Multiplexed Cancer Progression Analysis

Using the nCounter® PanCancer Progression Panel

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Introduction

Ninety percent of cancer related deaths are due to metastasis[1]. Even after a primary tumor has been excised, metastatic colonies can remain within a patient for years without reactivation, potentially leading to distant recurrence[2]. While the collective understanding of cancer progression grows, answers to key questions - such as why certain patients are prone to metastasis, why metastatic lesions predominantly occur in particular organs, and why only certain cells successfully transition into a secondary tumor - remain elusive[3]. This report introduces a universal gene expression panel that can be used to investigate both the progression of a primary tumor as it expands as well as the factors influencing the establishment and development of tumors in secondary locations.

Cancer progression can be separated into two phases: the growth and dissemination of the primary tumor and the metastatic migration and colonization of neoplastic cells into new regions (**FIGURE 1**). All tumors actively modify their microenvironment in order to sustain growth by recruiting vasculature, remodeling the extracellular matrix (ECM), and altering their metabolism[4]. As tumors grow, cells on the periphery work to recruit additional oxygen and nutrient sources, activating angiogenic factors including the VEGFA signaling pathway[5,6]. Within the center of developing tumors, cells begin to experience hypoxic conditions, leading to the activation of the HIF1A signaling pathway[7,8] and a shift towards glycolytic metabolism[9,10]. Evaluating changes in these processes is crucial to understanding the natural progression of primary tumors as well as a cancer's response to the immune system and therapeutic regimens.

As cancer progresses in the primary tumor, select cells transition between epithelial and mesenchymal morphologies, a process known as the epithelial-mesenchymal transition (EMT). During this transition, cancer cells gain mesenchymal qualities, such as losing polarity and intercellular adhesion, that enable migration to distant regions[11]. In favorable conditions, clusters of cancer cells invade previously healthy tissue and form small nodules[12]. The cells within these nodules use the same processes as the primary tumor to expand, such as ECM remodeling and angiogenesis[13]. If these disseminating cells are successful in colonization, a secondary tumor will appear that functions separately from the primary tumor. Research into the understanding and treatment of cancer must not only focus on the primary tumor, but also the processes that support the proliferation, dissemination, and metastasis of tumor cells.

