PROTEIN NETWORK MAPPING OF BLADDER CANCER: AN ANALYSIS OF TUMOR COMPARTMENT AND THE SURROUNDING MICROENVIRONMENT

by

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DEDICATION

This is dedicated to my loving husband Anthony Hodge, and to my four wonderful children Glen, Austin, Anthony, and Vincent.

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I would like to thank all of my colleagues from the Center for Applied Proteomics and Molecular Medicine at George Mason University. Their guidance and support has been invaluable in this journey.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND/OR SYMBOLS

Abelson Murine Leukemia ..... ABL
Anaplastic Lymphoma Kinase ..... ALK
Adenosine Monophosphate-Activated Protein Kinase ..... AMPK
Apoptosis Signal-Regulating Kinase 1 ..... ASK1
Adenosine Triphosphate ..... ATP
Bladder Cancer ..... BC
B-cell Lymphoma 2 ..... BCL-2
Benign Prostatic Hyperplasia ..... BPH
Cancer –Associated Fibroblasts ..... CAF
Cleaved Caspase 7 ..... CC7
Cluster of Differentiation ..... CD
Carcinoma *in situ* ..... CIS
Collagen ..... COL
Cyclooxygenase-2 ..... Cox-2
cAMP Response Element Binding ..... CREB
Catalyzed Signal Amplification ..... CSA
Cytotoxic T Lymphocytes ..... CTL
Cytotoxic T-Lymphocyte Associated Protein-4 ..... CTLA-4
Coefficient of Variation CV
Damage-Associated Molecular Pattern ..... DAMP
Discoidin Domain Receptor ..... DDR
Deoxyribonucleic Acid ..... DNA
Extracellular Matrix ..... ECM
Epidermal Growth Factor ..... EGF
Epidermal Growth Factor Receptor ..... EGFR
Epithelial–Mesenchymal Transition ..... EMT
Focal Adhesion Kinase ..... FAK
Food and Drug Administration ..... FDA
Fibroblast Growth Factor 2 ..... FGF2
Fibroblast Growth Factor Receptors ..... FGFR
Granulocyte Macrophage Colony Stimulating Factor ..... GM-CSF
High Grade Papillary ..... HGP
Human Immunodeficiency Virus ..... HIV
Human Papilloma Virus ..... HPV
Interferon ..... IFN
Insulin-Like Growth Factor Receptor ..... IGFR

Immunohistochemistry ..... IHC
Invasive High Grade Urinary Carcinoma ..... IHGUC
Interleukin IL
Insulin Receptor ..... IR
Interferon Regulatory Factor-3 ..... IRF3
Janus Kinase ..... JAK
c-Jun N-Terminal Kinase ..... JNK
Laser Capture Microdissection ..... LCM
Lipopolysaccharide ..... LPS
Mitogen-Activated Protein Kinase ..... MAPK
Michigan Cancer Foundation-7 ..... MCF7
Muscle Invasive Bladder Cancer ..... MIBC
Matrix Metalloproteinase ..... MMP
Mechanistic Target of Rapamycin ..... mTOR
Normal ..... N
Nuclear Factor-κB ..... NFκB
Non-Muscle Invasive Bladder Cancer ..... NMIBC
Nucleophosmin ..... NPM
Non-Receptor Tyrosine Kinase ..... NRTK
Non-Small Cell Lung Carcinoma ..... NSCLC
Optimal Cutting Temperature ..... OCT
p70 Ribosomal S6 kinase p70S6K
p90 Ribosomal S6 Kinase p90RSK
Pathogen-Associated Molecular Pattern ..... PAMP
Phosphate-Buffered Saline ..... PBS
Programmed Death 1 ..... PD-1
Programmed Death Ligand 1 ..... PD-L1
Programmed Death-Ligand 2 ..... PD-L2
Platelet-Derived Growth Factor ..... PDGF
Platelet-Derived Growth Factor Receptors ..... PDGFR
Phosphoinositide 3-Kinase/ Protein Kinase B PI3K/AKT
Protein Kinase C ..... PKC
Proline-Rich Akt Substrate of 40 kDa PRAS40
Pattern-Recognition Receptor ..... PRR
Prostate Specific Antigen ..... PSA
Phosphatase and Tensin Homolog ..... PTEN
Ribonucleic Acid ..... RNA
Reverse Phase Protein Microarrays ..... RPPA
Receptor Tyrosine Kinase ..... RTK
Stress Activated Protein Kinase/Jun N-Terminal Kinase ..... SAPK/JNK
Sodium Dodecyl Sulfate ..... SDS
Sarcoma-Family Kinases ..... SRC

Signal Transducer and Activator of Transcription.....STAT
The Cancer Genome Atlas.....TCGA
Transforming Growth Factor- β.....TGFβ
Tyrosine Kinase.....TK
Tyrosine Kinase Inhibitor.....TKI
Toll-Like Receptor.....TLR
Tumor Necrosis Factor Alpha.....TNFα
Tissue Protein Extraction Reagent.....TPER
Tumor-Specific Antigen.....TSA
Vascular Endothelial Growth Factor Receptor.....VEGFR

ABSTRACT

PROTEIN NETWORK MAPPING OF BLADDER CANCER: AN ANALYSIS OF TUMOR COMPARTMENT AND THE SURROUNDING MICROENVIRONMENT

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George Mason University, 2017

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The interaction between tumor and stroma has become of intense interest in the field of oncology in order to better understand the driving forces behind tumor onset and progression, metastasization and the responsiveness to therapy. These interactions are hard to capture using in vitro techniques and the extent to which in vivo models really recapitulate the tumor-stroma interactions of human tissues is still under investigation. For these reasons, human specimens still remain optimal input material for exploring these interactions. Laser capture microdissection (LCM) coupled with reverse phase protein microarrays (RPPA) are ideal technologies for isolating different cell compartments from heterogeneous tissues and for exploring protein signaling network of human tissue specimens. This study explored the protein signaling network of 23 bladder cancer samples within the epithelium and the surrounding stroma across different histotypes including normal, carcinoma in

situ (CIS), papillary, and invasive cellular compartments. Pure tumor epithelium and surrounding stroma from each sample were first isolated with LCM followed by RPPA that allows for the measurement of hundreds of proteins and phosphoproteins. The analysis of the epithelium compartments collected from patients affected by invasive and non-invasive (papillary) bladder cancer revealed different pathway involvement driving the tumor, with invasive cases showing a phenotype of immune/ inflammation, proliferation and survival in contrast to papillary tumors which were characterized by wound healing and metabolism. While the stroma surrounding both invasive and papillary tumors showed high correlation between the different members of the PI3K/AKT pathway, only invasive tumors presented with interconnection between proteins involved in the immune/ inflammation response. Receptor tyrosine kinases (RTKs) in the epithelium appeared to be highly correlated with RTKs in the stroma as well as their downstream targets suggesting a cross-talk between the two compartments. Finally, programmed death ligand 1 (PD-L1), an important drug target immune checkpoint protein, was increased significantly in invasive epithelium when compared to papillary epithelium. This PD-L1 expression offers a promising therapeutic target against invasive bladder cancer, which has had minimal treatment advances in more than a decade.

CHAPTER ONE

According to the American Cancer Society the estimates for bladder cancer (BC) in the United States for 2015 are approximately 74,000 new cases, of which 56,320 will be men and 17,680 will be women. Overall, the chance men will develop this cancer during their life is about 1 in 26, and for women, the chance is about 1 in 90.[1] Five year survival rates vary drastically depending on the stage of BC. The 5-year survival rate at stage 1 is 88%, whereas at stage 4, the rate is 15%. Patients present with painless hematuria, and physicians diagnose patients based on urine cytology and tumor biopsy. Treatment of BC is one of the most expensive cancer treatments, putting an enormous burden on healthcare systems, since it requires intensive vigilance in terms of conducting cystoscopy at regular intervals, urinary cytology, radiological examinations to monitor the disease and frequent tumor resections under anaesthesia.[2]

Urinary bladder tumors comprise two major groups, based on the extent of invasion into the urinary bladder wall and adjacent structures.[3] Non-muscle-invasive bladder cancer (NMIBCs) that have not invaded the urinary bladder's smooth muscle, and muscle-invasive bladder cancer (MIBCs) which invade beyond the muscle layer into nearby organs or structures. Approximately two thirds of newly diagnosed bladder tumors are NMIBCs with a 5-year survival rate of over 88%.[4] However, between 10% and 70% of them recur at the same site or in other areas of the urinary bladder during a

period of 5 years, with a risk of progression to invasive disease state estimated in 10–25% of cases.[5] MIBCs represent one third of new initial diagnoses of BC, and only around 50% of patients with MIBCs respond to cisplatin-based chemotherapy, which remains the first-line treatment in various clinical settings. The average age of diagnosis of patients with BC is 73, which makes the current harsh, non-selective chemotherapy a suboptimal treatment option.[4] This older age group of patients is at a higher risk of nosocomial infection due to multiple hospital stays, leading to higher healthcare costs, and overall doesn't recover well from surgeries. Long-term survivors are a minority, with a 5-year survival rate below 50% for non-metastatic MIBCs and 10% for metastatic MIBCs, with a median survival time of 14 months for metastatic MIBC.[6] Bladder cancer is an area in much need of further investigation and new treatment ideas beyond traditional non-selective chemotherapy.

Proteomics in Cancer

Cancer is a protein network disease characterized by a disruption in balance between cell proliferation and death. There has been an increase in discovery of genetic alterations in cancer, but studying the effects of a single mutation or a variation in copy number is not sufficient to fully understand the driving force behind the cancer.[7] Although the causes of cancer lie in mutations or epigenetic changes at the genetic level, their molecular manifestation is the dysfunction of biochemical pathways at the protein level.[8] Cells are exposed to several extracellular signals simultaneously, and these signals must be internalized, amplified, and then translated into the appropriate physiological response through signal transduction.[9] Extracellular signaling molecules

activate specific receptors that are located on the cell surface or inside the cell, which triggers a biochemical ripple effect inside the cell that creates a response, allowing one signaling molecule to cause many responses.[9] The molecules involved in signal transduction are largely regulated by phosphorylation and de-phosphorylation events that serve to either activate or deactivate these molecules. Protein phosphorylation is a reversible post-translational modification that plays a key role in physiological processes, and can be deregulated in cancer.[10]

Protein phosphorylation is the most widespread type of post-translational modification in signal transduction, and affects metabolism, growth, division, differentiation, and apoptosis.[11] Cellular signaling events are driven by protein-protein interactions, post-translational modifications, and enzymatic activities.[12] Protein kinases are enzymes that catalyze the transfer of phosphate from adenosine triphosphate (ATP) to amino acids in the protein substrate, usually on serine, threonine, and tyrosine residues, and these modifications provide sites in which specific protein interactions drive cellular signaling cascades.[11 12]

Proteomics is a large-scale comprehensive study of a specific proteome, including information on protein abundances, their variations and modifications, along with their interacting partners and networks, in order to understand cellular communication.[13] Only through the study of proteins can protein modifications be characterized and the targets of drugs identified, because proteins are responsible for the phenotypes of cells. There are vast amounts of DNA sequences in databases, but genomic data alone, is not sufficient to shed light on true biological function.[14] There is not a strict linear relationship between

genes and the proteins of a cell.[14] Proteomics focuses on the gene products, because the proteins are the active agents in cells, and are the targets of almost all FDA approved drugs.[14]

Receptor Tyrosine Kinases (RTKs)

