INSIGHT TNBCtype™

DESCRIPTION

Insight TNBCtype is a gene expression profiling test intended for analyzing tumor biopsies after diagnosis of triple-negative breast cancer (TNBC: ER, PR, and HER2 negative) in order to assign a subtype within this population. *Insight TNBCtype* processes gene expression data to classify TNBC into five molecularly-distinct subtypes:

- Basal-like 1 (BL1)
- Basal-like 2 (BL2)
- Luminal Androgen Receptor (LAR)
- Mesenchymal (M)
- Mesenchymal Stem-like (MSL)

Additionally, *Insight TNBCtype* provides information on whether the tumor exhibits an immunomodulatory environment (IM-positive or IM-negative).

SPECIFICATIONS

Turnaround Time	10-20 Business Days
Results Output	Assignment of *Insight TNBCtype* subtype and IM status
Specimen	Formalin-fixed, paraffin-embedded (FFPE) blocks/slides • Minimum of three, 5-micron sections ○ Storage: Ambient (4˚C preferred) • Sections should be freshly prepared or not more than 3 months old • Tissue content should contain greater than 30% tumor • Pathology report confirming TNBC status
Causes for Rejection	RNA quality may be compromised due to nucleic acid fragmentation, and/or modification by chemical reactions between formaldehyde and nucleic acids which include crosslinking with proteins or other biomolecules. Specimens with insufficient RNA quantity as measured by fluorescence or low quality as measured by insufficient library amplification or sequencing depth will be deemed as Quantity/Quality Not Sufficient (QNS) and noted as such.
Limit of Detection	Each specimen requires 2.08 million reads passing filter (validated as the minimum number of reads acceptable to provide a reproducible subtype call with 95% confidence in FFPE TNBC tumor tissue)
Method	Next-Generation Sequencing of RNA (RNAseq) from FFPE tumor tissue
How to Order	Download and complete the Insight Molecular Labs requisition form at www.insightmdx.com. Fax completed form to (615) 255-1330. For any questions, please call client services at (615) 255-8880.

Note: Testing performed at I.G. Laboratories acting as Insight Molecular Labs

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SUBTYPE DETAILS

Subtype: BL1
BL1 subtypes have higher expression of cell cycle and DNA damage response genes, and representative cell lines preferentially responded to cisplatin.
BL1: Preclinical Evidence
Cell lines representative of the BL1 subtype have been shown to have high expression of proliferation and DNA damage response genes and preferentially respond to platinum compounds and PARP inhibitors (Lehmann, 2011). Wathieu *et al*. also demonstrated in cell growth assays that a BL1 cell line was responsive to the proteasome inhibitor Bortezomib and susceptible to the pan-Aurora kinase inhibitor, AMG900 (Wathieu, 2017).
BL1: Clinical Evidence
Lehmann et al. retrospectively evaluated chemotherapy response of over 300 TNBC patients from pretreatment biopsies subtyped using either the intrinsic (PAM50) or the original TNBCtype approaches (Lehmann BD, 2016). Combined analysis of TNBC patients demonstrated that TNBC subtypes significantly differ in response to similar neoadjuvant chemotherapy with 41% of BL1 patients achieving a pathological complete response compared to 18% for BL2 and 29% for LAR with 95% confidence intervals. In addition, subtyping analysis of 130 patients who received neoadjuvant taxane and anthracycline-based therapy revealed the BL1 subtype to have the highest pCR rate (52%) and BL2 to have the lowest pCR rate (0%) (Masuda, 2013). Clinical responses to both neoadjuvant treatment arms, found BL2 to be significantly associated with poor response (Odds Ratio (OR) =0.12, p =0.03 for the 2188-gene model; OR = 0.23, p < 0.03 for the 101-gene model). Additionally, while the BL1 subtype trended towards significance in the 2188-gene model (OR = 1.91, p = 0.14), the 101-gene model demonstrated significant association with improved response in patients with the BL1 subtype (OR = 3.59, p = 0.02) (Ring, 2016). It also appears that BL1 patients may benefit from standard chemotherapy while BL2 patients may not benefit while still experiencing harmful side effects. Lastly, Severson et al. found the BL1 and Mesenchymal categories are most associated with a BRCA1-like status and could therefore respond to PARP inhibitors (Severson, 2015).
Subtype: BL2
Similar to BL1, BL2 subtypes have higher expression of cell cycle and DNA damage response genes.
BL2: Preclinical Evidence
Wathieu et al. 2017 demonstrated in cell growth assays that a BL2 cell line was susceptible to the pan-Aurora kinase inhibitor, AMG900.
BL2: Clinical Evidence
It appears that BL1 patients may benefit from standard chemotherapy while BL2 patients may not benefit while still experiencing harmful side effects. In addition, Severson et al., 2015 found the basal-like subtypes and Mesenchymal categories are most associated with a BRCA1-like status and could therefore respond to PARP inhibitors.
Subtype: LAR
The LAR subtype is enriched for hormonally regulated pathways and is dependent on androgen receptor (AR) signaling. Although AR can be expressed in multiple molecular subtypes of TNBC, the LAR subtype has the highest level of AR expression. The LAR subtype is predominantly subclassified in the non-basal subgroup and represents a novel subtype of TNBC with a distinct prognosis that offers an opportunity to develop targeted therapeutics.
LAR: Preclinical Evidence
Cell lines of the LAR subtype, the growth of which is driven by androgen receptor signaling, are uniquely sensitive to AR antagonists. The Pietenpol group discovered that LAR tumors and cell lines also have a high frequency (~40%) of

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PIK3CA mutations that confer responsiveness to PI3K inhibitors (Lehmann B. D., 2014) and may benefit from PI3K inhibitor treatment. Similar to BL1 cell lines, a LAR cell line also appeared to be susceptible to the proteasome inhibitor bortezomib.

