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Breast Cancer Theme Issue

The American Journal of
PATHOLOGY

ajp.amjpathol.org

REVIEW

Triple-Negative Breast Cancer

Next-Generation Sequencing for Target Identification

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Accepted for publication
May 23, 2017.

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Presently, the ability to study disease at the most fundamental molecular level has led to a reclassification of human cancers into numerous subtypes that vary in disease progression and response to therapy. Similar to most solid tumors, breast cancer is a heterogeneous disease with considerable variation in histologic and biological features. Triple-negative breast cancer (TNBC) is a subtype of breast cancer in which the estrogen receptor and progesterone receptor are not expressed, and human epidermal growth factor—receptor 2 is not amplified or overexpressed. Data derived from highly complex molecular technologies, such as microarrays and next-generation sequencing, have identified gene expression and somatic mutation profile subsets of TNBC that reflect biological behavior more accurately and may lead to further effective therapeutic targets, better prognosis, and improved outcomes. Herein, we review the genomic findings of TNBC and discuss current efforts in precision medicine as they relate to TNBC. (*Am J Pathol* 2017, 187: 2133–2138; <http://dx.doi.org/10.1016/j.ajpath.2017.05.018>)

Although breast cancer is no longer considered the most common human cancer in women in the United States, it is still responsible for significant morbidity and mortality.¹ Triple-negative breast cancer (TNBC) is a subtype of breast cancer that may account for up to 24% of all newly diagnosed cases.^{2,3} TNBC does not express estrogen receptor and progesterone receptor, and human epidermal growth factor receptor 2 is not amplified or overexpressed. These biological markers, along with the histologic characteristics of the tumor, tumor size, lymph node involvement, and the presence or absence of metastases (tumor node metastases staging) have been used to determine prognosis and therapeutic management strategies. Lack of expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 makes TNBC an orphan disease when considering standard therapeutic regimens for breast cancer.

Before the announcements of the presidential Precision Medicine Initiative and the MD Anderson Cancer Moonshots Program, investigators were already reclassifying

tumor cells based on their molecular characteristics. Today, highly complex clinical laboratory testing techniques such as microarray analysis and next-generation sequencing (NGS) are available to help classify many different human cancer types further and influence patient management. NGS has provided a way to identify the presence of complex genomic abnormalities simultaneously such as single-nucleotide variants, short insertions/deletions, copy number variations, and fusions in multiple genes and multiple patient samples.^{4–8} Routine detection of genomic alterations potentially allows for determination of tumors with the best chance of responding to traditional cytotoxic chemotherapy regimens, as well as more novel monoclonal antibody therapies, small molecular tyrosine kinase

Supported by the Department of Pathology and Laboratory Medicine, Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire.

Disclosures: None declared.

This article is part of a review series on next-generation breast cancer omics.

inhibitors, and immunotherapies. In addition, identification of genomic abnormalities can lead to drug development against new proteins involved in alternative signaling pathways.

TNBC Characteristics

TNBCs are seen more commonly in younger women (age, <50 years), particularly in those of African American descent, and often present as interval cancers. Radiologically, TNBCs are frequently identified as hyperdense masses without associated calcifications.⁹ The majority of TNBCs are histologically classified as high-grade, invasive, ductal carcinomas of no special type with basal-like features. Central necrosis, pushing tumor borders, a conspicuous lymphocytic infiltrate, and fibrosis are common histologic features.^{10,11} TNBCs with basal-like features lack expression of the biomarkers estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2, but commonly express high-molecular-weight basal cytokeratins (cytokeratin 5/6, cytokeratin 14, and cytokeratin 17), epidermal growth factor receptor (EGFR), vimentin, p-cadherin, α B-crystallin, fascin, and caveolins 1 and 2.¹² Overall, TNBCs show an aggressive phenotype with a shorter time to relapse and metastatic disease than the more common hormone-receptor–positive invasive ductal carcinomas.¹³ Triple-negative T1N0 patients have been shown to have a greater recurrence risk despite more aggressive therapy by both number treated and adjuvant chemotherapy type even in this node-negative category.¹³ However, it should be noted that not all TNBCs are basal-like. Rare histologic types with distinct morphology such as secretory carcinoma, adenoid cystic carcinoma, and adenosquamous carcinoma generally have a more favorable prognosis compared with basal-like TNBC.