Phosphorylation is a post-translational modification that is a pivotal component of normal cellular communication and maintenance of homeostasis.[15] Tyrosine kinases (TKs) are a family of enzymes, which catalyzes phosphorylation of select tyrosine residues in target proteins, using ATP. Tyrosine kinases are classified as receptor tyrosine kinases (RTKs), and non-receptor tyrosine kinases (NRTKs). Examples of RTKs are EGFR, PDGFR, FGFR and IR. NRTKs include SRC, ABL, FAK and JAK.[16] The RTKs are not only cell surface transmembrane receptors, but are also enzymes having kinase activity. All RTKs have a similar molecular structure, with an extracellular domain that contains a ligand-binding region, a single transmembrane helix, and a cytoplasmic region that contains the protein TK domain plus additional carboxy terminals and juxtamembrane regulatory regions.[17] RTKs play fundamental roles in cellular processes, including cell proliferation, migration, metabolism, differentiation, survival, and regulating intercellular communication during development.[18] In normal cells, RTK activity is tightly controlled, however when they are mutated or structurally altered, RTKs can become oncoproteins.[18] TKs are enzymes that serve as switches that can turn cellular functions on or off.[16] There are several mechanisms by which TKs can acquire transforming functions, and the result is the constitutive activation of normally controlled pathways leading to the activation of other signaling proteins and secondary messengers

which can disrupt the regulatory functions in cellular responses like cell division, growth and cell death.[16] Constitutive oncogenic activation in cancer cells can be blocked by selective tyrosine kinase inhibitors (TKI) and these inhibitors are considered a promising way of therapeutically targeting the RTK.[16]

Immunity

The immune system is the collection of cells, tissues and molecules that protects the body from numerous pathogenic microbes and toxins in the environment.[19] The immune system consists of innate and adaptive immunity. The innate immune system consists of nonspecific defense mechanisms that act immediately or within hours of an antigen's appearance in the body. Neutrophils, macrophages, dendritic cells, and mast cells are the types of cells that can be involved in innate immunity, and these cells either succeed in clearing the infection or contain it while an adaptive response develops.[19] In adaptive immunity there is an antigen-specific immune response that is more complex than the innate, and slower because the antigen first must be processed and recognized by the immune system.[20] Once an antigen has been recognized, the adaptive immune system creates immune cells specifically designed to attack that antigen. The cells of the adaptive immune system include B cells that make antibodies and T cells that can mature into either $CD4^+$ helper cells or $CD8^+$ killer cells.[21] With a large supply of diversified receptors that match the antigens, the immune response of the adaptive immune system is able to increase in strength with repeated exposure to the antigen.[20]

Innate Immunity

In order to protect against infection, the body must detect the presence of microorganisms. The body does this by recognizing molecules unique to microorganisms that are not associated with human cells. These unique molecules are called pathogen-associated molecular patterns (PAMPs).[19] Examples of PAMPs include lipopolysaccharide (LPS), peptidoglycan, and double-stranded RNA.[22] The body's defense cells have receptors on their surface called pattern-recognition receptors (PRRs) to recognize the PAMPs and damage-associated molecular patterns (DAMPs), which are associated with cell components that are released during cell damage or death.[22] PRRs are capable of binding specifically to conserved portions of these molecules. Toll-like receptors (TLR) are one type of PRR that play a major role in innate immunity and the induction of adaptive immunity.[22] The binding of a microbial molecule to its transmembrane TLR transmits a signal to the cell's nucleus inducing the expression of genes coding for the synthesis of intracellular regulatory molecules called cytokines.[22] After binding, TLRs activate two major signaling pathways. The core pathway utilized by most TLRs leads to activation of the transcription factor NF-kB (Nuclear Factor-kB) and the MAPKs (Mitogen-Activated Protein Kinases) p38 and JNK (c-Jun N-terminal Kinase).[23] The second pathway involves TLR3 and TLR4 and leads to activation of both NF-kB and IRF3 (Interferon Regulatory Factor-3), allowing for an additional set of genes to be induced, including anti-viral genes such as IFN-β.[23]

Adaptive Immunity

The adaptive immune response is much more sophisticated than the innate response, and can provide long lasting protection. TLRs also play a role not only play a

role in innate immunity, but adaptive immunity as well. TLRs can lead to the activation of several intracellular signaling pathways, and are expressed on the membranes of dendritic cells, macrophages, natural killer cells, cells of the adaptive immunity (T and B lymphocytes) and non-immune cells, including epithelial, and endothelial cells and fibroblasts.[24] TLR activation leads to regulation of gene expression, cell proliferation, differentiation, mitosis, cell-cycle regulation, and apoptosis.[25]

Reverse Phase Protein Arrays (RPPA)

Dysregulation of cellular signaling pathways due to genetic and/ or proteomic alterations leads to cancer.[26] RPPA is a quantitative technology that allows a mapping of the signaling network by measuring not only the unmodified protein but also their phosphorylated form as well as multiple proteins in specific pathways to create a dynamic guide to the activation status of the clinical sample. [26] This multiplex approach leads to effective tailored therapy that avoids unnecessary expensive treatments that will not be effective and cause patients significant side effects for no benefit. Although genes are the set of instructions for the potential protein makeup of an organism, gene analysis can't measure a very influential post-translational modification such as phosphorylation. The increasing use of monoclonal antibodies and kinase inhibitors in targeted therapeutics highlights the importance of being able to accurately measure phosphorylation in clinical samples.[27] The ability to measure phosphorylation can assist with stratifying patients for the most effective therapy, and be able to monitor the targeted therapy's effectiveness once administed.[27]

RPPA are miniaturized immunoassays that can contain hundreds of samples on a single array. A patient's sample is immobilized on a spot, and single proteins can be quantified across a set of samples, and spotted on the same array under the same experimental conditions.[26] RPPA compares the activation state of proteins from multiple samples within the same array, allowing for quantitation of proteins along with their level of phosphorylation, and the status of proteins downstream in the cellular network.

RPPA is a reproducible application that allows high-throughput testing with excellent sensitivity. Paweletz et al. tested the precision and linearity of the RPPA using prostate specific antigen (PSA) over seven different slides and found a sensitivity in the femtomolar range.[28] They then focused on the linearity and reproducibility in microdissected tissue from esophageal normal epithelial cells, and found from sample to sample R^2 of 0.952.[28] In addition, Rapkiewicz et al. tested the sensitivity and correlation of RPPA using an MCF7 cell line model of breast adenocarcinoma and found the sensitivity was in the femtomolar range with a coefficient of variance <13.5% for the most dilute samples.[29] Interslide precision was determined by testing the MCF7 cell line, which is known to overexpress epidermal growth factor receptor (EGFR) on 5 slides printed in triplicate and immunostained with anti-EGFR.[29] They found a linear correlation of $R^2 = 0.9821$ across the data set, and their coefficient of variation (CV) for interslide reproducibility ranged from 5.1% for the most undiluted sample, to 4.1% for the 1:2 dilution, and up to 13.5% for the 1:16 dilution.[29] Samples prepared from 5,000 to 20,000 cells are sufficient to analyze hundreds of different protein targets, which enables the analysis of a much larger number of proteins from each sample and makes this

technique suitable to map the activating signaling network in bladder cancer and identify what pathways are becoming activated or deactivated in human samples.

Animal Models and Cell Culture

Animal models are limited in their ability to mimic the complex process of human carcinogenesis, physiology and progression.[30] The major pre-clinical tools for new-agent screening prior to clinical testing are experimental tumors grown in rodents, which are poor models for the majority of human diseases, because of the differences in molecular, immunologic and cellular differences between humans and mice.[31] An example of a successful animal model that did not translate into clinical trials was the TGN1412 trial.[32] The drug TGN1412, was an immunomodulatory humanized agonistic anti-CD28 monoclonal antibody developed for the treatment of immunological diseases such as multiple sclerosis, rheumatoid arthritis and certain cancers.[32] TGN1412 was tested on different animals including mice, to ensure safety and efficacy in preclinical animal models, and the toxicity studies showed that doses a hundred times higher than that administered to humans did not induce any toxic reactions.[32] However, after the first infusion of a dose 500 times smaller than that found safe in animal studies, all six human volunteers faced life-threatening conditions involving multi-organ failure for which they were moved to intensive care unit.[33]

Animal models have also shown little success even when the clinical trial took on a more targeted molecular approach. A Phase II randomized clinical trial of the Hedgehog pathway antagonist IPI-926 in patients with advanced chondrosarcoma was stopped early for futility.[34] The Hedgehog pathway is dysregulated in a variety of solid

tumors and provides key growth and survival signals to tumor cells. The Phase II clinical trial for IPI-926 translated from a successful animal model of IPI-926 on a malignant solid brain tumor.[35] Mice treated with IPI-929 with advanced brain tumors gained a fivefold increase in survival.[35] However, IPI-926 showed no effect compared to placebo in the human trial.[34]

The most studied cell line in all of biology is HeLa, cultured in 1951 from the cervical cancer of a woman named Henrietta Lacks. The HeLa cell line is robust and has allowed researchers to study polio, measles, human papilloma virus (HPV), HIV and tuberculosis. It was used to create the first human-mouse cell hybrid, and it was sent into space.[36] It has played a role in more than 70,000 studies. HeLa is also the most common cell line contaminant, responsible for more than 20 percent of contaminated cell lines.[36] Cell repositories in the U.S., U.K., Germany and Japan have estimated that 18% to 36% of cancer cell lines are incorrectly identified.[37]

Even if cell cultures could be determined to be 100% contamination free, there is still the issue that cells grown in a cell culture will not always give the same results as cells in the body. Cells in the *in vivo* environment are surrounded by other cells and extracellular matrix (ECM). When cultured *in vitro,* cancer cells can lose some of their *in vivo* features, because of the lack of environmental signals present in native tumors.[38] In 2D culture, cells are deprived of the tissue matrix that is known to regulate rumor progression. The lack of cell matrix interactions that are involved in native tumors can lead to changes in cell phenotypes, resulting in misleading data for *in vivo* responses.[39] Currently, in drug discovery, the standard procedure of screening compounds starts with

the cell culture-based tests, followed by animal model tests, to clinical trials. Only about 10% of the compounds progress successfully through clinical development.[39]

Epithelial – Stroma Connection

Epithelial tumors do not exist in isolation, and are not made up solely of tumor epithelial cells, but rather are comprised of many different types of cells that co-evolve within the tumor. The tumor microenvironment includes stromal cells, which consist of fibroblasts, glial, epithelial, fat, immune, vascular, smooth muscle, immune cells, and the extra-cellular matrix (ECM).[40] The main function of the stroma to provide support, structure and anchoring. Significant amounts of research have been focused on the role of cancer –associated fibroblasts (CAF), because fibroblasts make up a large portion of the stroma, and these CAFs are functionally and phenotypically different from normal fibroblasts that are in the same tissue.[40] In normal tissue, the primary function of the fibroblast is to provide structural integrity within the connective tissue as well as wound healing, and it is responsible for making the ECM and collagen.[41] The stromal environment, although not as dynamic as the tumor epithelium, does undergo remodeling through the process of ECM protein production and degradation by matrix metalloproteinases (MMPs).[40] Fibroblasts become activated during wound repair and serve as a scaffold for cell proliferation, and secrete growth and chemotactic factors coordinating the incoming inflammatory and vascular cells.[40] There is dynamic cross-talk that exists between fibroblasts and injured epithelium. Factors such as, fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), epidermal growth factor (EGF),

and transforming growth factor- β (TGF-β) are released from the injured area and play a role in transforming a resting fibroblast into an activated one.[40]

Several research groups, highlighting the ability of stroma to interact with the tumor, have demonstrated the connection between the two. Hayward et al. used the microenvironment to permanently transform the non-tumorigenic human prostatic epithelial cell line, BPH-1, to become tumorigenic.[42] Hayward et al. brought about this transformation through recombination of the BPH-1 with human prostatic CAF, demonstrating that the stroma can have a determining effect on other tissue types.[42] Kurtova et al. explored the relationship between CAFs and invasive bladder cancer, and if one could influence the other with regards to metastasis.[43] Kurtova et al. isolated bladder cancer CAFs, and co-transplanted them with bladder cancer cells as xenograft tumors, and found high collagen I (COL1) deposition in the tumors that formed.[43] Kurtova et al. then pre-stimulated bladder cancer cells with COL1, which enhanced metastatic colonization of bladder cancer cells to the lung, and their molecular analysis of the pre-stimulated cells showed the up-regulation of the collagen receptor discoidin domain receptor (DDR1).[43] DDRs are the only RTK that specifically bind to, and are activated by collagen.[44] DDRs are part of the signaling network that transfers information throughout the ECM.[44] Olumi et al. showed that prostatic CAFs stimulated tumor progression by combining fibroblastic and epithelial cells both *in vivo* and *in vitro*.[45] The CAFs were capable of stimulating both initiation and progression in the tumor, when grown with initiated non-tumorigenic epithelial cells.[45]

In order to determine how the tumor epithelium is affecting the stroma and vice versa, the ability to separate and analyze the two cellular compartments is paramount. Analyzing signaling networks in human samples that include tumor epithelium and surrounding stroma for each patient can provide a personalized cross-talk signature that facilitates accurate identification of druggable targets.

