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Applications of Cellular Systems Biology in Breast Cancer Patient Stratification and Diagnostics

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Abstract

Tumors are complex structures of malignant cells and stromal cells that function as an integrated system that promotes tumor progression. Immune cells and other stromal components serve vital cooperative functions that often support tumor growth and metastasis; stromal content and function are strongly associated with disease progression and clinical outcome in cancer patients. Cellular systems biology considers tissues and tumors, and the cells within them, as integrated and interactive networks that function in concert as a system. Assessment of tumors as a “system” within the system of a patient using the cellular systems biology approach has the potential to improve on the current diagnostic tools for breast cancer by creating high content profiles of an individual patient’s tumor. The application of cellular systems biology (CSBTM) profiling to early drug discovery using cellular models of disease [1] and to drug development using the CellCiphrTM Cytotoxicity Profiling panels [2] can optimize the efficacy and decrease the potential toxicity of compounds taken into pre-clinical trials. However, it has become clear that patient sub-populations can respond differently to drug candidates in clinical trials due to patient variability. Therefore, cellular systems biology can also be a powerful approach to patient stratification for clinical trials and could become an important diagnostic tool.

This review describes how the cellular systems biology approach can be applied to patient stratification and diagnostics in breast cancer, focusing on the advantages of quantifying functional biomarkers representing key tumor system processes in intact tissues from patients in order to make highly specific and sensitive predictions towards development of individualized medicine for breast cancer. We discuss the state-of-the-art of multiplexing of functional biomarkers in tissues and the practical utilization of the cellular systems biology approach in creating classifiers for patient stratification and diagnostics.

Key words: Cellular Systems Biology, Breast Cancer, Diagnostics, Biomarkers, Tissue
Microarrays.

I. Tumors as a System within the Host System

Tumors are an integrated system of interacting malignant cells, cancer stem cells, and stromal components. Key tumor cell interaction partners include leukocytes, fibroblasts and vascular and lymphatic endothelial cells. The majority of the leukocytes within the tumor microenvironment are macrophages, but lymphocytes, granulocytes and dendritic cells are also present. These cells are attracted into the tumor system by chemokines and often can become commensal in performing tumor-promoting or -protective functions. Alternatively, these cells can be rendered nonresponsive (anergic) or driven into apoptosis. Tumor-associated macrophages perform a dominant role in supporting tumor progression by producing growth factors that support tumor growth, e.g. EGF [3, 4]; angiogenic factors such as VEGF and angiogenic chemokines [5-8]; tissue remodeling factors that aid tumor cell migration, e.g. matrix metalloproteinases [9, 10]; and immunosuppressive factors that maintain host tolerance to the tumor, such as IL-10 and COX-2 [11-13]. Regulatory T cells ($CD4^+ CD25^{\text{high}} FoxP3^+$) are a small yet potent tumor-protecting population of T cells within the tumor system, and also in draining lymph nodes and peripheral blood [14-16]. Natural regulatory T cells (Tregs) and naïve T cells lacking cytotoxic activity against tumor cells are attracted into tumors via chemokines such as CCL17, 18 and 22 [17-20]. T cells are induced to differentiate into $CD4^+$ Tregs or $CD8^+$ suppressor T cells by immunosuppressive factors and dysfunctional antigen presenting cells or myeloid-derived suppressor cells within tumors [21-24]. Non-regulatory T cells specific for tumor-associated antigens infiltrate into tumors but are rendered non-responsive or driven into apoptosis via interactions with tumor cells [25, 26]. The cross-talk between tumor cells and stromal fibroblasts is also essential to tumor progression. Fibroblasts are the most abundant stromal cell type within tumor masses and perform critical tumor-promoting functions [27]. Carcinoma-associated

