Decoding the heterogeneity of breast cancer with molecular biomarkers has shown to be an effective approach to understanding cancer and selecting personalized therapies for patients. However, despite the success of chemotherapy and endocrine therapies in specific subtypes of breast cancer there are still many patients that do not benefit from the standard of care and many patients that are overtreated when they have a low risk of disease recurrence.

The Breast Cancer 360 assay provides an approach for addressing this challenge which is aimed at providing an organized in-depth view of the biological information that underlies breast cancer that can be measured and integrated in one single test to further explore effective drug selection from a mechanistic point of view. NanoString has developed the Breast Cancer 360 panel, which includes a number of different RNA expression signatures describing the biology of breast cancer, to facilitate the decoding of this complex problem.

**Breast Cancer Subtyping**

**PAM50 Molecular Subtype Signature** – PAM50 - This 50-gene signature measures a gene expression profile that allows for the classification of breast cancer into four biologically distinct subtypes and identify genomic risk.

- This signature is trained and clinically validated to output Luminal A, Luminal B, HER2-Enriched, and Basal-like subtype calls.
- This signature is used to determine the genomic risk score.

**Claudin-Low Subtype Signature** – This molecular subtype is characterized by low levels of luminal differentiation markers, high enrichment for epithelial-to-mesenchymal transition markers, immune response and cancer stem cell-like genes.

**Triple Negative Breast Cancer Subtype Signature** – TNBC - This signature identifies four distinct TNBC subtypes: Luminal/AR subtype 1, characterized by AR, ER, prolactin and ErbB4 signaling; Mesenchymal subtype 2, characterized by cell cycle, mismatch repair, and DNA damage networks; Basal-like Immune-Suppressed subtype 3, characterized by downregulation of B, T and NK-cells immune-regulating and cytokine pathways; Basal-like Immune Activated subtype 4, characterized by upregulation of B, T and NK cells immune-regulating pathways, and activation of STAT.

**Breast Cancer Receptor Signaling**

**Estrogen Receptor Gene Expression** - ESR1 - This gene encodes an estrogen receptor, a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription. The associated ER protein is a key pathological marker of breast cancer.

**Progestosterone Receptor Gene Expression** - PGR - This gene encodes a member of the steroid receptor superfamily. The encoded protein mediates the physiological effects of progesterone, which plays a central role in reproductive events and the associated protein is a key pathological marker of breast cancer.

**HER2 Receptor Gene Expression** - ERBB2 - This gene encodes a member of the EGF receptor family of receptor tyrosine kinases. This protein has no ligand binding domain of its own and therefore cannot bind growth factors. However, it does bind tightly to other ligand-bound EGF receptor family members to form a heterodimer, stabilizing ligand binding and enhancing kinase-mediated activation of downstream signaling pathways. Amplification and overexpression are well established in breast cancer and the associated protein is a key pathological marker.

**Estrogen Receptor Signaling** - ER Signaling - Estrogen-binding systems associate with various proteins that direct cell cycle signaling, proliferation and survival. This signature captures ER-mediated signaling pathways to elucidate how ER modulates activity of key transcription factors through stabilizing DNA-protein complexes and recruiting co-activators. This signature also captures the impact to other signaling pathways induced by the binding of estrogens in the nuclear causing conformational changes in receptors.

**Tumor Responsiveness**

**Antigen processing machinery** – APM – This signature measures abundance of genes in the MHC Class I antigen presentation pathway and key genes involved in processing antigens prior to presentation.

- Typically, antigens from the cell cytoplasm are presented on Class I and recognized by the TCR on cytolytic CD8+ T cells. MHC Class I is expressed by all nucleated cells in the body, but downregulation of Class I MHC pathways is an evasion strategy that can be employed by tumor cells. An effective anti-tumor immune response depends on cytolytic T cells encountering neoantigens presented on the tumor cell surface.
- Strong anti-tumor immune responses are typically accompanied by high antigen presentation gene expression.
**Tumor Responsiveness (cont.)**

**HRD Signature** – HRD Signature - This signature is used to functionally assess Homologous Recombination Repair status, with potential to predict sensitivity to DNA-damage repair inhibitors such as PARP inhibitors.

- This signature captures cell cycle regulation, DNA damage, DNA replication, and DNA recombination and repair pathways.
- This signature can be used to predict overall survival in breast cancer.

**BRCA Signature** – BRCA Signature - This signature captures breast cancer biology representative that is informative as to defects in the DNA damage repair genes BRCA1 and BRCA2. Similar to our Homologous Recombination Deficiency signature this captures breakdown in BRCA-related DNA damage repair.

**p53 Signature** – p53 Signature - This signature categorizes p53 status by mutant-like vs wild-type-like and the signature is significantly associated with overall survival in breast cancer, identifying a group with high unmet need.

**Tumor Regulation**

**Apoptosis** – Apoptosis - This signature captures genes associated with apoptotic processes, specifically with genes involved in mitochondrial membrane integrity. It includes both pro- and anti-apoptotic genes.

**Tumor proliferation** – Proliferation – This signature measures genes involved in tumor proliferation.

- A highly proliferative tumor can overcome an immune response if replication exceeds immune mediated detection and elimination.

**Differentiation** – Differentiation - This signature assigns a score of differentiation to the sample. Well-differentiated tumors that is phenotypically more similar to normal cells or tissue will grow and spread at a slow rate compared with poorly differentiated tumors, these present with abnormal cells that often grow rapidly.

**FOXA1 Gene Expression** – FOXA1 – This transcription factor is involved in the regulation of gene expression in differentiated tissues. Sometimes associated with BRCA1 through cell cycle regulation. Also involved in ESR-1 mediated transcription and required for ESR1 binding to the NKX2-1 promoter in breast cancer.