Laser Capture Microdissection (LCM)

LCM is a technique that allows the user to isolate only the cell population of interest in order to specifically detect the molecular signaling of that population. This technology has greatly improved the quality of data with regards to working with heterogeneous tissue in the field of genomics and proteomics.[46] LCM involves a light microscope and a near-infrared laser that transfers energy to a thermolabile polymer cap, which is placed on the tissue.[46] The operator uses laser pulses on the specific portion of tissue that is of interest, and then the cap is removed and the cells of interest are attached to the cap, and the remaining unwanted portion of the tissue is left behind.[46]

Many studies have shown the importance of using LCM to isolate cell populations instead of simply cutting a section of tissue and lysing the entire section, which can include fibroblasts, immune cells, nerve cells, and normal cells.[47] Each of the numerous cells found in a whole piece of tissue express different proteins and have their own signaling network, which can confound results and hinder the goal of finding a specific, effective druggable target for that individual patient.

Baldelli et al. analyzed 15 non-small cell lung carcinoma (NSCLC) tumors by having each sample represented by a whole tissue lysate and a matching LCM sample for

26 proteins, and found that 93% of the matched pairs clustered separately when analyzed by unsupervised clustering analysis.[48] Baldelli et al. also looked at EGFR, IGFR, MAPK, AKT-mTOR, and ALK pathway components, because these proteins are of significance in NSCLC.[48] They found 40% of the samples showed a difference equal to or greater than two quartiles when testing for EGFR Y1148, IGF-1R Y1135/ IR Y1146, and VEGFR Y951.[48] The percentage of starting tumor in each sample, regardless of how large, still impacted the accuracy of the signaling data, and they found that the surrounding microenvironment might strongly impact the overall cellular signaling measured.[48] Baldelli et al. also determined that the LCM process itself does not cause any difference in signaling by testing 15 analytes in 5 different tissues where the whole tissue was microdissected versus a whole tissue lysate that was not microdissected.[48] They found under unsupervised hierarchical clustering that the matched samples clustered together, which showed that the LCM process does not alter the signaling state of the sample.[48]

The comparison of microdissected and non-microdissected tissue has been studied in multiple types of cancers. Mueller et al. analyzed 133 signaling proteins in tumors from a study set from The Cancer Genome Atlas (TCGA) of 39 samples of glioblastoma to determine if non-microdissected material gave as accurate of results as microdissected material. They found that data from non-microdissected glioblastoma tumor is either masked or not accurate, producing correlations between genomic and proteomic data that lead to false classifications when stratifying patients for therapy.[47] Mueller et al. found 44% of the analytes tested differed between the non-microdissected and the microdissected samples, and of this 44% there were several targets for clinically

important inhibitors, such as phosphorylated mTOR, AKT, STAT1, VEGFR2, and BCL2.[47] Mueller et al. also determined that even samples with >90% tumor content, in 28% of the cases a patient would be falsely stratified and given the incorrect drug.[47] The ability to select the right patients for the right drug is one of the hallmarks of personalized medicine and LCM has been continually shown to be an integral step in the process of characterizing a patient's tumor.

Objectives of the Study

The goal of this study was to map the activating protein signaling network in papillary (non-invasive) and invasive bladder cancer and to identify what protein pathways were becoming activated or deactivated as the cells transition from non-invasive to invasive. In addition to comparing papillary (non-invasive) and invasive BC, LCM was used to separate the surrounding stroma next to the tumor epithelium in order to begin to identify which signaling pathways were activated in each of the tumor stroma and epithelial compartments within the same tumor background. Each BC case had an epithelium sample and a stroma sample that came from one patient.

CHAPTER TWO:MATERIALS AND METHODS

Tissue Collection

Twenty-three urothelium samples were provided by Dr. Donna Hansel, at the Cleveland Clinic in Cleveland, Ohio. Three of the samples were normal urothelium (N), four samples were carcinoma-in-situ (CIS), eight samples were high grade papillary (HGP), and eight samples were invasive high grade urothelial carcinoma (IHGUC). These samples were received in optimal cutting temperature compound (OCT) with dry ice, and then kept at -80°C while in storage.

Table 1 - Bladder Cancer Sample List

Histotype	Number of Samples
Normal	3
Carcinoma-in-situ	4
High Grade Papillary	8
Invasive High Grade Urinary Carcinoma	8
Total	23

Tissue Staining

Forty sections were cut using the CM1850UV cryostat (Leica, Buffalo Grove, IL) at an 8.0μm thickness and placed on plain, uncharged glass slides (Fisher, Pittsburgh, PA) and frozen at -80°C. For each sample, one slide was stained with Hematoxylin

(Sigma, St. Louis, MO) and Eosin (Sigma, St. Louis, MO) and examined by a certified pathologist, Dr. Lance Liotta, to confirm the presence of malignant, premalignant and normal cells along with a suitable amount of surrounding stroma.

Slides were fixed in 70% ethanol, rinsed with deionized water, stained with Hematoxylin (Sigma Aldrich, St. Louis, MO) and Scott's Tap Water (Electron Microscopy Sciences, Hatfield, PA), and dehydrated in increasing concentrations of ethanol (70%, 95%, and 100%) and xylene. Complete mini protease inhibitors (Roche Applied Science, Indianapolis, IN) were added to both 70% ethanol staining solutions, both deionized water solutions, and the hematoxylin and Scott's tap water solutions. Complete mini protease inhibitors (Roche Applied Science, Indianapolis, IN) are necessary to block proteolytic and phospholytic enzymes that breakdown proteins in the cell and will cause the loss of the phosphorylation site.

Tissue Microdissection

Laser capture microdissection (LCM) was performed using a PixCell II (Arcturus Bioscience, Mountain View, CA). For each sample, a Macro LCM cap (Applied Biosystems (Foster, CA) was used to collect approximately 15,000 cells from the tumor, and a separate Macro LCM cap was used to collect approximately 7,500 cells from the stroma. Caps were stored at -80°C until lysed. Microdissected cells were lysed on the Macro LCM cap using extraction buffer consisting of 50% tissue protein extraction reagent (TPER) (Thermo Scientific, Rockford, IL), 47.5% 2X tris-glycine SDS sample buffer (Invitrogen, Carlsbad, CA), and 2.5% β-mercaptoethanol (Fisher, Pittsburgh, PA).

TPER is a tissue cell lysis reagent that utilizes a proprietary detergent to maximize the efficiency of protein solubilization from mammalian tissue. Detergents break the lipid barrier surrounding cells by solubilizing proteins and disrupting lipid protein interactions.[49] Sodium Dodecyl Sulfate (SDS) is an anionic detergent that breaks down secondary structures like alpha-helices and beta-sheets, which are both primarily comprised of hydrogen bonds as well as many tertiary structures.[49] β-mercaptoethanol is a reducing agent that cleaves disulfide bonds and aids in the aqueous solubilization of proteins. Differences in cell morphology and dimension made it necessary to lyse cells from the different cell compartments in different amounts of buffer to reach a similar protein concentration across all samples of 0.125 – 0.25µg/µl.

Reverse Phase Protein Arrays (RPPA)

Cell lysates were immobilized onto nitrocellulose coated slides (Grace Bio-Labs, Bend, OR) using an Aushon 2470 arrayer (Aushon BioSystems, Billerica, MA). Each sample was printed in triplicate along with standard curves for internal quality control. Selected arrays were stained with Sypro Ruby Protein Blot Stain (Life Technologies, Eugene, OR) following manufacturing instructions in order to quantify the amount of protein present in each sample.[26] The remaining arrays were treated for 15 minutes at room temperature with the mild stripping reagent, Reblot Antibody Stripping solution (Millipore, Temecula, CA), in order to expose antigenic sites prior to antibody staining. The arrays were then washed twice for 5 minutes at room temperature in phosphate-buffered saline (PBS) (Life Technologies, Grand Island, NY), and incubated for 5 hours

in I-Block (Applied Biosystems, Foster, CA) in order to block non-specific binding sites on the nitrocellulose. Using a Dako Autostainer Universal Staining System (Dako Cytomation, Carpinteria, CA), arrays are first probed with 3% hydrogen peroxide, biotin blocking system (Dako Cytomation, Carpinteria, CA), and an additional serum free protein block (Dako Cytomation, Carpinteria, CA) to reduce non-specific binding between endogenous proteins and the detection system. Arrays were probed with 110 antibodies, of which 95 were phosphorylated. Antibodies were validated for their use on the array by Western Blot to determine antibody specificity. Only antibodies showing a single band at the expected molecular weight were used on the arrays.[50] Biotinylated anti-rabbit (Vector Laboratories, Inc., Burlingame, CA) or anti-mouse secondary antibody (CSA; Dako Cytomation, Carpinteria, CA) was used in conjunction with GenPoint™ kit (Dako Cytomation, Carpinteria, CA), a commercially available tyramide-based signal amplification system. The GenPoint kit amplification involves a streptavidin biotin complex binding to the biotinylated secondary antibody, which is attached to the primary antibody. Biotinyl tyramide then attaches to the the streptavidin biotin complex. Fluorescent detection was obtained through the use of IRDye 680RD Streptavidin (LI-COR Biosciences, Lincoln, NE) according to the manufacturer's recommendations. Antibody and Sypro Ruby stained slides were scanned on a Tecan laser scanner (TECAN, Mönnedorf, Switzerland) using the 620nm and 580nm weight length channel, respectively. Images were analyzed with MicroVigene Software Version 5.1.0.0 (Vigenetech, Carlisle, MA). The software performs spot finding along with subtraction of the local background and non-specific binding generated by the secondary antibody.

The program automatically normalizes each sample to the corresponding amount of protein derived from the Sypro Ruby stained slides and averages the triplicates.[51] The arrays are immunostained and the signal is amplified in order to generate a signal that is proportional to the concentration of the measured analyte.[26] Scanners are then used to acquire an image of the array, and software is used to generate a numeric value after spots are detected, intensity is measured, background is subtracted, and signal is normalized to total protein.