fibroblasts have chromosomal alterations that may stimulate tumor growth [28] and these cells interact with tumor cells and infiltrating immune cells via direct contact and soluble mediators, e.g. platelet-derived growth factor [29-31], TGF- β 1 [32, 33], matrix metalloproteinases [34] and chemokines, such as CXCL12 [35], to stimulate tumor growth and promote angiogenesis and to modulate immune cell function. Carcinoma-associated fibroblasts are also stimulated by tumor cells to express aromatase, a component of the estrogen biosynthetic pathway, resulting in estrogen production at the tumor site and stimulation of tumor cell proliferation [36-39]. Tumor cells and various stromal cells including fibroblasts and immune cells interact with endothelial cells via various adhesion molecules and chemoattractants to attract endothelial progenitor cells and to promote angiogenesis, tumor growth, infiltration of further immune cells and migration of tumor cells to new sites [40, 41]. Tumors also appear to induce neurogenesis, and thereby interact with nerve cells, which produce neurotransmitters to stimulate proliferation and migration of malignant cells [42, 43].

The complex structural network of the tumor system and the vital interactions of tumor cells with stromal and immune cells highlight the need for a cellular systems biology approach to cancer diagnostics, which combines multiplexed biomarker panels with informatics tools to produce a systemic readout relevant to patient prognosis.

II. Current Tools for Patient Stratification and Diagnostics in Breast Cancer

The current clinical diagnostic tools for breast cancer are valuable for patient stratification and for predicting risk of recurrence, disease-free survival and responses to therapy. The standard of care diagnostic tests for breast cancer are immunohistochemistry (IHC)-based measurements of

estrogen receptor (ER), progesterone receptor (PR) and HER2/neu [44], the latter of which is often further confirmed by fluorescence in situ hybridization. Clinicians use statistical decision making tools, e.g. Adjuvant! Online that combines clinical and histological parameters (e.g. patient age, tumor size, nodal involvement, etc.) to predict the risk of disease recurrence for an individual patient.

In addition to the basic single biomarker IHC measurements, there is a new generation of RT-PCR- and DNA microarray-based multigene tests that are prognostic for an individual patient's risk of disease recurrence or predictive of response to therapy for specific subtypes of breast cancer. These tests are based on gene expression signatures measured in breast tumor samples that have been assigned to specific clinical phenotypes with an associated risk of recurrence or therapeutic sensitivity, which can be more accurately measured by the combined expression of multiple genes than single genes or biomarkers. These tests performed with high accuracy in small studies and may provide more robust information than the clinicopathological prognostic models, particularly in distinguishing patients with ER-positive tumors who are likely to have good outcome and can be spared aggressive chemotherapy from those patients whose disease is likely to recur and should receive appropriate therapy. Two such tests that are farthest along in development are Oncotype DX (Genomic Health, Inc., CA, USA) and MammaPrint (Agendia, BV., Netherlands), which are currently being evaluated in phase III clinical trials to determine their utility in decision making for breast cancer patients. The Oncotype DX predictive test performs an RT-PCR-based analysis of twenty-one genes, including ER-regulated genes and HER2/neu, to assign a recurrence score of low, intermediate or high for lymph node-negative breast cancer patients with ER⁺ tumors treated with tamoxifen [45, 46]. The recurrence score

correlates with distant recurrence, survival and response to chemotherapy. Oncotype DX is currently the only multigene test recommended for clinical use by the American Society of Clinical Oncology (ASCO) 2007 Update of Recommendations for the Use of Tumor Markers in Breast Cancer [44]. This test is being further evaluated in the NCI-sponsored TAILORx trial (Trial Assigning Individualized Options for Treatment (**Rx**)) to determine whether such genomic profiling approaches can be translated into clinical decision making for breast cancer [47]. The trial will include over 10,000 patients at 900 different locations, limited to those patients with ER⁺ and PR⁺ (HER2/neu-) breast tumors with no positive lymph nodes, to examine whether this type of genomic profiling can accurately predict whether a patient will benefit from adjuvant chemotherapy, particularly for patients scored as intermediate risk by the Oncotype DX test. Mammaprint is a seventy-gene microarray assay that assigns a good or bad signature to ER⁺ lymph node-negative breast cancer cases to indicate risk of recurrence [48]. The precise clinical utility and appropriate application of this test is still under investigation [44] and the test is being prospectively evaluated in the MINDACT (Microarray **In** Node-negative **D**isease may **A**void **C**hemo**T**herapy) clinical trial, which will assess the utility of Mammaprint in assessing a patient's requirement for chemotherapy [49].