LAR: Clinical Evidence

Apocrine differentiation was associated with some but not all of the LAR subtype of TNBC with a better prognosis even with residual tumor after neoadjuvant chemotherapy. The LAR subtype alone did not predict DFS; however, LAR tumors with apocrine differentiation had a better prognosis than did LAR tumors without apocrine differentiation. Using a combination of morphologic and genomic testing may be helpful in determining the prognosis of patients with apocrine-positive TNBC tumors who have residual disease after NST. (Masuda et al., 2018 – SABC). Further, LAR tumors appear to respond poorly to conventional chemotherapy. We have reported a pCR rate of only 10% following sequential taxane and anthracycline neoadjuvant therapy (Masuda, 2013). Thus, LAR TNBCs need to be distinguished and different treatment approaches used specifically for them. The androgen receptor (AR) is a potent mitogenic driver of the LAR TNBC subtype (Fioretti, 2014). Data from the Pietenpol group indicate that LAR TNBC cell lines and xenografts are sensitive to AR antagonists (Lehmann B. D., 2011). Those findings suggested that simultaneous targeting of AR and the PI3K/mTOR pathway may be of clinical benefit for LAR TNBC patients, as this combination has been shown to be synergistic in AR-dependent prostate cancer cells. Collectively, these and other corroborating data have prompted several clinical trials seeking to confirm the efficacy of AR antagonist therapy in LAR TNBC.

Subtype: M

It has been shown that the presence of the immune-suppressive T regulatory cells (Tregs) in the tumor microenvironment play a role in the escape of immune control. A meta-analysis using the ratio CD8+ cells to FoxP3+ cells, as surrogates to the ratio of activated T-cells to Tregs, gave a more impressive hazard ratio (HR) for OS than CD8+ cells alone (Mao, 2016). In relation, our collective data shows that the M subtype - which is so named because of high expression of genes associated with the epithelial to mesenchymal transition (EMT) - appears in only 9% of patients who have a single subtype versus 80% of patients who have a dual subtype *Insight TNBCtype* can identify multiple subtypes over a defined threshold and may imply the latter is identifying tumors undergoing EMT. Furthermore, the inverse relationship previously seen between the M subtype and positive IM status when using the 2188-gene algorithm is observed when using the 101-gene algorithm. This holds true even when M is one of dual subtypes and the other subtype has a higher correlation coefficient (Grigoriadis, et al., 2016; Harano, 2018).

M: Clinical Evidence

In addition to the genes associated with EMT, the M gene expression signature is enriched for genes associated with the extracellular matrix (ECM) and the TGF-ß signaling pathway (Lehmann BD, 2016). Given that the secretion of TGF-ß is an anti-inflammatory mediator that inhibit dendritic cells and T-cells (Kim, 2007), the EMT may represent an additional immune escape mechanism whereby tumor-infiltrating lymphocytes (TILs) lose their aggression toward the tumor independent of PD-L1 inhibition. This supposition has at least one observation to support it: a group of melanoma patients resistant to PD-L1 inhibitors were characterized by genes of which a subset associate with EMT and ECM, both of which are characterized by the TNBC M signature (Meng, 2015). Interestingly, this study noted that the genes that distinguish the basal subtype - collectively BL1 and BL2 in the two algorithms - from mesenchymal tissue in breast cancer are down regulated in the resistant patients. Lastly, as stated above, Severson et al., 2015 found the BL1 and Mesenchymal categories are most associated with a BRCA1-like status and could therefore respond to PARP inhibitors (Severson, 2015).

Subtype: MSL

MSL subtypes appear to correlate with cellular heterogeneity and has been shown to result from the presence of tumor-associated stromal cells. Consequently, the MSL subtype may be a reflection of surrounding stromal tissue and

not a bona fide subtype (Lehmann BD, 2016).

Subtype Modifier: IM

While the obvious biomarker for immune checkpoint inhibitor would be an antibody that detects PD-L1, this biomarker has, at times, failed to provide a desirable objective response rate with the various agents (Sica GL, 2017). Therefore, various alternatives, including DNA mutations and gene expression signatures, have been evaluated as alternatives (Meng, 2015; Hugo W, 2016). *Insight TNBCtype* was used as an investigative diagnostic in a recently published case report that described a patient which tested **negative for PD-L1 by IHC, but** as **IM-positive** by *Insight TNBCtype*. The patient had received exhaustive chemotherapy and therefore had few other treatment options, so partially based on the positive IM result, the patient was approved for pembrolizumab treatment and experienced a complete radiologic response after 4 cycles of pembrolizumab (Bhatti, 2017).

Follow-up Testing

Recently, 89 TNBC patients who received neoadjuvant chemotherapy (NAC) and did not have a pathological complete response (pCR) were analyzed by the *Insight TNBCtype*. Matched pairs of pre-NAC and post-NAC FFPE tumor samples were collected. It was observed that 41 (51%) had a change in molecular subtype after NAC. The most frequent subtype change was from the BL1 to the M subtype (32%), followed by from LAR to BL2 (27%) and from BL2 to LAR (25%). Patients who had subtype changes had better OS than those who did not have subtype changes (P=0.0195; 2-year OS, 84% and 60%, respectively). Multivariate analysis showed that subtype change was associated with improved OS (hazard ratio 0.425; P=0.0189). Ultimately it appears that TNBC subtypes frequently change after NAC without pCR, and the change is associated with good prognosis. If tumor of a predominant subtype is effectively damaged by NAC, the remaining tumor may show a different subtype. It may therefore benefit the patient to have follow-up monitoring of the tumor (Harano, 2018).

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Note: Testing performed at I.G. Laboratories acting as Insight Molecular Labs