Table 2 - RPPA Antibodies (110 total)

Protein	Company	Catalog Number
4E-BP1 S65	Cell Signaling	9451
4E-BP1 T70	Cell Signaling	9455
Acetyl CoA Carboxylase S79	Cell Signaling	3661
AKT S473	Cell Signaling	9271
AKT T308	Cell Signaling	9275
ALK Y1586	Cell Signaling	3348
AMPKα1 S485	Cell Signaling	4184
AMPKβ1 S108	Cell Signaling	4181
A-Raf S299	Cell Signaling	4431
ASK1 S83	Cell Signaling	3761
B-Raf S445	Cell Signaling	2696
Bad S112	Cell Signaling	9291
Bad S155	Cell Signaling	9297
Bax	Cell Signaling	2772
β-Catenin T41 S45	Cell Signaling	9565
Bcl-2 S70	Cell Signaling	2827
BIM	Cell Signaling	2933
C-Raf S338	Cell Signaling	9427
Caspase 3 cleaved D175	Cell Signaling	9661
Caspase 6 cleaved D162	Cell Signaling	9761
Caspase 7 cleaved D198	Cell Signaling	9491
Caveolin 1	Santa Cruz	sc-984
CHK1 S345	Cell Signaling	2341

cKit Y703	Cell Signaling	3073
cKit Y719	Cell Signaling	3391
cMet	Abcam	ab51067
Cofilin S3	Cell Signaling	3313
Cox2	Cell Signaling	610203
cPLA2 S505	Cell Signaling	2831
CREB S133	Cell Signaling	9191
Cyclin B1	Cell Signaling	4135
Cyclin D1	Cell Signaling	2926
Cytochrome C	Stressgen	AAM-175
E-Cadherin	Cell Signaling	4065
EGFR	Cell Signaling	2232
EGFR Y1068	Cell Signaling	2234
EGFR Y1148	BioSource	44-792
EGFR Y1173	BioSource	44-794
Elk1 S383	Cell Signaling	9181
ERα	DAKO	M7047
ERα S118	Cell Signaling	2511
ErbB2 Y1248	Imgenex	90189
ErbB3 Y1289	Cell Signaling	4791
ERK T202 Y204	Cell Signaling	9101
FADD S194	Cell Signaling	2781
FGFR1 Y653 Y654	Cell Signaling	3471
FKHR S256	Cell Signaling	9461
FKHR T24 FKHRL1 T32	Cell Signaling	9464
FRS2α Y436	Cell Signaling	3861
GSK3αβ S21S9	Cell Signaling	9331
Hif1α	BD	610958
HSP90α (T5/7)	Cell Signaling	3488
IGF1Rβ Y1135 Y1136 IRβ Y1150 Y1151	Cell Signaling	3024
IκB alpha S32 36	Cell Signaling	9246
IL-10	Abcam	ab52909
IL-6	Biovision	5143-100
IRS1 S612	Cell Signaling	2386
JAK1 Y1022 1023	Cell Signaling	3331
LC3B	Cell Signaling	2775
LIMK1 T508 LIMK2 T505	Cell Signaling	3841
LKB1 S428	Cell Signaling	3051
MDM2 S166	Cell Signaling	3521
MEK 1/2 S217 S221	Cell Signaling	9121

Met Y1234 Y1235	Cell Signaling	3126
MGMT	Cell Signaling	2739
MMP9	Cell Signaling	3852
mTOR S2448	Cell Signaling	2971
NFkB p65 S536	Cell Signaling	3031
NPM T199	Cell Signaling	3541
p38 MAPK T180 Y182	Cell Signaling	9211
p53	Cell Signaling	9282
p53 S15	Cell Signaling	9284
p70S6K T412	Upstate	07-018
p90RSK S380	Cell Signaling	9341
PAK1 S199 S204 PAK2 S192 S197	Cell Signaling	2605
PARP Cleaved D214	Cell Signaling	9541
Paxillin Y118	Cell Signaling	2541
PD-L1	Cell Signaling	13684
PDGFRα Y754	Cell Signaling	2992
PDGFRβ	Upstate	06-498
PDGFRβ Y716	Upstate	07-021
PKCαβ T638 T641	Cell Signaling	9375
PKCζλ T410 403	Cell Signaling	9378
PLCγ1 Y783	Cell Signaling	2821
PRAS40 T246	Biosource	44-1100
PTEN	Cell Signaling	9552
PTEN S380	Cell Signaling	9551
Raf S259	Cell Signaling	9421
Ras GRF1 S916	Cell Signaling	3321
Rb S780	Cell Signaling	3590
Ret Y905	Cell Signaling	3221
Ron Y1353	Epitomics	5176-1
S6 Ribosomal Protein S235 S236	Cell Signaling	4856
S6 Ribosomal Protein S240 S244	Cell Signaling	2215
SAPK JNK T183 Y185	Cell Signaling	9251
SGK1 S78	Cell Signaling	5599
Shc Y317	Upstate	07-206
Smad 1/5/8 SS	Cell Signaling	9511
Snail	Cell Signaling	4719
Src Fam Y416	Cell Signaling	2101
Src Y527	Cell Signaling	2105
Stat3 S727	Cell Signaling	9134
Stat5 Y694	Cell Signaling	9351
TGFβ	Cell Signaling	3709

TNFα	Abcam	ab9635
TWIST	Santa Cruz	sc-81417
VEGFR2 Y1175	Cell Signaling	2478
VEGFR2 Y996	Cell Signaling	2474
Vimentin	Cell Signaling	3295
Zap70 Y319/ Syk Y352	Cell Signaling	2701

Data Analysis

Unsupervised hierarchical clustering analysis was utilized to examine whether histotypes of bladder cancer would group together, and to identify if proteins of a certain pathway were a driving force in those histotypes. The Wilcoxon-rank sum test was the nonparametric test used in the mean comparison to analyze the changes in the phosphorylation and expression levels of individual proteins in comparison of papillary epithelium and invasive epithelium in bladder cancer. Mean comparison was also used in comparison of papillary stroma and invasive stroma. All p values <0.05 were considered significant. Pairwise comparisons between all combinations of proteins tested was performed by Spearman Rho nonparametric correlation analysis to explore the interactions between the proteins within and across tumor epithelium and stroma. Correlation matrices were generated with JMP version 5.1 for:1) papillary tumor epithelium; 2) invasive tumor epithelium: 3) papillary stroma; 4) invasive stroma; 5) invasive epithelium and stroma. Correlation maps were then created using Gephi version 0.8.2. Only associations with a correlation coefficient ≥ 0.90 were included in the correlation maps. Correlation maps were created to display the protein interactions

within the cell compartments and in the case of the invasive epithelium, the protein interaction between the tumor epithelium and the stroma.

CHAPTER 3: RESULTS

Unsupervised hierarchical clustering analysis was first used to broadly explore the signaling network of the 2 different cellular compartment (tumor epithelium and surrounding stroma) across normal-appearing bladder tissue, CIS, papillary lesions, and invasive tumors.

Unsupervised Hierarchical Clustering of the Epithelium and Stroma across Histotypes

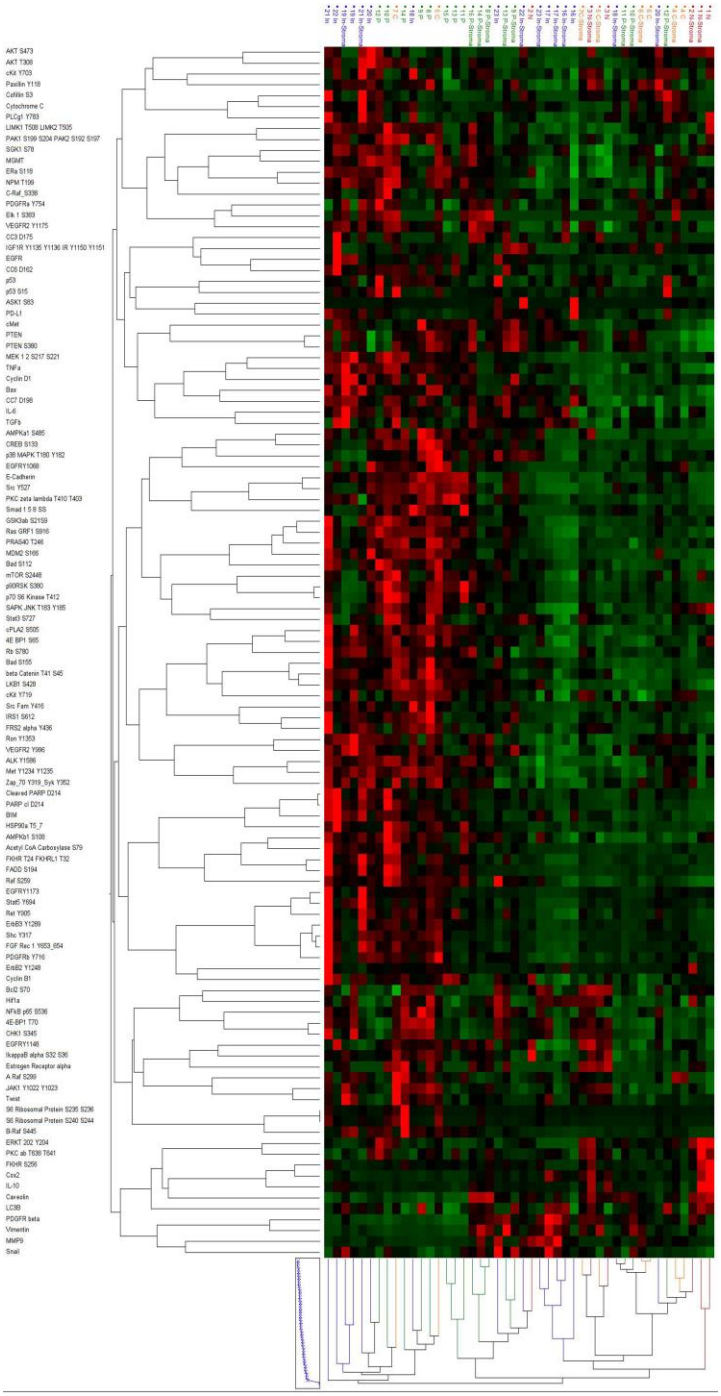


Figure 1 - Unsupervised hierarchical clustering analysis including all samples (normal bladder tissue: red; CIS: orange; papillary: green; invasive: blue) and cellular compartment (epithelium and stroma)

The unsupervised clustering analysis shows two major different clusters on initial separation (Figure 1). The first group consisted of high activation with the proteins tested, whereas the second group had a lower activation profile. The first high activation group consisted mainly of epithelium samples with a few invasive stroma cases (14.3%). Papillary and invasive histotypes did not separate from one another. Finally, some cases (n=2) showed both the epithelium and the stroma grouping together in the first high activation group (e.g. 19In and 19In-Stroma), whereas in other cases the epithelium did not group with its respective stroma (e.g. 22In in group 1 and 22In-Stroma in group 2).