**Inhibitory Tumor Mechanisms**

**IDO1 gene expression** – IDO1 – Indoleamine 2,3-dioxygenase 1 (IDO1) is expressed by tumor, immune, and stromal cells and is the rate limiting enzyme of tryptophan catabolism. By catalyzing the degradation of tryptophan, which is necessary for cytolytic T cell proliferation and activity, IDO1 inhibits anti-tumor immune responses.

**PD-L1 gene expression** – PD-L1 – Program cell death ligand-1 (PD-L1, CD274) is a ligand for PD-1 and negative regulator of T cell activity that is expressed on both tumor and immune cells.

**Stromal Factors**

**Endothelial cells** – This signature measures genes associated with vascular tissue and angiogenesis.

- Angiogenesis is important for nutrient trafficking to the tumor and proper oxygenation for tumor growth. Tumor angiogenesis forms leaky inefficient vessels that can reduce efficiency of lymphocyte trafficking to tumors.
- Changes in endothelial cell frequency can help identify drug mechanism of action.

**Stromal Tissue Abundance** – Stroma – This signature measures stromal components in the tumor microenvironment.

- The tumor stroma is the collection of non-cancerous and nonimmune tissue components surrounding the tumor. Stroma can act as a physical barrier that excludes immune cells from the tumor, preventing effective anti-tumor immunity even when tumor-associated antigens have induced immune cell priming and activation. These cells can also secrete important signals to the tumor, affecting tumor biology and response to the immune system.

**Inhibitory Metabolism**

**Hypoxia** – Hypoxia - This signature measured genes associated with reduced oxygenation in the tumor.

- Hypoxia can induce expression of many cancer promoting processes (e.g. invasion, motility, metabolic reprogramming) and can promote resistance to immune cell-mediated cytolysis and reduced cytolytic activity in NK and CD8+ T cells.
Anti-Tumor Immune Activity

Tumor Inflammation Signature – TIS – TIS measures the abundance of a peripherally suppressed adaptive immune response within the tumor.

• This signature trained to predict response to anti-PD1 therapy (pembrolizumab). It consists of genes related to Interferon gamma signaling, antigen presentation, natural killer and T cells and inhibitory pathways. It also consists of normalization genes that have been selected to give consistent expression levels across most tissue or tumor types.

• This signature is useful for predicting response to anti-PD1 therapy and determining hot and cold immune status across multiple cancer types.

Cytotoxicity – Cytotoxicity - This signature measures the molecules used by natural killer (NK) and CD8+ T cells to mount a cytolytic attack on tumor cells.

• Cytotoxic cells, both NK and CD8+ T cells, use a number of molecules, including perforin, granzymes and granulysin to penetrate and kill infection cells and tumors. Cytotoxic activity is the mechanism by which the immune system most effectively kills tumor cells.

Interferon gamma signaling – IFN gamma – This signature tracks the canonical response to IFN gamma, including the most universal components of that response.

• Interferon gamma is a critical component of a natural killer cell, CD8+ and TH1 CD4+ T cell-mediated adaptive anti-tumor immune response. IFNγ induces macrophage and NK cell activation, increased antigen presentation, and induces gene transcription patterns that can lead to immune cell recruitment to the tumor.

MHC class II antigen presentation – MHC2 – This signature measures the major human leukocyte antigens (HLA) involved in MHC Class II antigen presentation.

• Professional antigen presenting cells (dendritic cells, macrophages and B cells) use the class II MHC to present extracellular antigens to CD4+ T cells. Activation of CD4+ T cells induces expression of cytokines that can promote cytotoxic T cell activation and effective anti-tumor adaptive immune responses.

• Presence of MHC Class II molecules is associated with improved patient outcome.

Inhibitory Immune Signaling

Inflammatory chemokines -- Inflammatory chemokines recruit both myeloid and lymphoid populations to the tumor microenvironment.

PD-1 gene expression – PD1 – Program cell death receptor 1 (PD-1, PDCD1, CD279) is expressed predominantly on lymphocytes, it is upregulated upon activation and becomes a negative regulator of activation by preventing proliferation and cytokine secretion. PD-1 expression has been shown to be associated with tumor-specific T cells.

TIGIT gene expression – TIGIT – T cell immunoreceptor and Ig and ITIM domains (TIGIT) is an immune checkpoint molecule that suppresses anti-tumor immune activity in CDB+ T cells.

Immune Cell Population Abundance

Cytotoxic cell abundance – Cytotoxic cells – This signature measures the abundance of cytotoxic cells in the tumor microenvironment.

• Cytotoxic cells, both NK and CD8+ T cells, use a number of molecules, including perforin, granzymes and killer cell lectinlike receptor (KLRG) family members to recognize, penetrate and kill infection cells and tumors. Cytotoxic activity is the mechanism by which the immune system most effectively kills tumor cells.

CD8+ T cell abundance – CD8 T cells – This signature measures the abundance of CD8+ T cells in the tumor microenvironment.

Macrophage abundance – Macrophages – This signature measures the abundance of macrophages in the tumor microenvironment.

• Macrophages can either augment tumor immunity (e.g. by presenting antigen) or suppress tumor immunity (e.g. by releasing immunosuppressive cytokines).

Mast cell abundance – Mast cells – This signature measures the abundance of mast cells in the tumor microenvironment.

Treg abundance – Treg – Regulatory T cell (Treg) abundance is measured by gene expression of Forkhead box P3 (FOXP3). FOXP3 is the canonical transcription factor that defines the regulatory T cell (Treg) population and is used to measure Treg abundance. Regulatory T cells suppress other T cell activities through a variety of mechanisms.

For more information, or to receive a copy of the gene list please visit nanostring.com/BC360