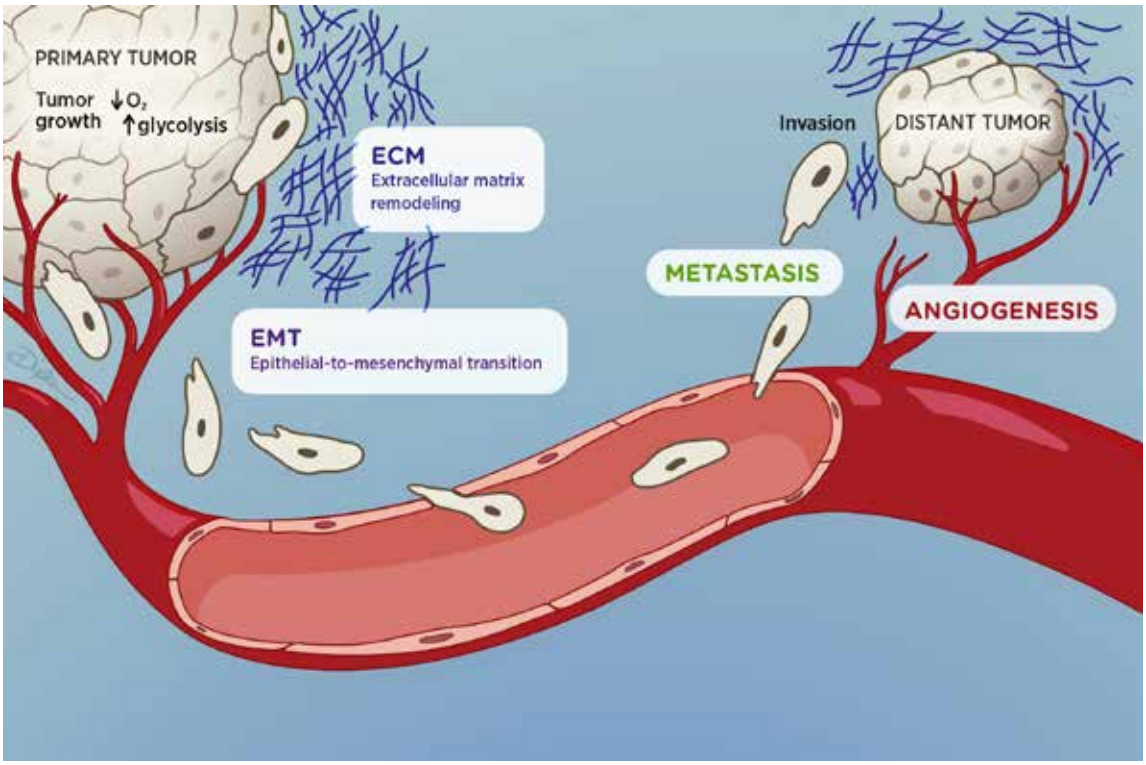

FIGURE 1 Biological overview of cancer progression. The processes of the primary tumor that lead to changes in microenvironment, tumor growth, and cell dissemination are shown, such as hypoxic conditions, metabolism changes, and ECM remodeling. After tumor cells undergo EMT and invade a new site, additional metastatic hurdles need to be overcome, including enabling angiogenesis and avoiding tumor dormancy.

Features in PanCancer Progression

The nCounter PanCancer Progression Panel covers each of the major aspects of tumor progression (**FIGURE 2**). The panel contains a broad collection of genes associated with critical aspects of cancer progression and the necessary controls to enable:

- Measurement of angiogenesis and response to inhibitors.
- Assessment of extracellular matrix components and remodeling mechanisms.
- Detection of key genes that mark the epithelial to mesenchymal transition.
- Evaluation of metastatic growth and suppression genes throughout tumor progression.
- Robust sample-to-sample normalization via uniformly expressed housekeeping genes.

The nCounter PanCancer Progression Panel is designed to measure the expression of 770 genes with a variety of sample types, including fresh-frozen (FF) tissue, formalin-fixed paraffin-embedded (FFPE) tumor sections, whole blood, PBMCs, and cell lysates. The panel may be used in conjunction with nCounter Panel-Plus products, which allow for the addition of up to 30 user-defined genes to customize the panel and add flexibility to experimental design.

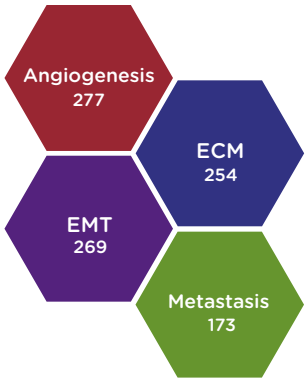

FIGURE 2 Distribution of genes in the PanCancer Progression panel, comprising of angiogenesis, extracellular matrix, epithelial-mesenchymal transition, and metastasis.

As cancer progression involves multiple processes, the PanCancer Progression panel enables researchers to target the essential genes in each of the aspects of tumor progression. Each of those themes is detailed below:

Angiogenesis

Angiogenesis is the biological process of generating new blood vessels from pre-existing vasculature. In healthy patients, angiogenesis is only activated in wound healing, organ regeneration, and the female reproductive cycle[14]. However in the disease state, angiogenesis is induced quite early in cancer development, a process often referred to as turning on the "angiogenic switch"[15]. The **277** angiogenesis genes in this panel provide coverage of numerous processes, including the primary angiogenesis response, sprouting angiogenesis, VEGF signaling, and coagulation. Angiogenesis is of particular interest in cancer progression considering that distant tumors cannot grow more than 2-3 cubic millimeters without vasculature[16]. Numerous therapies have been developed to starve the tumor cells by targeting circulating angiogenic factors, such as Bevacizumab (Avastin) and Aflibercept (Zaltrap)[17].