Analysis of the epithelium compartment across papillary and invasive bladder cancer

Invasive and Papillary Epithelium Unsupervised Hierarchical Clustering

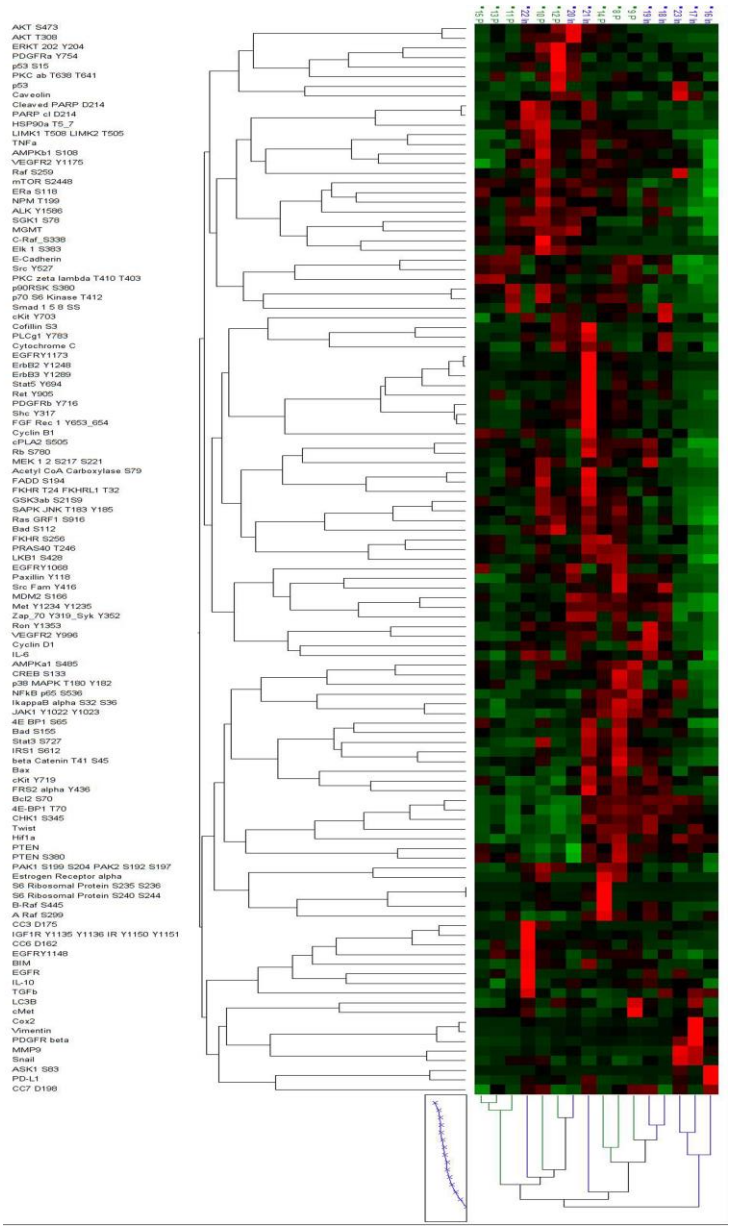


Figure 2 - Unsupervised hierarchical clustering analysis including papillary (green) and invasive (blue) epithelium only

The unsupervised clustering analysis showed two groups with initial separation also when the analysis was limited to the two most representative histotypes of the study set (Figure 2). One cluster showing overall low activation contained only invasive epithelium (n=3), while papillary and invasive epithelium comingled together in the second cluster. The first cluster, although characterized by low activation across the 110 proteins measured, show higher activation in eight proteins involved in angiogenesis (e.g. Cox2 and PDGFRβ) apoptosis (e.g. ASK1 S83 and CC7 D198), immunity (e.g. PD-L1) and EMT (e.g. Snail and Vimentin).

Mean comparison analysis of Papillary and Invasive Epithelium

Table 3 - p Value Significant (<0.05) Proteins in Invasive and Papillary Epithelium

Protein	Papillary (n=8)	Invasive (n=8)	p value
PD-L1		↑	0.0148
PDGFRβ		↑	0.0281
p90RSK S380	↑		0.006
PKCζλ T410 T403	↑		0.0104
Src Y527	↑		0.0281
SAPK JNK T183 Y185	↑		0.0303
AMPKα1 S485	↑		0.0047
E-Cadherin	↑		0.016
CREB S133	↑		0.0104
EGFR Y1148	↑		0.0194
p38 MAPK T180 Y182	↑		0.0281
p70S6K T412	↑		0.0009
PRAS40 T246	↑		0.0499
NPM T199	↑		0.0463

Of the 110 proteins measured in this analysis, 14 were statistically different between papillary and invasive lesions (Table 3) Specifically, PD-L1 and PDGFRβ expression was highest in the invasive tumors compared to the papillary lesions (p= 0.015 and p=0.028 respectively). PD-L1 and PDGFRβ expression, when compared over all histotypes, shows a clear increasing trend from normal through invasive, which raises the possibility that as the cancer becomes more invasive PD-L1 and PDGFRβ increase proportionally (Figure 3).

On the other hand, the MAPK signaling pathway was significantly higher in papillary tumor (e.g. EGFR Y1148 p = 0.019, p90RSK S380 p=0.006, and PKC ζλ T410 T403 p=0.01) along with E-Cadherin (p = 0.016), and the metabolic modulator AMPKα1 S485 (p < 0.01).

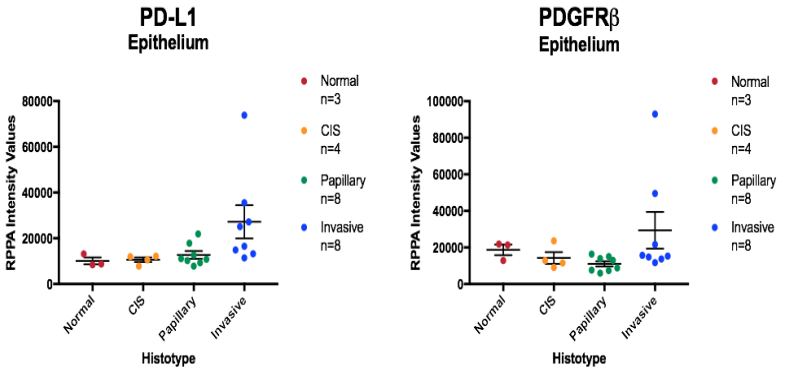


Figure 3 - PD-L1 and PDGFRb expression across all four histotypes

Correlation analysis and pathway maps of the epithelium of papillary and invasive bladder cancers

Non parametric Spearman Rho analysis was used to identify proteins that were strongly correlated within papillary and invasive tumors. Proteins with correlation coefficient greater than 0.9 were visualized using correlations maps as shown in figure 4 and 5.

Invasive Epithelium Correlation

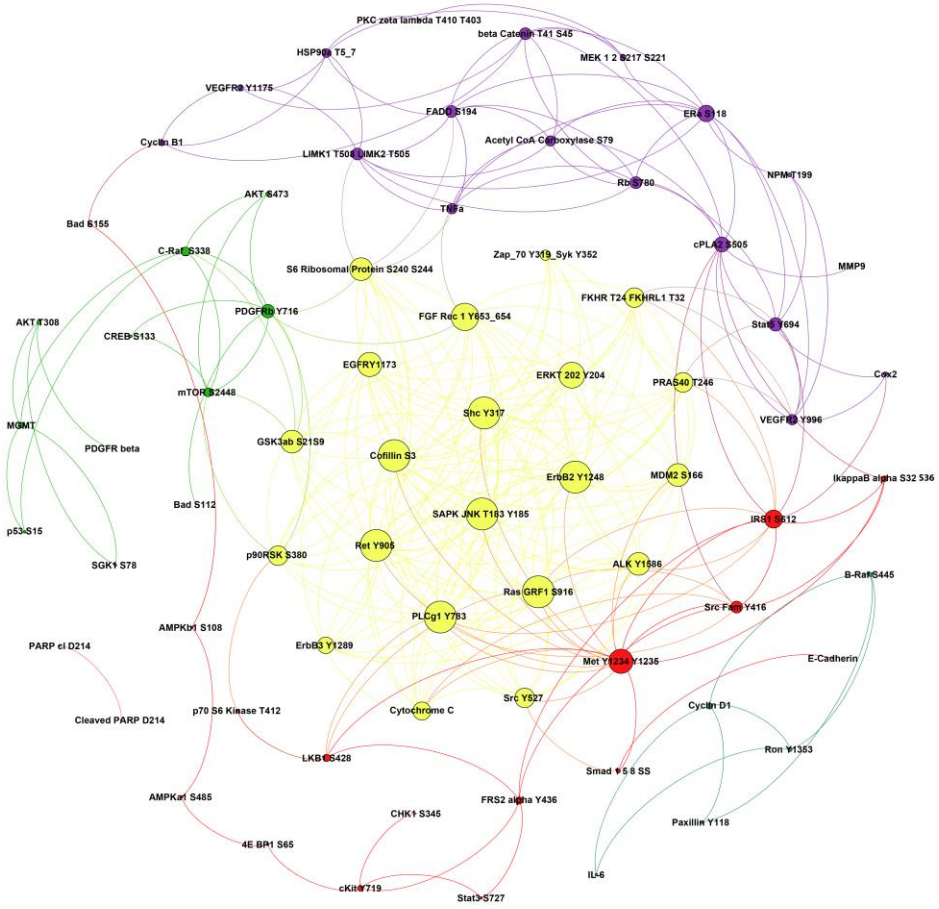


Figure 4 - Correlation maps for interconnection with a correlation coefficient >0.9 in the invasive epithelium

The correlation analyses identified a more active network, with a greater number of interconnection reaching correlation coefficient greater than 0.9, in the invasive tumors compared to the papillary lesions. Four major clusters were identified for the invasive (Figure 4) with the MAPK pathways appearing as a central knot, highly interconnected with a number of different pathways. The main cluster (yellow cluster) and a smaller cluster (red cluster) contained a number of interconnection between members of the MAPK and PI3K pathways, indicating a phenotype of proliferation and survival for this subgroup of tumors (Table 4). A third subgroup (purple cluster) was dominated with cross-talk between the MAPK pathway and proteins involved in apoptosis and angiogenesis (Table 4). Lastly, the green group was a mixture of MAPK proteins, PI3K, angiogenesis, and DNA proteins (Table 4). Each of the four main groups included proteins involved in immunity/inflammation (Table 4).

Table 4 - Pathways in Invasive Epithelium Correlation Colored Sections

Protein	Pathway	Immunity/ Inflammation	Protein	Pathway	Immunity/ Inflammation
EGFR Y1173	MAPK		ERα S118	MAPK	
ErbB3 Y1289	MAPK		MEK 1/2 S217/S221	MAPK	
ERK T202/Y204	MAPK	✓	Rb S780	MAPK	
p90RSK S380	MAPK		PKCζλ T410 T403	MAPK	✓
Ras GRF1 S916	MAPK	✓	Cox 2	Angiogenesis	✓
Ret Y905	MAPK		VEGFR2 Y1175	Angiogenesis	✓
SAPK JNK T183/Y185	MAPK	✓	VEGFR2 Y996	Angiogenesis	✓
Shc Y317	MAPK		FADD S194	Apoptosis	✓
Src Y527	MAPK	✓	STAT5 Y694	Apoptosis	✓
FKHR T24/FKHRL1 T32	PI3/AKT	✓	TNFα	Apoptosis	✓
GSK3ab S21/S9	PI3/AKT	✓	cPLA2 S505	Immunity	
PRAS 40	PI3/AKT		MMP9	Immunity	✓
S6 Rib Pro S240/S244	PI3/AKT		Cyclin B1	DNA	
ZAP70 Y319/SykY352	Immunity	✓	Acetyl CoA Carboxylase S79	Metabolism	
PLCγ1 Y783	Immunity	✓	β-Catenin T41/S45	Motility	
Cofillin S3	Motility		HSP90a T5/7	Other	
FGFR1 Y653/Y654	Angiogenesis		NPM T199	Other	
Cytochrome C	Apoptosis		Smad 1/5/8 SS	MAPK	
MDM2 S166	DNA		Src Fam Y416	MAPK	✓
ALK Y1586	Other		Met Y1234/Y1235	MAPK/PI3K	
C-Raf S338	MAPK		p70S6K T412	PI3/AKT	✓
CREB S133	MAPK		4EBP1 S65	PI3/AKT	
AKT S473	PI3/AKT	✓	AMPKα1 S485	Metabolism	✓
AKT T308	PI3/AKT	✓	AMPKβ1 S108	Metabolism	✓
mTOR S2448	PI3/AKT	✓	IRS1 S612	Metabolism	
SGK1 S78	PI3K		LKB1 S428	Metabolism	
PDGFRβ	Angiogenesis		Bad S155	Apoptosis	
PDGFRβ Y716	Angiogenesis		STAT3 S727	Apoptosis	✓
p53 S15	DNA		p53 S15	DNA	
MGMT	DNA		MGMT	DNA	
Bad S112	Apoptosis		CHK1 S345	DNA	
			IkBα S32/S36	Immunity	✓
			E-Cadherin	Motility	
			cKit Y719	OTHER	
			FRS2α Y436	OTHER	