TNBC Subclassification

It was not until Perou et al¹⁴ used gene expression patterns to classify breast cancer into four main intrinsic subtypes (luminal, human epidermal growth factor receptor 2–enriched, basal-like, and normal breast-like) that the true heterogeneity of breast cancer began to be elucidated further. Shortly thereafter, a follow-up study identified two additional subgroups within the luminal group, luminal A and luminal B.¹⁵ An additional molecular subtype, the claudin-low type, was identified in 2007 and was characterized by decreased expression of genes involved with tight junctions and cell adhesion.¹⁶

Similar molecular approaches have been used to better subclassify TNBCs. Lehmann et al¹⁷ originally identified six subtypes of TNBC: basal-like 1 and 2 (BL1 and BL2), immunomodulatory (IM), mesenchymal, mesenchymal stem-like (MSL), and luminal androgen receptor (LAR). The BL1 subtype included genes involved in the cell cycle

and DNA damage repair, whereas the BL2 subtype was defined by higher expression of growth factor pathway genes. The IM subtype involved genes responsible for immune cell processes. The mesenchymal and MSL subtypes expressed genes responsible for cell motility and cellular differentiation (epithelial–mesenchymal transition), and the LAR subtype was characterized by androgen-receptor signaling. Many BL1 and BL2 tumors are associated with *BRCA* mutations and can be classified within the intrinsic basal-like subtype described by Perou et al.¹⁴

After closely examining the contribution of stromal and immune cells to TNBC molecular subclassification by using a combination of histologic evaluation, laser-capture microdissection, RNA isolation, and gene expression analysis, Lehmann et al¹⁸ refined their original TNBC typing. They provided convincing evidence that infiltrating lymphocytes and associated tumor stromal (mesenchymal) cells were largely responsible for the gene expression profiles of the IM and MSL subtypes, respectively. Therefore, a revised four-subtype TNBC classification (BL1, BL2, mesenchymal, and LAR) was proposed, with the IM and MSL profiles to be considered as subtype descriptors.¹⁸

Burstein et al¹⁹ also subclassified TNBC into four subgroups: LAR, mesenchymal, basal-like immunosuppressed, and basal-like immune activated. Although not identical, some overlap exists between the Lehmann et al¹⁸ and Burstein et al¹⁹ subclassification groups. For example, similar transcriptional profiles and clinical outcomes were evident between MSL and mesenchymal, IM and BL1 with basal-like immune activated, mesenchymal with basal-like immunosuppressed, and the two LAR subtypes.²⁰

Analysis of the TNBC subtypes has shown differing clinical outcomes and varying responses to therapy, both in the neoadjuvant and postsurgical settings. These inherent differences related to TNBC subclassification have resulted in a renewed effort to identify driver mutations and more appropriate targeted treatment. Table 1 summarizes the molecular classification of TNBC and lists potential matched therapies.

Mutation Analysis

Rapid advances in NGS technology have made it possible to refine the analysis of TNBC subtypes at the genomic level with the aim of identifying novel targetable mutant proteins or pathways that could allow a tailored selection of therapy, or combination therapies, for optimal patient management and outcomes.

NGS has increased the identification of previously unrecognized genes that also may be associated with improved therapeutic response and development of resistance to therapies. This is an important technical attribute because TNBCs are known not to have a high frequency of driver mutations.²¹ Recent studies have found that

Table 1 Molecular Subclassification of Triple-Negative Breast Cancer

TNBC subtype ¹⁷	Genes expressed	Intrinsic subtype ^{14,16}	Relative overall survival	Potential therapies
BL1	Cell cycle DNA damage repair	Basal-like	++	PARP Platinum
BL2	Growth factor Myoepithelial	Basal-like	+	PARP Platinum
M	EMT Growth factor	Normal-like Claudin-low	+	Tyrosine kinase inhibitors PI3K/mTOR inhibitors
MSL	EMT Growth factor Proliferation (decreased)	Basal-like Claudin-low	++	Tyrosine kinase inhibitors PI3K/mTOR inhibitors
IM	Immune signal	Basal-like	+++	Anti-PD-L1 inhibitors
LAR	AR	Luminal HER2	+	AR-targeted PI3K inhibitors

+++ , Best survival; ++ , intermediate survival; + , worst survival.

AR, androgen receptor; BL1, basal-like 1; BL2, basal-like 2; EMT, epithelial-mesenchymal transition; HER2, human epidermal growth factor receptor 2; IM, immunomodulatory; LAR, luminal androgen receptor; M, mesenchymal; MSL, mesenchymal stem-like; mTOR, mechanistic target of rapamycin; PARP, poly-ADP ribose polymerase; PD-L1, programmed death-ligand 1; PI3K, phosphoinositide 3-kinase; TNBC, triple-negative breast cancer.

mutation profiles across breast cancer subtypes commonly include somatic mutations in the *TP53*, *PIK3CA*, and *GATA3* genes⁶; however, the frequency of mutations in these genes differs among the different breast cancer subtypes. For example, TNBCs had a higher prevalence of *TP53* mutations (80%) versus luminal A breast cancers (12%).⁶ In this same study, *PIK3CA* was the second most commonly mutated gene in TNBCs.