ECM

The extracellular matrix (ECM) is an assembly of proteins and sugars that surrounds cells in solid tissues, primarily providing mechanical structure. Each of the different layers of the ECM is represented in this panel as well as structural components including both fibular and non-fibular collagens, integrins and glycoproteins. The ECM must be remodeled in order to accommodate tumor growth. Tumor cells rely on the MMP and LOX gene families to change the surrounding ECM environment[18]. Lysyl oxidase (LOX) modifies the structural proteins collagen and elastin, and acts as a catalyst during fiber crosslinking[19,20]. If the LOX family becomes hyperactive, collagen accumulation occurs leading to ECM stiffening and tissue fibrosis, one of the biggest intrinsic drivers of metastasis[21]. The matrix metalloproteinase (MMP) family plays a variety of roles within the tumor microenvironment[22], including degradation of the ECM structure[23]. Several of these remodeling genes have further roles in progression, acting as transcription factors in metastatic growth[24]. ECM structure and remodeling processes are covered by **254** genes in this panel.

EMT

The epithelial-mesenchymal transition (EMT) is a process that cancer cells undergo which promotes metastatic progression. Epithelial cells are defined by their ability to laterally tether to each other in sheets using intercellular junctions[25], whereas mesenchymal cells are more elongated for motility and rely on focal adhesions for attachment[26]. The epithelial-mesenchymal transition (EMT) is known to be a dynamic spectrum between these two states that can be reversible depending upon intra- and extra-cellular factors[27]. There are 269 panel EMT genes, including several genes described by Tan et al.[27] that have been extensively validated in a pan-cancer setting. Traditional cell migration and adhesion signaling pathways are incorporated as well. Many of EMT genes have further roles in cancer progression, functioning as transcription factors in ECM remodeling[28].

Metastasis

Metastasis is a collection of cellular processes encompassing cell migration from the primary tumor to the successful development of a distant tumor. While all of the general processes noted above occur in secondary tumors, several of the processes that are not included in the major three themes have been grouped into the term 'metastasis'. This term includes common cell growth signaling pathways, hypoxia response, and metabolic changes. Additionally, there are a set of metastasis suppressor genes that each potential new tumor site must avoid[29]. While researchers have identified these pivotal genes, their exact biological mechanisms for suppressing metastasis remain unclear[30]. The **173** metastasis related genes fall into cell growth, hypoxia, metabolism, and metastasis suppressor genes.

Normalization

Housekeeping genes are included in the previous PanCancer panels to aid with data normalization. Using data from The Cancer Genome Atlas (TCGA) data (http://cancergenome.nih.gov/), genes with low variance across multiple cancer types were selected. The **30** genes included in this panel cover a wide range of expression values.

Angiogenesis Response
Blood Coagulation
Negative Regulation of Angiogenesis
Regulation of Angiogenesis
Sprouting Angiogenesis
Positive Regulation of Angiogenesis
Vasculogenesis
VEGFA Signaling

Basal Lamina
Basement Membrane
Collagen Family
ECM Structure
Fibrosis
Integral to Membrane
Plasma Membrane
ECM Receptor Interaction
LOX Remodeling
MMP Remodeling

EMT
Cell Differentiation
Cell Motility
Cell Adhesion
EMT to Metastasis
TGFB Signaling

Metastasis Response
Metastasis Suppressors
HIF1A Signaling
Hypoxia Response
Regulation of Metabolism
Carbon Cancer Metabolism
Cell Proliferation
Cell Growth Factors
Choline Cancer Metabolism
Cell Cycle

Fundamental Cancer Progression Research Inquiries

The nCounter PanCancer Progression panel enables many types of research and helps address critical questions surrounding cancer progression listed below:

- Why do some cancer cells metastasize while others do not?
- How can metastasis be prevented?
- Why do distant tumors predominantly occur in particular organs depending on cancer type?
- What is the biological mechanism behind the tumor suppressor genes in metastasis?
- How does modulation of the immune system impact metastasis?
- Why are some patients more susceptible to metastasis than others?
- What are the determining factors for successful metastasis?
- How do different therapies affect the progression of cancer?