An intra-operative RT-PCR-based assay to detect nodal metastases in breast cancer patients has also been developed. The GeneSearch Breast Lymph Node Assay [50] (Veridex, LLC, NJ, USA) measures the mRNA levels of mammaglobin and cytokeratin 19, which are expressed by breast tumor cells, in sentinel lymph nodes from breast cancer patients undergoing surgery. This fast assay gives a positive, negative or invalid reading for the presence of metastases of greater than 0.2mm within the sentinel lymph node to guide intra-operative decisions on dissection of axillary lymph nodes. Novel tests have also been developed to detect circulating tumor cells in breast

cancer patients. The CellSearch System (Veridex, LLC) detects circulating tumor cells of epithelial origin and is prognostic for progression-free survival and overall survival in patients with metastatic breast cancer [51].

While these tests are valuable, the cellular systems biology approach of assaying multiplexed, functional biomarkers to produce a functional readout of the tumor system will likely become a powerful improvement to these clinical tools. The multiplexing of key tumor process biomarkers to produce a functional readout of the tumor system will enable the creation of classifiers to stratify patients according to clinical phenotype and subtype, and to assess risk of recurrence, outcome and to predict response to therapy with higher specificity and sensitivity on a continuous scale. This cellular systems biology approach has recently been successfully applied to cytotoxicity profiling [2]. The goal is to choose the optimal panel of functional biomarkers employed in a multiplexed profile, coupled with classifier software to translate data sets into indices that can be used for patient stratification and diagnostics.

III. Current Unmet Needs in Breast Cancer Patient Stratification and Diagnostics

Although the current diagnostic tools are valuable, over 40,000 women die of breast cancer each year in the US alone [52]. There is clearly a need for improved diagnostic tools that are highly sensitive and specific to stratify patients and predict risk of recurrence and therapeutic sensitivities on a continuous scale to aid individualized decision making for the treatment of breast cancer patients. The improvements in screening have resulted in increased numbers of patients diagnosed with early stage breast cancer. For all subtypes of breast cancer, diagnostic tools are needed for these early stage patients to identify high risk patients who require

aggressive therapy and monitoring to prevent recurrence. Risk of cancer progression and prediction of therapy response is critical since the treatment options for patients who progress to stages III and IV are limited and not curative. For patients with ER⁺ breast tumors, there is a subset of patients whose outcome is poor and these patients may be undertreated based on the results of current diagnostic tests. An accurate diagnostic test is needed that distinguishes these patients as higher risk and requiring more aggressive therapy than low risk patients. Conversely, there are patients with ER⁺ tumors and positive lymph nodes who have good outcome and very low risk of recurrence and these patients could be spared aggressive chemotherapy. For HER2/neu⁺ cases, the response rate to trastuzumab as a single agent is approximately 35%, whereas there is a group of approximately 20% of HER2/neu⁺ patients whose disease very rapidly progresses despite ongoing treatment with trastuzumab. There is an acute need to assess other biomarkers in addition to HER2/neu relating to the response of HER2/neu⁺ tumors to trastuzumab to more confidently distinguish those patients likely to respond from resistant patients who would benefit from alternate therapy.

There is also a requirement for predictive tools to guide therapy choices for the subgroup of 'triple-negative' breast cancer patients whose tumors are negative for ER, PR and HER2/neu. Specific molecular targets for this subgroup have not been identified and hence directed therapies are unavailable. Triple-negative tumors may have higher sensitivity to chemotherapy than ER⁺ and HER2/neu-amplified tumors, however, as a group these patients have poor prognosis and need accurate predictors of therapy response to ensure the most appropriate first-line treatment is chosen.