Weisman et al²² used the Integrated Mutation Profiling of Actionable Cancer Targets platform, an NGS assay targeting all coding regions of 229 common cancer-related genes, to examine tumor and normal tissue from a cohort of TNBC patients. This study identified an average of 3.33 nonsynonymous mutations per case. The most frequently mutated genes in this patient cohort were *TP53* (74%), *MLL2* (15%), and *PIK3CA* (10%).²² Mutations in the *KDM6A*, *JAK1*, and *PIK3R1* genes also were identified. The Integrated Mutation Profiling of Actionable Cancer Targets platform also detected copy number variations that included amplification of the *MYC*, *MCL1*, and *DDR2* genes, as well as deletion of the *CDKN2A/CDKN2B* gene. Among histologic subtypes of TNBC, this study showed that *PIK3CA* mutations were found predominantly in carcinomas that showed apocrine differentiation and expressed androgen receptor (presumably of the LAR molecular subtype).²²

Recently, additional genetic variants were identified in TNBC using an expanded 358-gene NGS assay on the Illumina HiSeq 2500 or MiSeq sequencers (Illumina, San Diego, CA).²³ The assay used in this study, JAX-CTP, detects actionable variants in the form of single-nucleotide variations, small insertions and deletions (≤ 50 bp), and copy number variations. In this study, which was enriched with tumors containing basal-like histologic features including a prominent stromal lymphocytic response (Figure 1A), 90% of TNBCs contained at least one somatic mutation detected (mean, 2.6 alterations per sample).

Amplification of the *MYC* oncogene was the most common abnormality, present in 75% of TNBCs tested. Amplification of six additional genes including *BCL2*, *CCDN1*, *EGFR*, *FGFR2*, *ID2*, and *NOTCH3*, was also identified. Mutations in the *TP53*, *AURKA*, and *KDR* genes were each present in 30% of cases and somatic mutations were also seen in the *PALB2*, *MET*, *PIK3CA*, *JAK3*, *FGFR*, *RET*, and *RB1* genes. One of two TNBCs with apocrine morphology in this cohort contained a *PIK3CA* mutation (Figure 1B), which is similar to the findings of Weisman et al,²² discussed earlier. Although the case number was small, this study identified a significant number of clinically actionable gene alterations in TNBCs that increases the potential for new therapeutic interventions and clinical trial eligibility.²³

Confirmation of these gene mutations and findings of other alterations in different TNBC cohorts have also been reported.^{24–27} Gewinner et al²⁴ described the loss of *INPP4B* and *PTPN12* in up to 56% and 23%, respectively, of TNBCs. Shaver et al²⁷ used segmental transcript analysis to identify novel gene rearrangements in a cohort of TNBC cases. They identified a novel rearrangement between the *MERTK* and *TMEM87B* genes, resulting in increased expression of the *MERTK* intracellular kinase. Similarly, they identified an *FFR3–TACC3* gene fusion that had been identified previously in glioblastoma. In a prospective study, Individualized Molecular Analysis Guide Efforts, Parsons et al²⁸ used the liquid biopsy approach to study TNBC mutation profiles. Tissue resection or biopsy, as compared with circulating cell-free DNA, has inherent limitations including sampling error associated with tumor cell heterogeneity, unreliable representation of tumor biological activity weeks or months after the procedure, and invasiveness and cost of procedures. Parsons et al²⁸ used a NGS assay for 236 cancer-related genes to test DNA isolated from tissue samples and an NGS assay for 27 genes to test cell-free DNA isolated from plasma. The NGS results for

Figure 1 Morphologic variants of triple-negative breast cancer (TNBC) with different genetic alterations. **A:** TNBC with basal-like histologic features containing a prominent stromal lymphocytic infiltrate; this tumor had *MYC* amplification. **B:** TNBC with apocrine differentiation and a *PI3KCA* mutation. The tumor cells have abundant eosinophilic cytoplasm, round nuclei, and prominent nucleoli. Scale bars: 100 μ m. Original magnification with hematoxylin and eosin: $\times 520$ (**A**); $\times 560$ (**B**).

patients who had adequate tissue and cell-free DNA were 70% concordant.

Therapeutic Intervention and Clinical Trials

Current management of TNBC often includes a combination of therapies including surgery, radiation, and chemotherapy. The most common chemotherapeutic regimen consists of anthracycline–taxane chemotherapy, either in the neoadjuvant or adjuvant setting.²⁹ TNBCs have shown a higher response rate to neoadjuvant therapy compared with estrogen receptor–positive tumors.³⁰ However, residual disease in TNBC patients is associated with a poorer prognosis and shorter recurrence-free survival when compared with other types of breast cancer with residual disease. The lack of identification of significant genomic driver alterations in TNBC, and the degree of tumor cell heterogeneity, has limited a targeted approach to management. It has been suggested that treatment with chemotherapy might select for clones of tumor cells harboring genomic alterations that subsequently can be targeted with select agents.^{3,19,30}