Identification of PanCancer Progression Genes

After delineating the primary aspects of cancer progression i.e. angiogenesis, ECM, EMT, and metastasis, a variety of sources were utilized to create comprehensive coverage of these different molecular processes. Each of the main topics can be partitioned into several smaller categories; for example, angiogenesis can be further divided into general angiogenesis response, regulation of angiogenesis, etc. Content in these categories were generated using publicly available data sources (KEGG www.genome.jp/kegg, Reactome www.reactome.org, GO www.geneontology.org)[31–33] as well as a thorough review of the current scientific literature.

Genes within each of the four major themes were selected using a variety of statistical indicators from The Cancer Genome Atlas (TCGA) data (www.cancergenome.nih.gov). Candidate genes first were screened for biologically interesting behavior. All selected genes were statistically significant for: change in survival, consistently differentially expressed between tumors and matched normal tissue, and/or displayed bimodality within multiple cancer types. Additional genes for each category were selected for their ability to capture maximal expression variability across different annotated TCGA cancer types. After this data-driven approach, the resulting genes were verified using an in-depth review from the cancer progression research community.

The PanCancer Progression panel gene list encompasses over 30 major biological processes and pathways (**FIGURE 3**). The complete list of genes in the nCounter PanCancer Progression Panel and their biological annotations is available at **www.nanostring.com/progression**. The annotated list contains additional information such as the HUGO Gene Nomenclature Committee (www.genenames.org) name, RefSeq accession, commonly used aliases, and probe target sequence.

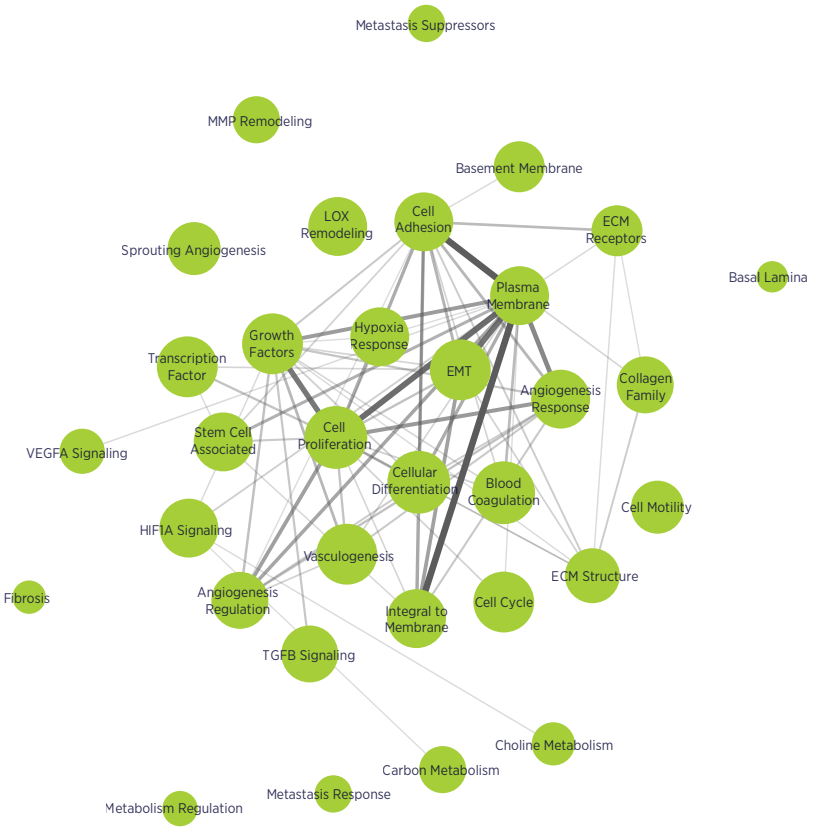

FIGURE 3 Network map of biological process and pathway annotations for the content genes in the PanCancer Progression panel. Each biological process is represented by a node with lines between nodes to denote shared classifications for a gene. The thickness of the line is representative of the total number of genes with a shared classification.