Papillary Epithelium Correlation

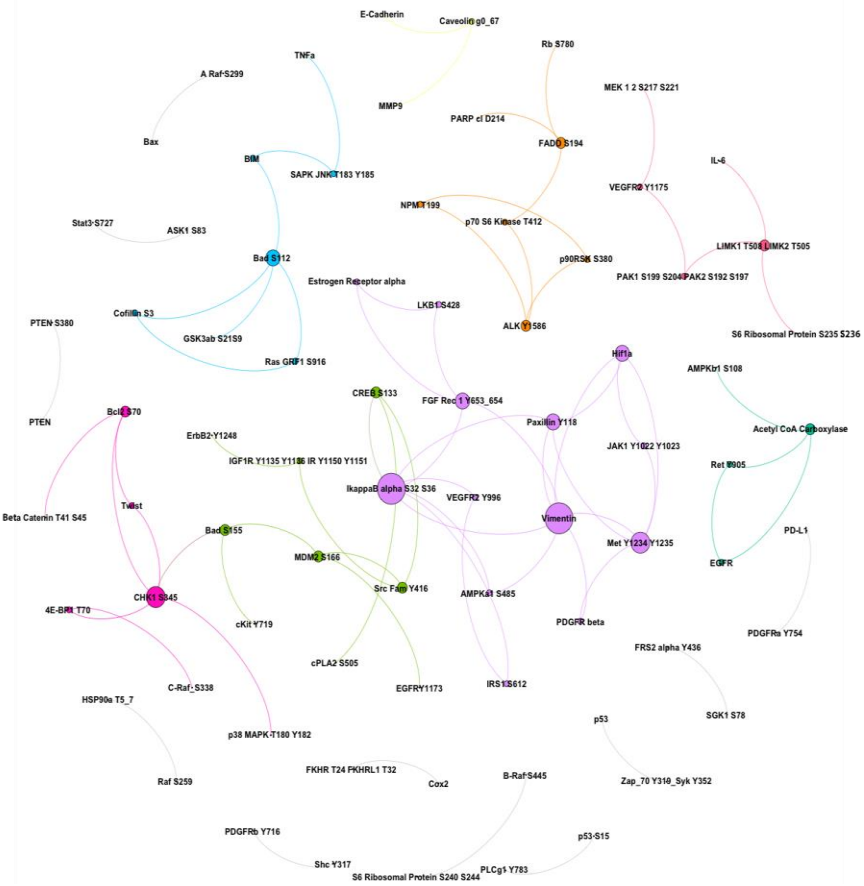


Figure 5 - Papillary Epithelium Correlation

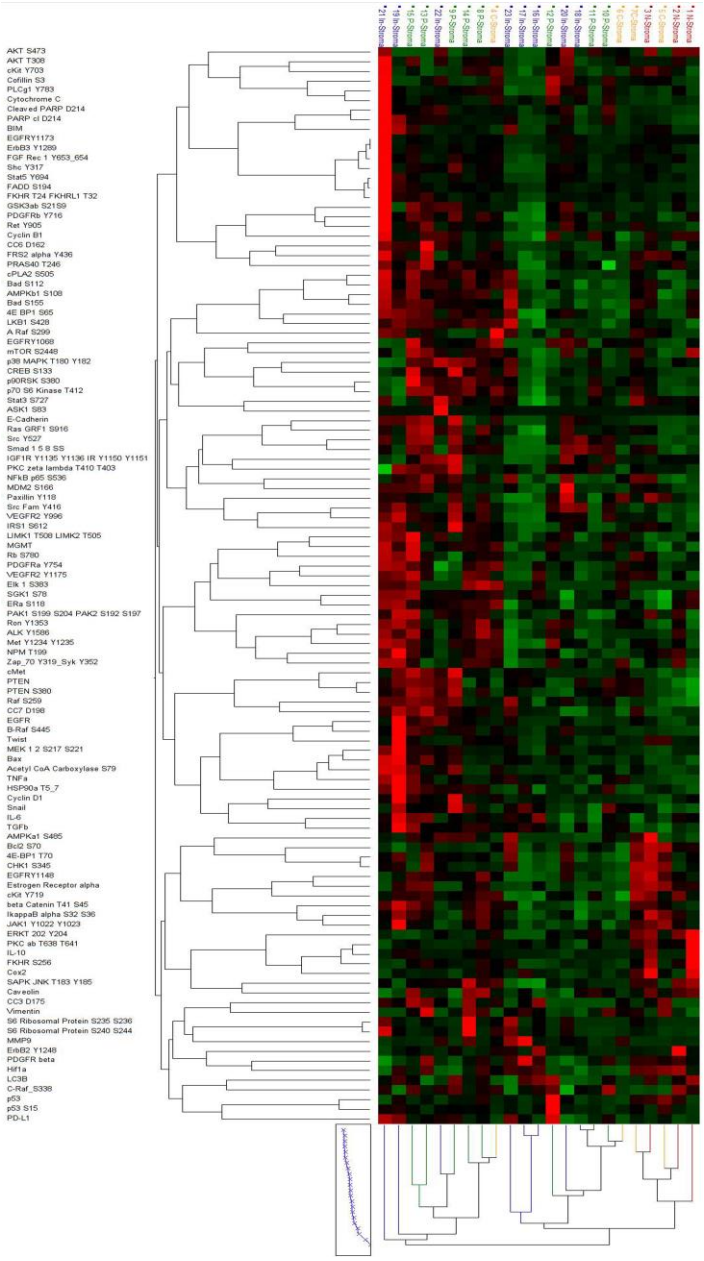
The papillary epithelium showed a less extensive cross-talk network compared to what was seen in the invasive epithelium correlation (Figure 5). The main group of proteins in the papillary epithelium were associated with wound healing and metabolism phenotype (Table 5).

Table 5 - Pathway in Papillary Epithelium Correlation Purple Subgroup

Protein	Pathway	Protein	Pathway
IkappaB α S32 S36	Wound Healing	LKB1 S428	Metabolism
Hif1α	Wound Healing	AMPKα S485	Metabolism
VEGFR Y996	Wound Healing	IRS1 S612	Metabolism
Vimentin	Wound Healing	JAK1 Y1022 Y1023	Angiogenesis
Paxillin Y118	Wound Healing	PDGFRβ	Angiogenesis
FGFR1 Y653 654	Wound Healing	MET Y1234 1235	Cytoskeletal
		Estrogen Receptor α	MAPK

Analysis of the stroma compartment across papillary and invasive bladder cancer

All Histotypes Stroma Unsupervised Hierarchical Clustering



When the stroma of all histotypes was analyzed using unsupervised hierarchical clustering analysis, the normal and CIS cases primarily grouped away from papillary and invasive cases, while the papillary and invasive stroma were comingled together showing no separation. This indicated that the stroma of malignant lesions has unique characteristics compared to the normal and in situ tissues.

Figure 6 - All Histotypes Stroma - Normal (Red) CIS (Orange) Papillary (Green) Invasive (Blue)

Mean comparison analysis of Papillary and Invasive Stroma

Of the 110 proteins measured in this analysis, 6 were statistically different between papillary and invasive lesions (Table 6). Specifically, MMP9 was the only protein with greater expression in the invasive compared to the papillary tumors (p=0.05). Of interest the level of MMP9 increased progressively with the increase of severity of the lesion (from normal lesion to invasive cancer) indicating that this protein may play an important role in bladder cancer carcinogenesis and tumor progression (Figure 7). On the other hand, downstream effectors of the MAPK pathway like SAPK/JNK T183/Y185 and p90RSK S380 were less activated in the invasive stroma (p=0.05 and 0.003 respectively). Of interest SAPK/JNK T183/Y185 was inversely proportional to the severity of the lesion (Figure 7).

Table 6 - p Value Significant (<0.05) Proteins in Papillary and Invasive Stroma

Protein	Papillary (n=8)	Invasive (n=8)	p Value
MMP9		↑	0.05
SAPK JNK T183 Y185	↑		0.05
Vimentin	↑		0.028
cKit Y719	↑		0.022
p70S6K T412	↑		0.01
p90RSK S380	↑		0.003

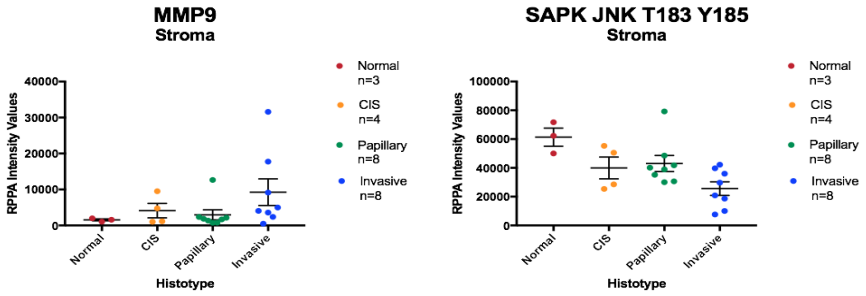


Figure 7 - All Histotypes - MMP9 and SAPK JNK T183 Y185 in Stroma

Correlation analysis and pathway maps of the epithelium of papillary and invasive bladder cancers

Non parametric Spearman Rho analysis was used to identify proteins that were strongly correlated within the papillary and invasive stroma. Proteins with correlation coefficient greater than 0.9 were visualized using correlations maps as shown in Figure 8 and 9.

Invasive Stroma Correlation

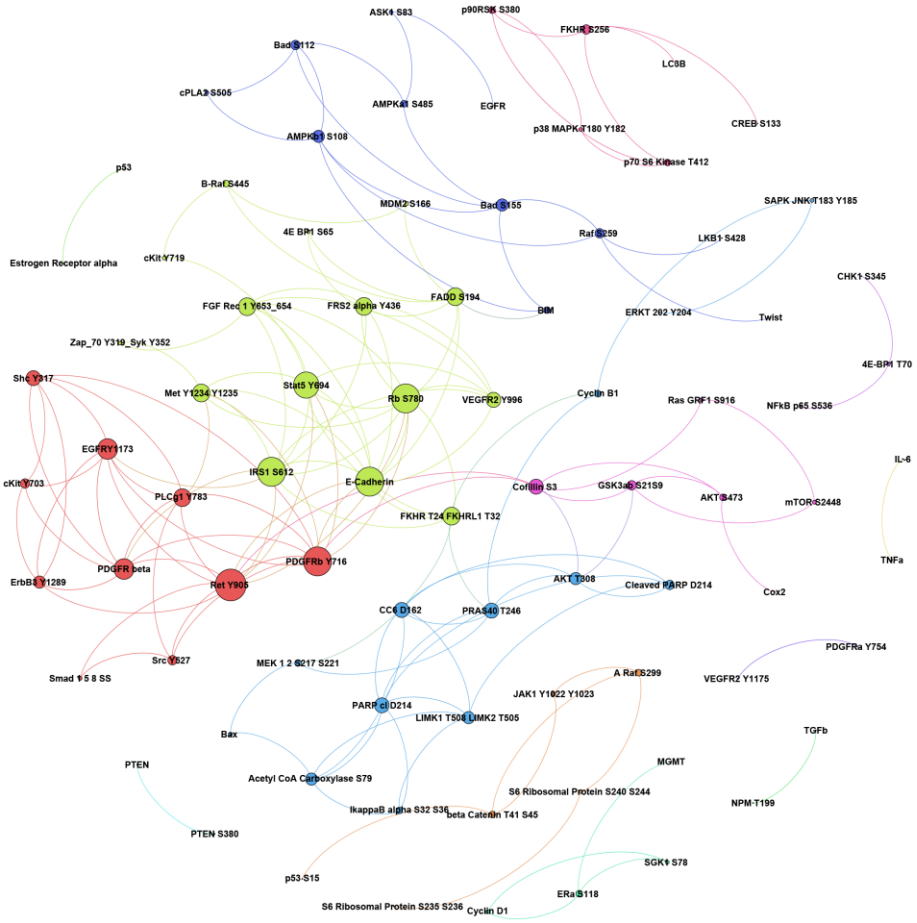


Figure 8 - Invasive Stroma Correlation

The correlation analysis showed more interconnections in the stroma of the invasive (Figure 8), than in the stroma of the papillary (Figure 9). Both invasive and papillary stroma (Figure 8 and 9) showed MAPK proteins in the red and yellow subgroups, respectively (Table 7). Invasive stroma showed more proteins involved with immunity/ inflammation (Table 7), which was lacking in the papillary stroma.