IV. Breast Cancer Biomarkers and Options for Measuring Cellular Systems Parameters

Many breast cancer-associated biomarkers with prognostic or predictive value have been described. These can be divided into categories relating to their role in the tumor system or their utility in breast cancer patient stratification and are summarized in Table I.

The rationale for measuring functional biomarkers in intact tissues by automated light microscopy is based on the findings for many biomarkers that the protein level, activation status, cellular localization and tissue localization are important parameters relevant to their use as biomarkers in cancer. Examples for each of these parameters are discussed below:

i) Protein Level

Several cancer biomarkers are downregulated at the protein level without changes in gene expression. An example is the CD3 ζ chain of the T cell receptor signaling complex, an immune biomarker in cancer. Upon ligation of the T cell receptor, tyrosine kinases p56 lck and p59 fyn phosphorylate CD3 ζ , triggering further activation of kinases ZAP-70 and Syk, resulting in activation of NF- κ B [53]. In cancer patients, CD3 ζ expressed by T cells in tumors, draining lymph nodes and blood is degraded at the protein level without alteration at the mRNA level, which contributes to general anergy of T cells in cancer patients [54, 55]. This is associated with several tumor-related activities: i) tumor-secreted soluble factors that may activate intracellular peptidases in T cells or directly degrade CD3 ζ protein [55], ii) chronic stimulation of immune cells in cancer leading to accumulation of terminally differentiated effector cells in which CD3 ζ is replaced by FcR γ , iii) apoptosis of immune cells via interaction with death ligands on the surface of tumors or on tumor microvesicles in the sera [56, 57], iv) reactive oxygen intermediates produced by activated macrophages or granulocytes [58] and v) interactions with

myeloid suppressor cells [59]. Another immune biomarker in cancer that is downregulated at the protein level is NKG2D, a natural cytotoxicity receptor that is expressed on natural killer (NK) cells and T cells. NKG2D is endocytosed and degraded after interaction with NKG2D ligands such as MICA and UL-16 binding proteins that are overexpressed on the surface of tumor cells and on tumor-derived microvesicles [60-62].

ii) Activation Status

Biomarkers whose protein level or function is more relevant to patient prognosis and response to therapy than the mRNA level include NF- κ B, which is constitutively activated in various cancer types and is associated with aggressive forms of inflammatory breast cancer [63]. The activated state of NF- κ B is maintained by autocrine loops of cytokines and growth factors expressed by tumor cells and stromal cells, and many NF- κ B-target genes are also cancer biomarkers [64, 65]. The activation state of signal transduction and activation of transcription (STAT) proteins is also relevant to outcome in breast cancer. High levels of phosphorylated STAT1, STAT3 and STAT5a/b have been correlated with good survival in breast cancer patients, and loss of phosphorylated STATs is observed with disease progression [66-68]. Many apoptosis-related cancer biomarkers have been described and increased levels of apoptosis within breast tumors are correlated with higher tumor grade and poor outcome [69]. The activation status of these apoptosis biomarkers may be more relevant than their mRNA expression, as shown by the association of the activated and precursor forms of caspase 3, but not the mRNA level, with higher rates of apoptosis in tumors and poor survival in breast cancer [70, 71].

iii) Cellular Localization

For many cancer biomarkers both their expression and their cellular localization are relevant to prognostic and predictive value. An example is β -catenin, which is involved in cell-cell adhesion and *wnt* signaling and is regulated by phosphorylation. Mutation of β -catenin is very rare in breast cancer, however, loss of membrane localization of β -catenin and accumulation of phosphorylated β -catenin in the nucleus is strongly associated with poor outcome in breast cancer [72, 73]. p21 is a key regulator of the cell cycle and survival. Nuclear localization of p21 is strongly correlated with the inhibitory effect of p21 on cell growth, whereas cytoplasmic localization is associated with the p21-mediated protection of cells from apoptosis [74-76]. Both cytoplasmic location and raised levels of phospho-p21 are associated with poor overall survival in breast cancer [77, 78]. The chemokine receptor CXCR4 is a well-established biomarker of tumor cell migration and metastasis in various cancer types [79]. In breast cancer, cytoplasmic CXCR4 in tumor cells has been associated with tumor aggressiveness and positive-lymph nodes, whereas nuclear expression of CXCR4 has been associated with negative-lymph nodes and disease stage [80, 81].