Pathway-Based Analyses of Tumor Data

To demonstrate the capabilities of the PanCancer Progression panel, a subset of publicly available gene expression information was analyzed from 1059 breast samples available at TCGA. Breast cancer is known to include four subtypes: Luminal A, Luminal B, HER2-enriched, and Basal-like[34]. The PAM50 algorithm[35] was used to estimate the "intrinsic subtype" of every sample in the dataset. Additionally, differential expression of the panel genes was examined with respect to overall survival, tumor state from American Joint Committee on Cancer (AJCC), and AJCC nodal status[36]. Tumor state was compressed to a single continuous variable, and patient node status was dichotomized as either positive or negative. These types of principled analyses can be performed in nSolver 2.5 with more detailed methodologies demonstrated in the optional analysis module[37].

Linear regression was used to estimate each gene's differential expression associated with PAM50 subtype, stage, and nodal status. Additionally, Cox regression was used to predict survival from gene expression and these same clinical variables. Between these two models, an estimate for each gene's association with each clinical variable of interest was calculated; associations were measured as log fold-change for subtype, stage, and nodal status and as log hazard ratios for survival. Subtype differences showed the strongest association with differential gene expression. 93% of the panel genes had differential expression with subtype detected with FDR < 0.05. Nodal status was the next most prominent variable with 22% of genes differentially expressed with FDR <0.05. Also with FDR < 0.05, 8.5% of panel genes were associated with stage and 0.4% of genes were associated with survival.

Directed global significance scores were calculated for each biological annotation associated with the cancer progression themes (**FIGURE 4**). High (low) directed global significance statistics indicate a tendency for a set of genes to be over (under) expressed with a variable. For example, upregulation of genes in the regulation of metabolism category is associated with both poor survival and the absence of nodes. These findings highlight the relevance of altered metabolism in primary tumor progression irrespective of metastasis and strengthen the need for targeted inhibitors of receptor tyrosine kinases and mTOR[38]. Moreover, expression of genes in the hypoxia response pathway is associated with poor survival, low stage, and absence of lymph node infiltrates. These results indicate that these genes may be an early marker of both primary tumors and metastatic colonies as well as resistance to treatment[39]. Overexpression of the vast majority of the processes examined by the panel are associated with positive nodal status (**FIGURE 5**), consistent with employing positive node status as an indicator of metastatic lesions[40]. These analyses illustrate the ability of the PanCancer Progression panel to elucidate biologically relevant findings related to cancer progression as well as provide a framework for interpretation of results.