Table 7 - MAPK Proteins in Invasive Stroma (Red) and Papillary Stroma (Yellow)

Invasive Stroma MAPK Proteins	Papillary Stroma MAPK Proteins
EGFR Y1173	CREB S133
ErbB3 Y1289	ErbB3 Y1289
Ret Y905	Ret Y905
Shc Y317	Shc Y317
Smad 1/5/8 SS	EGFR
Src Y527	cMet
PLCγ1 Y783	

Papillary Stroma Correlation

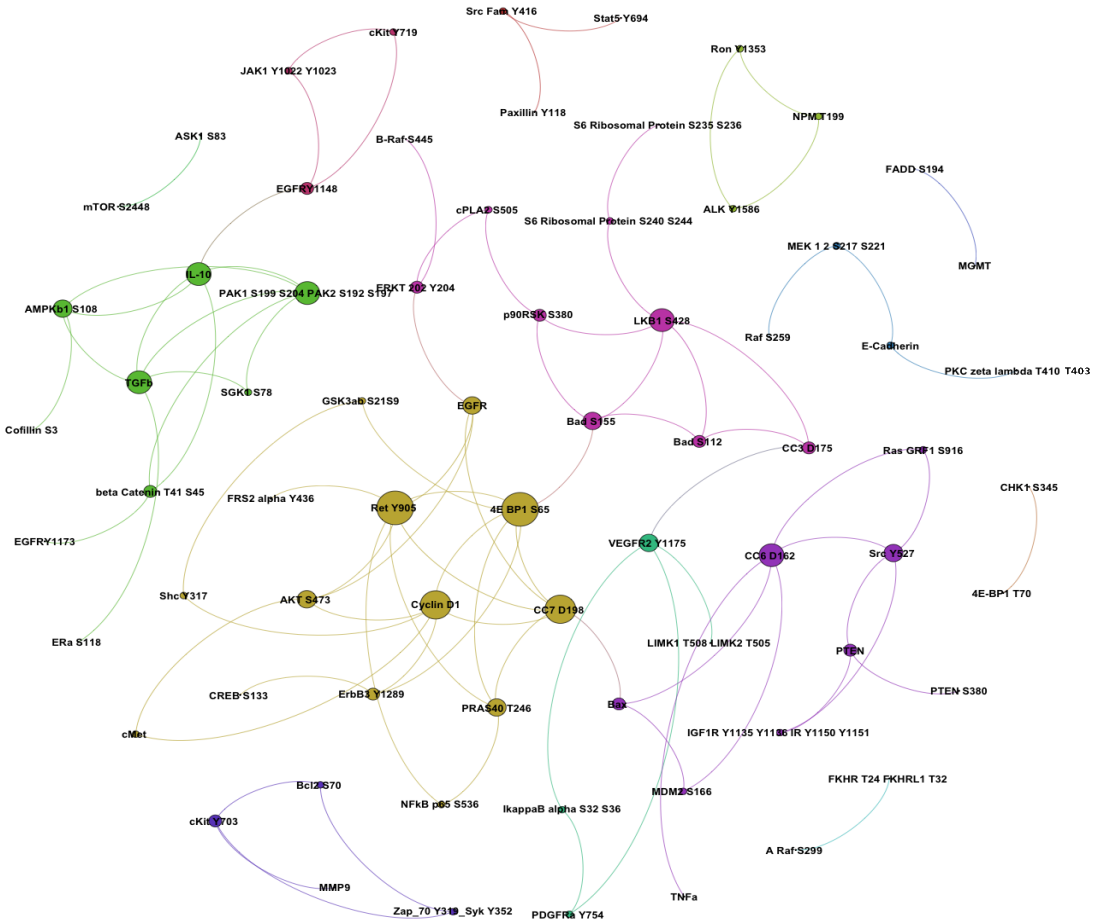


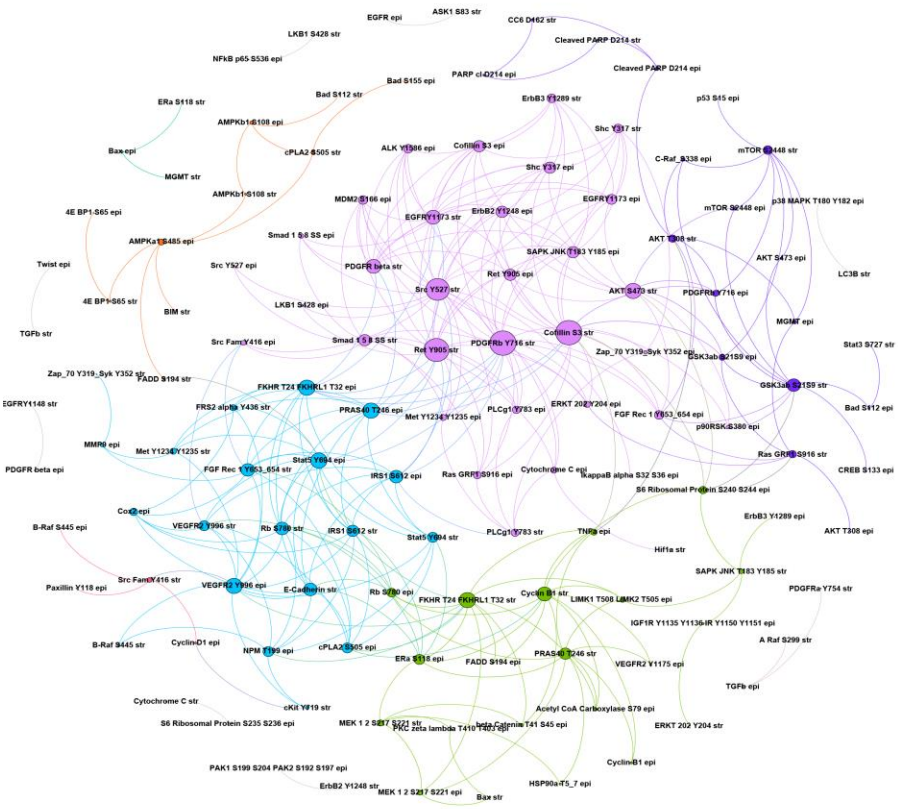
Figure 9 - Papillary Stroma Correlation

Table 8 - Pathways in Invasive Stroma Correlation Colored Subgroups

Protein	Pathway	Immunity/ Inflammation	Protein	Pathway	Immunity/ Inflammation
EGFR	MAPK		B-Raf S445	MAPK	
Raf S259	MAPK	✓	Rb S780	MAPK	
BAD S112	Apoptosis		4EBP1 S65	PI3K	
BAD S155	Apoptosis		FKHR T24 FKHRL1 T32	PI3K	✓
BIM	Apoptosis		Met Y1234/Y1235	MAPK/PI3K	
ASK S83	Apoptosis		VEGFR2 Y996	Angiogenesis	✓
AMPKα S485	Metabolism	✓	FGFR1	Angiogenesis	

			Y653/Y654		
AMPKβ1 S108	Metabolism	✓	FADD S194	Apoptosis	✓
LKB1 S428	Metabolism		Stat5 Y694	Apoptosis	✓
cPLA2 S505	Immunity	✓	MDM2 S166	DNA	
Twist	Motility		Zap70 Y319/ Syk Y352	Immunity	✓
EGFR Y1173	MAPK		IRS1 S612	Metabolism	
ErbB3 Y1289	MAPK		E-cadherin	Motility	
Ret Y905	MAPK		cKit Y719	OTHER	
Shc Y317	MAPK		FRS2α Y436	OTHER	
Smad 1/5/8 SS	MAPK		ERK T202/Y204	MAPK	✓
Src Y527	MAPK	✓	MEK 1/2 S217/S221	MAPK	
PLCγ1 Y783	MAPK	✓	SAPK JNK T183/Y185	MAPK	✓
PDGFRβ	Angiogenesis		AKT T308	PI3K	✓
PDGFRβ Y716	Angiogenesis		PRAS40 T246	PI3K	
ckit Y703	OTHER		Bax	Apoptosis	✓
Ras GRF1 S916	MAPK	✓	CC6 D162	Apoptosis	
AKT S473	PI3K	✓	Cyclin B1	DNA	
GSK3α/β S21/S9	PI3K	✓	PARP Cleaved D214	DNA	
mTOR S2448	PI3K	✓	Acetyl CoA Carboxylase S79	Metabolism	
Cox2	Angiogenesis	✓	IκBα S32/S36	Immunity	✓
			LIMK1 T508/LIMK2 T505	Motility	

Invasive Epithelium and Stroma Correlation



Finally, this work explored the interconnection between the tumor and the surrounding stroma of invasive bladder cancer, as shown in Figure 10. Only connections that were between stroma and epithelium were considered in analysis, and connections that were stroma-stroma or epithelium-epithelium

Figure 10 - Invasive Epithelium and Stroma Correlation

were not considered in order to only isolate possible cross-talk between the two compartments. The main purple cluster, seen in Figure 10, consisted of RTKs in both the epithelium and the stroma (Table 9). Furthermore, both cellular compartment RTKs have a connection with downstream targets in both the epithelium and stroma in the invasive cases.

Table 9 - Invasive Epithelium and Stroma Receptors and Down Stream Targets

Epithelium Receptors	Stroma Receptors	Epithelium Downstream Targets	Stroma Downstream Targets
Met Y1234 Y1235	Src Y527	PLCγ1 Y783	AKT S473
ErbB2 Y1248	PDGFRβ	ERK T202 Y204	PLCγ1 Y783
Ret Y905	PDGFRβ Y716	Shc Y317	Shc Y317
EGFR Y1173	ErbB3 Y1289	SAPK JNK T183 Y185	
ALK Y1586	Ret Y905	Ras GRF1 S916	
	EGFR Y1173	mTOR S2448	

CHAPTER FOUR: DISCUSSION

In this study, the goal was to map the activating protein signaling networks in papillary (non-invasive) and invasive bladder cancer and identify which pathways were becoming activated or deactivated as a consequence of the invasive process. These pathway changes could then be considered important drug target candidates. Isolation of the tumor epithelium compartment and stroma compartment from the surrounding microenvironment in order to evaluate two separate cell populations from each patient using LCM and RPPA allowed for an evaluation of possible cross-talk between the tumor epithelium and stroma cell compartments. The finding of an increased expression of PD-L1 in the invasive epithelium when compared to the papillary epithelium is of great interest since this is a key drug target for immunotherapeutics. This finding highlights the importance of determining how the immune system is reacting to the cancer in both the context of the epithelium and the stroma cellular compartments to determine the most effective druggable target.

Bladder cancer tumor epithelium

Upon analysis of the bladder epithelium, the invasive epithelium showed a much different phenotype than the papillary epithelium. Proliferation and survival proteins from the MAPK and PI3K pathways dominated the cross-talk in invasive epithelium, whereas in papillary epithelium, proteins that are involved in wound healing and

metabolism were more evident. Of the highly correlated proteins in the invasive epithelium, a number of proteins involved in regulating the immune system and inflammation were identified, which highlights the prospect of using immunotherapy drugs to effectively treat invasive bladder cancer. Additionally, the higher expression of PD-L1 and PDGFRβ in invasive epithelium versus papillary epithelium offer promising drug targets that are already approved by the FDA, e.g. Atezolizumab (Tecentriq, Genentech) and Sunitinib (Pfizer).