iv) Tissue Localization

Measurement of certain cancer biomarkers requires preservation of the tissue structure, which is lost in diagnostic approaches that digest tissue to extract cells, proteins or nucleic acids. The morphology of the nucleus, particularly large nuclear area, and also DNA mass in tumor cells have been described to have prognostic power in breast cancer [82]. Nuclear morphology can be

imaged in high resolution by microscopy and analyzed by software to objectively assess features relevant to patient outcome.

The localization of certain immune cells in the tumor microenvironment is related to their prognostic value for patients. Dendritic cells (DCs) are important antigen-presenting cells that initiate and modulate both anti-tumor and tumor-protective T cell responses. The density and maturation state of DCs in tumors are significant prognostic markers in cancer. The location of DCs also reflects the maturation status of DCs within tumors; immature DCs are mainly located in tumor cell nests and stromal areas, whereas mature DCs are confined to peritumoral areas [83, 84]. The localization of CD4 T cells has also been shown to be relevant to prognosis in cancer; stromal localization, rather than tumor cell nest localization in tumors is associated with improved patient outcome [85].

v) Cell Type Enumeration

An additional and important advantage of immunostaining-microscopy-based approaches to cancer diagnostics is in the enumeration of cell types that are relevant to patient outcome, particularly for those cell types that require multiple biomarkers for identification. Treg cells ($CD4^+ CD25^{high} FoxP3^+$) are an example of tumor-associated cells with predictive power in cancer. High density of Tregs in tumors identifies patients with high risk of relapse [86].

Detection of multiplexed immunostaining by microscopy and image analysis is necessary to correctly identify and quantify these cells since three markers are required for identification of Tregs, and these markers are expressed by other cell types. Assessment of other T cell

phenotypes also requires multiple markers, e.g. naïve T cells (CD27⁺ CD45RA⁺), effector T cells (CD45RA⁺ CD27⁻) and memory T cells (CD27⁺ CD45RA⁻).

Cancer stem cells also require multiple markers for accurate detection and distinction from non-stem tumor cells. In breast cancer, a population of cytokeratin⁺ CD44⁺ CD24^{-/low} tumor cells has been characterized as consisting of cancer stem cells with prognostic value [87]. These cells are highly invasive and produce cytokines that promote metastasis of tumor cells.

Myeloid-derived suppressor cells have been shown to accumulate in breast tumors. These cells can be characterized by at least five biomarkers (e.g. CD34⁺ CD33⁺ CD13⁺ CD15⁺ HLA-DR⁻) to distinguish them from other phenotypes of immune cells in the tumor environment. These tumor-associated suppressor cells produce growth factors to support tumor growth and neoangiogenesis and also inhibit immune responses against tumors, while promoting the function of regulatory T cells [21, 88].

V. State-of-the-art of Multiplexing Biomarkers in Tissues for Greater Predictive Power

Individual biomarkers contain only limited predictive information of the activity of the network or system in which they function. A more accurate assessment of the disease state in breast cancer may be made by simultaneous measurement of multiple biomarkers representing various key processes in the whole tumor system. This has been demonstrated for various combinations of biomarkers in cancer. p21^{Cip/WAF1} and HER2/*neu* are functionally related in breast cancer. p21 is a downstream substrate of the AKT pathway, which is activated by HER2/*neu*.