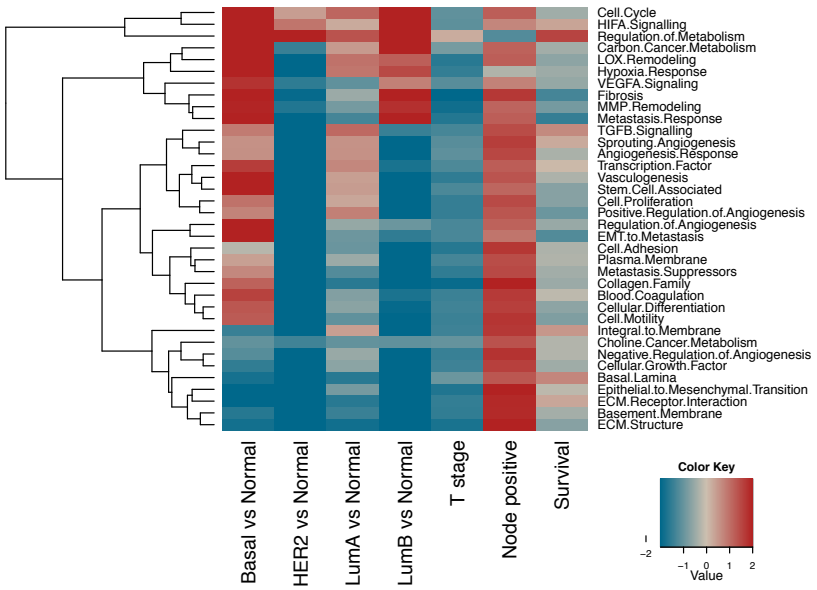

FIGURE 4 Heatmap of differential expression results organized by cancer progression biological annotations. Red indicates largely positive associations between a variable and the genes involved in a biological process; blue indicates largely negative associations. Variables surveyed include the contrast between normal-like PAM50 subtype samples and each of the other PAM50 subtypes, AJCC node status greater than zero, AJCC tumor stage, and time to death.

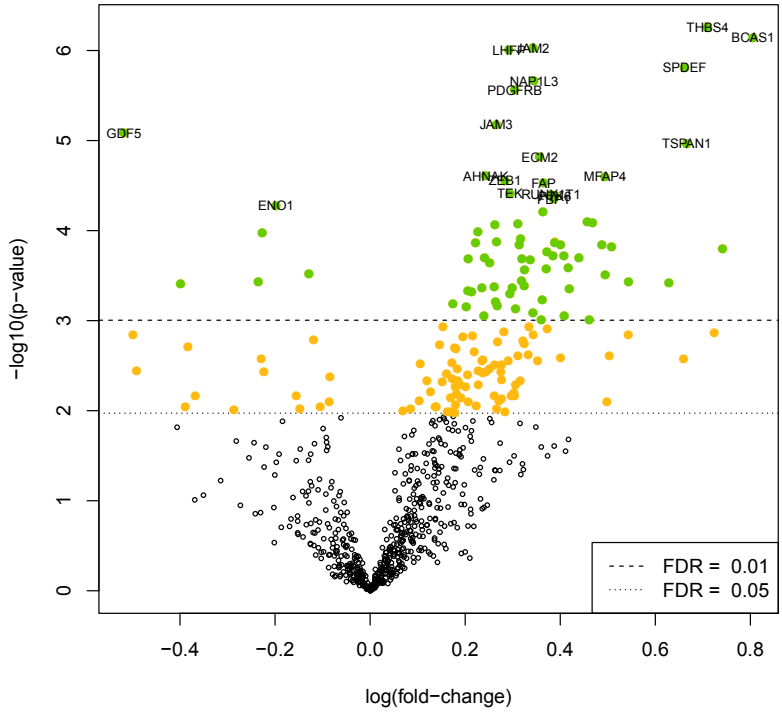

FIGURE 5 Volcano plot illustrating differential gene expression between node positive and node negative patients. Differential expression in log fold-change is on the x-axis and statistical significance measured by −log10 p-values is on the y-axis. The top twenty most differentially expressed genes are labeled and the genes are colored according to their false-discovery rates (FDR).

Conclusion

Metastatic progression of cancer is not an efficient process; multiple conditions must be met in order for a tumor cell to grow successfully[41]. The primary tumor must undergo ECM remodeling and survive hypoxic conditions before individual cells commit to a slow epithelial-mesenchymal transition step[42]. A tumor cell must survive migration to a new area, attach to a surface, invade the surrounding region, and stimulate cell growth, environment remodeling, and angiogenesis[43]. Cancer metastasis is a rate-limiting process; if one of the many steps to progression is terminated, the patient will not develop secondary tumor sites[44]. Cancer progression is not a single event but a collection of independent processes and mechanisms that are highly interconnected.

