metastatic TNBC

Distinct TNBC subtypes appear to display different propensities in terms of their metastatic potential as well as metastatic sites. LAR TNBC is a significant predictor for lymph node metastasis; in a study, nearly half (47%) of the patients with this subtype were found to have regional spread to the lymph nodes (versus 32%, 33%, and 21% in the BL1, BL2, and M subtypes, respectively, with $p = 0.02$) (58).The metastatic site incidence may also differ, with the M and LAR subtypes showing a significantly higher frequency of lung and bone metastasis, respectively.

The frequency of TNBC subtypes appears to differ between the primary and metastatic settings. This is not surprising, given that the most prevalent subtype of TNBC (i.e., BLIA) is sensitive to chemotherapy, and a large contingent of patients with this subtype are currently cured by the current mainstay of neoadjuvant chemotherapy. This subtype, therefore, appears to be relatively decreased in the metastatic setting as compared with primary TNBCs (58).

The repertoire of somatic genetic alterations in metastatic TNBCs contains the drivers of the primary TNBC, including *TP53* mutations (144). The analysis of metastatic triple-negative disease, however, has revealed an enrichment for *PIK3CA*, *GATA3*, *CDH1, MAP3K1, PTEN*, and *PIK3R1* as compared with primary TNBCs (88, 145). These somatic genetic alterations have been shown to be more frequently found in ER-positive breast cancers as well as LAR cancers and, to a lesser extent, M TNBCs. These observations would be consistent with the notion that the LAR and M TNBC subtypes would be enriched in the metastatic setting, given their relative chemoresistance as compared with the BLIA subtype. In addition, conversion of ER-positive or HER2-positive breast cancers in the primary setting to triple-negative disease in the metastatic setting has been described (146); hence, another hypothesis for the differences in the repertoire of somatic mutations in primary versus metastatic TNBCs is that a subset of metastatic lesions represents the conversion of ER-positive to triple-negative disease.

Recent transcriptomic analyses have shown significantly decreased immune-activating gene expression signatures and TILs in metastatic TNBCs when compared with paired primary TNBCs (10, 147). This finding is consistent with the notion that metastatic TNBCs may display a more subdued immune response than primary disease and to some extent temper the enthusiasm for the use of immune checkpoint blockade in late stages of metastatic triple-negative disease. The pathogenesis of immune depletion in metastatic TNBC is not yet clear, and further studies are warranted to define the biological basis of this phenomenon.

CONCLUSION

TNBC is merely an operational term that stemmed from the fact that, in the mid-2000s, the only systemic therapy available for patients with ER-, PR-, and HER2-negative disease was chemotherapy. This term has been shown to encompass a collection of distinct diseases, which only happen to share the lack of ER, PR, and HER2 as a common denominator but are vastly different in terms of their histologic features, genomic characteristics, clinical behavior, and response to therapy.

The stratification of TNBCs into biological and/or clinical subgroups will be essential for the personalization of therapy for patients with triple-negative disease. The observation that a substantial proportion of TNBCs display genomic features of HRD will likely have an impact on the classification of these tumors from a therapeutic angle. Likewise, the development of antibody-drug conjugates targeting tumors with HER2-low expression may result in further stratification of TNBCs, given that a subset of TNBCs, although HER2 negative on the basis of the clinical definitions for HER2 overexpression/ gene amplification, still express HER2. In fact, approximately 35% of TNBCs were shown to be HER2 low (148), and approximately 13% of patients with HER2 immunohistochemistry 1+ and 10% of patients with HER2 immunohistochemistry 2+ without *HER2* gene amplification have been shown to have a triple-negative phenotype (148). Given that HER2 antibody-drug conjugates have been shown to have clinical activity in HER2-low breast cancers (149, 150), a subset of TNBCs may be reclassified as HER2-low disease in the not-so-distant future.

We would contend that embracing, rather than ignoring, the diversity and heterogeneity of TNBCs will be germane to the success of studies dissecting the pathogenesis and therapeutic dependencies of subsets of this collection of diseases. In the next decade, we foresee a scenario wherein the term TNBC either is no longer used or is solely employed to refer to a much smaller population of breast cancers for which the biological underpinnings and effective treatments have yet to be defined.

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