Phosphorylation of p21 by AKT results in cytoplasmic localization of p21, which suppresses the p21-mediated growth inhibition that occurs when p21 is located in the nucleus, and allows its function in the cytoplasm in protecting tumor cells from apoptosis [75, 76]. The combined

measurement of phosphorylated-p21 and HER2/*neu* has been shown to enable stratification of breast cancer patients with HER2/*neu*-amplified tumors to more precisely predict five-year survival, with high HER2/*neu* and cytoplasmic phosphorylated-p21 correlating with lowest patient survival and nuclear p21 correlating with low levels of HER2/*neu* and improved survival [77].

For ER⁺ tumors, the combination of immunostaining for p53, NDRG1, CEACAM5, SLC7A5 and HTF9C has been demonstrated to distinguish breast cancer patients with poor, moderate and good outcomes and may be an improvement on the standard of care biomarkers ER and PR [89]. A combination of multiple signaling protein activation profiles has also been shown to have high predictive power for breast cancer. The multiplexed analysis of the phosphorylated forms of HER2/*neu*, ER, EGFR, MAPK, p70S6K and IGFIR/In was shown to predict breast cancer patient survival and the same combination without IGFIR/In could be used to predict response to the combination of cyclophosphamide, methotrexate and 5-fluorouracil chemotherapy [90].

VI. Applying the Cellular Systems Biology Approach to Cancer Patient Stratification and Diagnostics

The application of the cellular systems biology approach to cancer diagnostics is illustrated in Fig. (1) and begins with the selection of biomarkers that represent key tumor processes and have prognostic and predictive value for patient stratification. Panels of candidate biomarkers can then be assessed in tumor tissues in the format of tissue microarrays. This high-throughput approach allows rapid assessment of biomarkers in large prospective training sets of tissue samples. Tissue microarrays are constructed from cores taken from archived formalin-fixed paraffin-embedded

tissue specimens or frozen tissues. The current standard core diameter is 0.6mm and analysis of two such cores per case from a tissue block has been shown to be highly correlated with analysis of an entire tissue section [91]. This method maximizes the use of limited human tissue samples, allowing high-throughput screening of biomarkers and standardization of experimental conditions and methods across many tissue samples. The current fluorescence scanning and imaging technologies, e.g. PM-2000TM (HistoRx, Inc., CT, USA), enable measurement of up to four multiplexed fluorophores by immunofluorescent staining in a single tissue section (Fig. (2)) and serial sections can be stained with additional panels of antibodies and/or hematoxylin and eosin for morphological analysis. Such fluorescence scanning systems are coupled to image analysis software such as AQUATM (HistoRx, Inc.) and Aureon M-PlexTM (Aureon Laboratories) to enable quantitative measurement of signal and localization to subcellular compartments, generating objective scoring of biomarkers as continuous variables. Combined with demographic, clinical and pathological data, these quantitative measurements of biomarkers can be assessed using multivariate statistical algorithms to determine the prognostic or predictive value of biomarkers in various combinations. Classifiers can be created to stratify patients according to risk of recurrence, progression-free survival and survival at specific time intervals (five, ten, twenty years), overall survival and responses to various therapies. Classifiers can then be validated in large independent tissue microarray cohorts of patient samples to determine whether the optimal panel of biomarkers that gives a systems readout of the tumor can be used to make clinically useful prognoses and predictions for individual patients.

Summary

In summary, the cellular systems biology approach to breast cancer patient stratification and diagnostics has the potential to make significant improvements on current single biomarker and multigene approaches by producing a readout of the function of key processes of the tumor system. The combination of multiplexed functional biomarkers and informatics tools will hasten the development of specific and sensitive diagnostic tools to more accurately stratify patients with breast cancer and to assess risk of recurrence, survival and to predict responses to specific therapy options.

Conflicts of Interest

D. Lansing Taylor and Rebecca. J. Critchley-Thorne are employees of Cellumen, Inc., a company that creates cellular systems biology products and services.