Given the morbidity rate associated with metastatic cancer and that one million tumor cells are shed per cubic centimeter of primary tumor mass per day[45], new tools must be developed to better understand the intricacies of cancer progression. NanoString Technologies used data-driven methods to select the critical genes in the main themes of cancer progression: angiogenesis, ECM, EMT, and metastasis. The PanCancer Progression panel includes carefully chosen genes to ensure the most comprehensive and meaningful coverage for a targeted discovery approach.

The development of the PanCancer Progression panel marks NanoString's continued commitment to creating valuable gene expression tools with functional biological annotations that can be translated into novel understanding. This panel has many uses in the cancer research field from identifying drug target interactions in the metastatic microenvironment to understanding how to prevent metastasis. Additionally, the panel brings the "Hallmarks of Cancer" full circle, facilitating the study of metastatic cancer progression. The nCounter PanCancer Progression Panel is a unique assay that allows researchers not only to dissect each of the major progression processes separately but also to quantify their expression together to fully investigate the overall state of tumor progression.

nCounter PanCancer Panels Encompassing the Hallmarks of Cancer

The "Hallmarks of Cancer: The Next-Generation Review"[46] is one of the most widely recognized organizing principles for the study of cancer today. NanoString has embraced the Hallmarks of Cancer philosophy and now provides multiplexed gene expression assays that comprehensively cover of all ten Hallmarks of Cancer characteristics (**FIGURE 6**). Each product included in the PanCancer panel collection consists of 770 genes with over 1,850 unique genes in the whole portfolio. These panels independently measure: (1) deregulation of canonical cancer pathways[47], (2) immune system response to tumor growth[48], and (3) cancer progression with the PanCancer Progression panel. Each of these panels singly illuminates valuable insights into the many aspects of cancer; together they enable a complete exploration of the Hallmarks of Cancer.

Overlapping and Unique Content in the PanCancer Panels

PanCancer Pathways Panel

770 cancer pathway genes

- 606 pathway genes for 13 canonical pathways
- 124 cancer driver genes
- 40 reference genes

PanCancer Immune Profiling Panel

770 cancer immune genes

- 24 different immune cell types
- 30 CT antigens
- > 500 genes covering innate and adaptive immune response
- 40 reference genes

PanCancer Progression Panel

770 cancer progression genes

- 277 angiogenesis genes
- 269 epithelial-mesenchymal transition genes
- 254 extracellular matrix genes
- 173 metastasis genes
- 30 reference genes

For gene lists describing the content of each of our PanCancer panels, please visit: www.nanostring.com

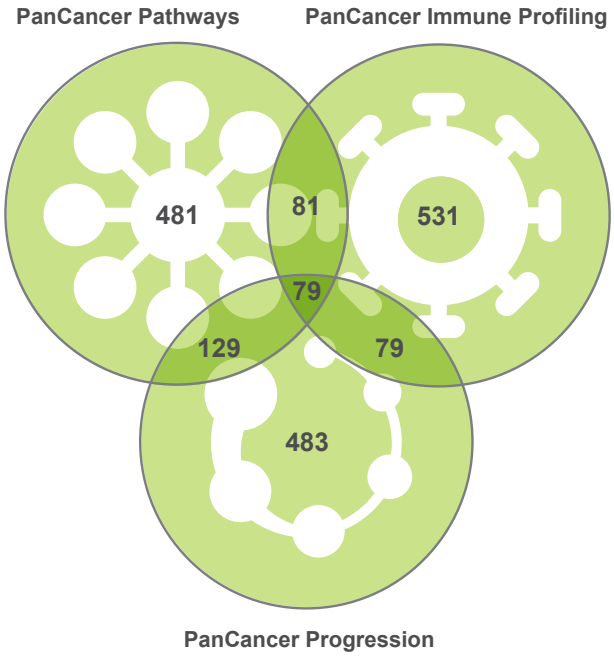

FIGURE 6 PanCancer Panels Venn diagram with >1,850 unique genes. The genes shared between these panels are members of the PI3K, JAK-STAT, and TGF-β pathways, as well as normalization genes.

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