The ultimate goal of precision medicine based on genomic analysis is to identify molecular alterations that allow for initiation of established targeted therapies or inclusion into available clinical trials, thus improving patient outcomes. Antibody–drug conjugates, the combination of monoclonal antibodies and effector molecules, currently are being studied for more accurate drug delivery. One such therapy, glembatumumab vedotin, is a combination of an antibody against the gpNMB glycoprotein, which is overexpressed in TNBC, and the cytotoxin drug monomethyl auristatin.^{31,32} Sacituzumab govitecan is a second novel antibody–drug conjugate that combines the active metabolite of irinotecan, SN-38, with an anti–Trop-2 monoclonal antibody specific to a glycoprotein found in TNBC.³³

A significant percentage of TNBCs are known to harbor *BRCA1/2* mutations, in which tumor cells are deficient in homologous recombination DNA repair mechanisms. As noted earlier, many of the BL1 and BL2 TNBC subtypes are associated with *BRCA* mutations. Poly-ADP ribose polymerase catalyzes the syntheses of components needed for

alternative pathways of DNA repair and thus inhibitors of poly-ADP ribose polymerase could prove effective in these subtypes of TNBC. Poly-ADP ribose polymerase inhibitors are currently in clinical trials and include veliparib and olaparib as monotherapies, which when combined with cisplatin have shown significant improvement in overall response rates.³⁴

Basal-like breast cancers frequently show *EGFR* expression, and, in 2014, Park et al³⁵ reported that although *EGFR* mutation was a rare event, high *EGFR* copy number was found to be an independent prognostic factor for poor disease-free survival in patients with TNBC. This finding could be important for selecting patients for anti-*EGFR*-targeted therapy, particularly patients with the Lehmann BL2 subtype.

Recent transcription studies have shown that the IM subtype of TNBC with high levels of tumor-infiltrating CD8⁺ T cells may be associated with chemotherapy sensitivity and improved prognosis, whereas tumor-associated macrophages have been associated with a relatively poor outcome in TNBC.³⁶ Programmed cell death-ligand 1 (PD-L1) is an immune checkpoint regulator that is expressed more commonly in TNBC compared with non-TNBC. Expression of PD-L1 varies according to cellular site: tumor cell membrane, 64%; cytoplasm, 80%; and stromal, 93%.³⁷ Beckers et al³⁷ showed that although cytoplasmic and stromal PD-L1 expression was associated with improved prognosis, membranous expression of PD-L1 was not. A recent clinical trial investigating pembrolizumab, an anti-PD-L1 antibody, in metastatic TNBC found an overall survival rate of 18.5%, reinforcing the potential of immune checkpoint therapies in TNBC.³⁸

A number of clinical trials using androgen receptor (AR)-targeting therapy for the treatment of AR-positive TNBC currently are underway.³⁹ These include AR antagonists (bicalutamide and enzalutamide), biosynthesis inhibitors (abiraterone acetate), small molecular inhibitors of AR function (ganetespib), and AR agonists (4-OH-testosterone). There also is emerging evidence that a combination of phosphoinositide 3-kinase inhibitors and AR antagonists show greater efficacy than monotherapy in AR-positive TNBC.⁴⁰ If AR-targeting therapy is determined to be

effective, evaluation of AR expression likely will become routine practice. Furthermore, gene expression profiling to identify LAR TNBC might better select patients for such treatment.

Limitations of NGS Testing

As genomic information becomes increasingly integrated into clinical practice, there are several limitations to the use of NGS that warrant further mention. Choosing the most appropriate assay and tissue sample, as well as ensuring sufficient percentage of tumor cell content, all are critical pre-analytic variables that require special attention. Variation in results between NGS studies may be the result of tumor heterogeneity and difference in the number of genes evaluated, extent of coverage within those genes, types of mutations tested, and limits of detection between NGS assays.^{4,5} Finally, genetic alterations identified by NGS might not be clinically actionable, detection of mutations does not guarantee therapeutic response, and many targeted inhibitors have yet to be proven clinically effective when matched with their specific mutation.

Conclusion

In summary, TNBC represents a heterogeneous subtype of breast cancer. Genomic analysis using NGS technologies recently has begun to illuminate the potential use of novel targeted therapy in TNBC. Although a simple genomic solution for the treatment of TNBC may not be possible, future investigations of the epigenome, transcriptome, and proteome may identify additional targets and/or novel therapeutic approaches.^{41,42}

Acknowledgments

The data presented in this manuscript were generated in part through Clinical Genomics and Advanced Technology in the Department of Pathology and Laboratory Medicine of the Geisel School of Medicine at Dartmouth, and the Norris Cotton Cancer Center at Dartmouth-Hitchcock Medical Center. We thank the staff of the Laboratory for Clinical Genomics and Advanced Technology.

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