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Table I. Summary of Breast Cancer Biomarkers		
Biomarker Category	Example Biomarkers	Reference
Breast Tumor Typing	ER, PR, HER2/neu	[92, 93]
Apoptosis	Fas, FasL, TRAIL receptors, activated caspases, pAKT, Survivin, MCL-1, Bcl-2	[94-101]
Cell Cycle Control	p53, p21, p27, p16, Cyclins D1, E	[77, 102-107]
Signal Transduction	STAT1, 3 and 5, c-kit, HSP90	[66-68, 108, 109]
Therapy Responses	MDR1, GST- π , pSR, HER2/neu	[93, 110-112]
Adhesion	E-cadherin, β -catenin, CD44, CD24, Claudin-1	[72, 113-116]
Migration	CXCR4	[81]
Angiogenesis	VEGF, Flt-4, HIF-1 α	[117-120]
Proliferation	Ki-67	[121]
Immune Response	CD3 ζ , PD-L1, Tregs, Macrophages, Cytokines	[86, 122-125]
Inflammation	NF- κ B, COX2, CSF-1R	[63, 126, 127]

Figures

Fig. (1). The series of processes involved in the cellular systems biology approach to patient stratification and diagnostics for breast cancer.

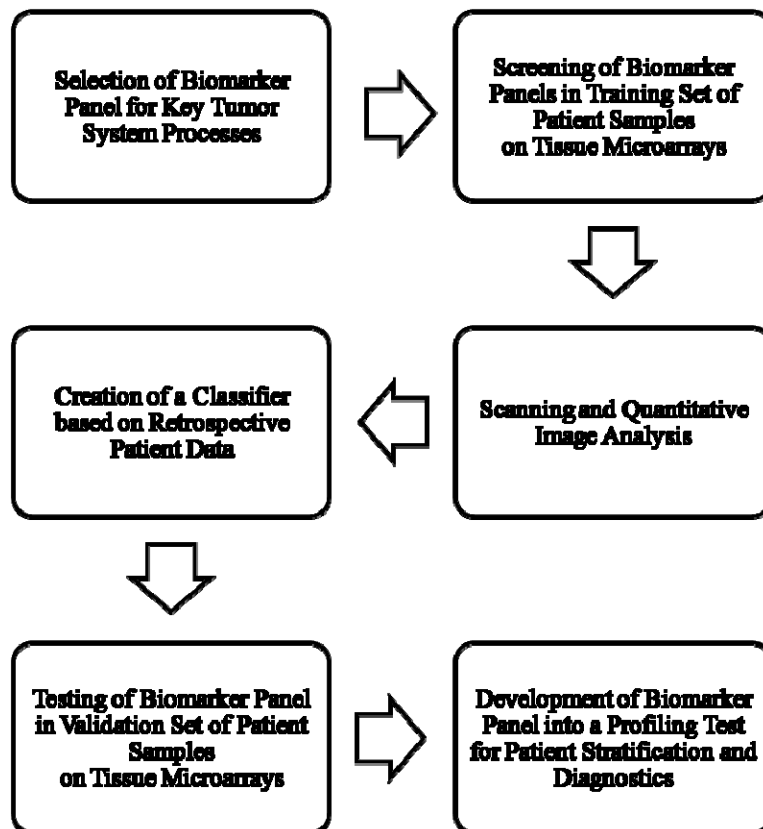


Fig. (2). Multiplexed Microscopy.

In this example, the top left panel is of a tissue core section stained with hematoxylin and eosin for traditional histological assessment. The other panels are from an adjacent section of the same tissue core. The section was immunostained with antibodies against HER2/neu (red) and epidermal growth factor receptor (EGFR) (green). Nuclei are stained with Hoechst 33342 (blue). The two lower panels are of individual signals for HER2/neu (red) on the left and EGFR (green) on the right with the nuclear signal (blue). The relative contributions of the two immunostains are easier to visualize when separated, especially electronically when one is much stronger than the other, as is the case here with HER2/neu masking the EGFR signal in the 4-color merged image in the upper right panel.