Programmed Death 1 (PD-1) and Programmed Death Ligand 1 (PD-L1)

The immune system must differentiate between normal cells in the body and foreign cells in order to destroy foreign cells, but not normal cells. There are molecules on some immune cells called checkpoint molecules that either have to be activated or inactivated before an immune response can begin.[52] PD-1 is a checkpoint protein on T cells that acts as an off switch that keeps T cells from attacking cells in the body. This is very beneficial during pregnancy, because the PD-1/ PD-L1 interaction is what protects the fetus from being attacked by the immune system.[53] In the body, there are cells continually being created and destroyed in a perfectly tuned balance that ensures damaged and mutated cells are not retained and new cells are only formed when needed. Cancer is an uncontrolled replication of cells, where the body is not destroying abnormal cells and is making more cells when it is not necessary. This surplus of cells continues to divide and form tumors. Cancer cells are able to recruit blood vessels into the tumor, which is called angiogenesis, and they are able grow and metastasize and divide indefinitely.[54] Tumor cells are also able to avoid immune surveillance which allows them

to not be eliminated from the host immune system.[53] In the 1940s and 1950s it was demonstrated that tumors have unique, tumor-specific antigens (TSAs).[55] [56] These studies showed that when inbred mice that had carcinogen-induced tumors underwent surgical resection, they were immune to subsequent re-challenge with the same tumor cells, but not with other distinct tumor cells.[55] [56] TSAs, also known as cancer neoantigens, are are not encoded in the normal host genome and can be either oncogenic viral proteins or abnormal proteins that arise from somatic mutations.[57] A somatic mutation is an alteration in DNA that occurs after conception, and can occur in any of the cells of the body except the germ cells, and therefore are not passed on to children.[57] During cancer initiation and progression, tumor cells acquire protein-altering mutations that are either responsible for transformation, which are driver mutations, or are a byproduct of the genomic instability that accompanies cellular transformation, which are passenger mutations.[58] These alterations can result in expression of mutant proteins that are perceived as foreign proteins by the immune system.[57] T cells in the immune system can recognize the cancer cells and then generate cytotoxic T lymphocytes (CTLs) to travel to and infiltrate the cancer cells.[53] The CTLs attach to the cancer cell and kill the cell.[53]

PD-1 is a cell surface molecule that regulates the adaptive immune response, and protects peripheral tissues from unnecessary inflammation.[53] PD-1 has two ligands PD-L1 and PD-L2, that transduces a signal that inhibits T-cell proliferation, cytokine production, and cytolytic function, which results in suppressing the immune system.[59] PD-L1 is found on resting T cells, B cells, macrophages, vascular endothelial cells and

dendritic cells. PD-L2 is found on dendritic cells and macrophages and is not as prevalent as PD-L1.[53] PD-L1 can be induced by IFN-γ, TNF-α, Lipopolysaccharides (LPS), Granulocyte macrophage colony stimulating factor (GM-CSF), VEGF, IL-4, and IL-10.[60] PD-L1 expression is suppressed by PTEN, but if the PTEN is mutated in the cancer then PD-L1 is no longer blocked, and the AKT pathway can be activated.

Immune Checkpoint Inhibitor Drugs

Advances in understanding the role of immune checkpoints in suppression of T-cell activation have led to the development of immune checkpoint inhibitors in the treatment of cancer. The blocking antibody against cytotoxic T-lymphocyte associated protein 4 (CTLA-4) ipilimumab (Yervoy, Bristol-Myers Squibb) and the antibodies against programmed death-1 (PD-1) nivolumab (Opdivo, Bristol-Myers Squibb) and pembrolizumab (Keytruda, Merck) have been approved by the United States Food and Drug Administration (FDA) to treat metastatic melanoma and non-small-cell lung cancer (NSCLC).[61] In May 2016, the FDA approved the first PD-L1 antagonist, atezolizumab (Tecentriq, Genentech) for the treatment of urothelial carcinoma, which is the most common type of bladder cancer.[62] PD-L1 has also been found in several types of cancers including melanoma, NSCLC, gastric cancer, and multiple myeloma.[63] [64] [65]

The use of Atezolizumab is approved for patients with locally advanced or metastatic urothelial carcinoma that has gotten worse after treatment with platinum chemotherapy, or during chemotherapy.[62] There has not been a new approved treatment for bladder cancer in the last 20 years.[62] Atezolizumab belongs to a class of

immunotherapy drugs known as check point inhibitors, and prevents PD-L1 on tumor cells, from binding to the receptor PD-1, on immune cells. By blocking this interaction, checkpoint inhibitors allow the immune system to attack tumors.[66]

The approval of atezolizumab was based on a study by Rosenberg JE et al., which included 310 patients with metastatic or locally advanced urothelial carcinoma whose cancers had intensified during or after treatment with platinum-containing chemotherapy or within 12 months of receiving platinum-containing chemotherapy, either before or after surgery.[67] Approximately 15% of patients had at least a partial shrinkage of their tumors, and this effect lasted from at least 2.1 months to more than 13.8 months.[67]

Bladder cancer stroma

MMP9, a protein associated with tumor invasion and progression, was increased in invasive stroma when compared to papillary stroma. This result correlates with other findings in the bladder cancer field that tumor invasion in the bladder is a process promoted by changes in the microenvironment, that includes downregulation of E-cadherin, and overexpression of MMP9.[68] Additionally, it was found that a more extensive cross-talk network exists in invasive stroma than in papillary stroma. Both histotypes used members of the MAPK pathway, however invasive stroma also had highly correlated proteins associated with immunity/ inflammation. Several RTKs in the invasive epithelium and stroma were identified along with downstream targets of those RTKs in the epithelium and stroma, which demonstrates that coupling LCM with RPPA in future larger studies could provide insight into how the tumor epithelium is affecting the stroma and vice versa.

Limitations of the Study

The human body has approximately 21,000 human protein coding genes, and the amount of proteins created by the body from those genes is at least three times that amount. The large number of proteins in the human proteome makes the selection of the appropriate panel of proteins for any signaling analysis very challenging. We chose our set of proteins and phosphoproteins based on their tumorigenic knowledge based roles in the defense mechanisms associated with the hallmarks of cancer. The amount of proteins known to be important in regulating innate and adaptive immunity could have been increased in order to get a more complete picture of the invasive signaling, and to try to determine which pathways were activating PD-L1. Although other groups have found evidence that the MAPK and PI3K pathway could play a role in activation of PD-L1 through the use of knock-out mice in melanoma, this study did not include enough samples to determine what activated PD-L1, only that PD-L1 expression was higher in invasive tumors in comparison to papillary tumors.[69] [70] An increase in the amount of samples in all histotypes of bladder cancer could also increase the statistical power of this study, and a higher number of normal cases could have allowed more insight into the change in signaling through the progression of the disease. With a limited number of normal cases it was only possible to look at trends in proteins such as PD-L1 and PDGFRβ, but mean comparison between normal and invasive cases was not possible. Clinical background information was also not available for this patient group, which

could have provided clinical outcomes, prior treatments received, and if any of the CIS or papillary patients progressed to invasive disease. Also, there is also no way to determine if the data was driven by age and gender, due to the lack of patient background information. Nonetheless, this study was the first of its kind to combine LCM with RPPA in the analysis of cross-talk between BC tumor epithelium and stroma compartments with the goal of elucidation of possible drug targets.

Limitations of Immunohistochemistry (IHC)

This is the first study in the field of bladder cancer to separate tumor epithelium from stroma in human tissues using LCM and map the signaling pathways using RPPA that provides a quantitative result for proteins both phosphorylated and non-phosphorylated in order to find FDA approved druggable targets that can be applied to the treatment of bladder cancer patients. Several studies have been conducted in the bladder cancer field that utilize IHC and tissue microarrays.[71] [72] [73] The biggest disadvantage of IHC is that it is an inherently subjective and semi-quantitative process, that depends on factors that are difficult to standardize.[74] For example, quality of microscope, illumination of microscope and individual human vision limitations.[74] Variability between pathologists within the same lab, or between different labs is a limitation for utilizing IHC to acquire reliable and reproducible results which is essential for assessing possible drug targets. In addition to variability, the technique is also limited by the scoring system it utilizes. The subjective determination of negative (0/1+), equivocal (2+), or positive (3+), does not allow for the subtle changes in protein expression level that can be found in patients.[75]

Conclusion

This study demonstrates that there is cross-talk occurring between the epithelium and stroma in invasive bladder cancer cases that is distinctive from non-invasive bladder cancer. The ability to use unique proteomic capabilities provided by LCM and RPPA technologies, allows us to interrogate clinical samples that provide information that animal models and cell lines can't provide. Furthermore, these methods lead us to identify a series of candidate proteins and phosphoproteins that appear to be highly activated in invasive bladder cancer and could serve as possible drug targets. Key findings in this study include an increased PD-L1 and PDGFRβ expression in the tumor epithelium of invasive bladder cancer when compared to papillary (non-invasive) bladder cancer. With the technical ability to isolate the stroma surrounding the tumor epithelium, we found increased expression of MMP9 in invasive bladder cancer when compared to papillary (non-invasive) bladder cancer. Additionally, we found that key pathways involved in invasive bladder cancer were not the same as papillary (non-invasive) bladder cancer in regards to both the tumor epithelium and the stroma. The tumor epithelium of the invasive cases was characterized by proteins involved in regulating the immune system and inflammation as well as proteins associated with proliferation and survival in the MAPK and PI3K/ AKT/ mTOR pathway. However, the papillary (non-invasive) bladder cases were characterized by proteins involved in wound healing and metabolism. Our proteomic analysis of the stroma in invasive and non-invasive bladder cancer cases revealed proteins involved in the MAPK pathway for both types of bladder cancer, however there was more extensive cross-talk observed in the invasive stroma. There were a large number of proteins found in the stroma surrounding the tumor epithelium in

invasive cases that were involved in immunity and inflammation. The key finding of proteins involved in the immune system in both the invasive tumor epithelium and the invasive stroma demonstrate the possibility of using immunotherapeutics to improve bladder cancer therapy for those patients with the most aggressive form of the disease. Also, our analysis of invasive tumor epithelium and the invasive stroma found RTKs in both the tumor epithelium and the stroma with affected downstream protein targets, uncovering possible cross-talk between the two cellular compartments. This finding highlights the possibility that matching the right drug to the right patient may require a combination therapy that counteracts the driving signaling networks the cancer is using in both the tumor epithelium and the surrounding stroma in order to minimize the mechanisms of resistance that the cancer can utilize to compensate for the effects of the drug. Most importantly, this study demonstrates how the utilization of proteomic techniques such as LCM and RPPA are uniquely suited for identifying the driving forces in a patient's cancer, selecting a targeted therapy, and delivering a long lasting personalized treatment.

REFERENCES

1. van der Horst, G., Bos, L. & van der Pluijm, G. Epithelial Plasticity, Cancer Stem Cells, and the Tumor-Supportive Stroma in Bladder Carcinoma. *Molecular Cancer Research* **10,** 995–1009 (2012).
2. Johnson, D. C., Greene, P. S. & Nielsen, M. E. Surgical Advances in Bladder Cancer: At What Cost? *Urologic Clinics of North America* **42,** 235–252 (2015).
3. Bladder cancer stages. Available at: http://www.cancer.org/cancer/bladdercancer/detailedguide/bladder-cancer-staging. (Accessed: 22nd November 2016)
4. Survival rates for bladder cancer. Available at: http://www.cancer.org/cancer/bladdercancer/detailedguide/bladder-cancer-survival-rates. (Accessed: 10th November 2016)
5. van Rhijn, B. W. G. *et al.* Recurrence and Progression of Disease in Non–Muscle-Invasive Bladder Cancer: From Epidemiology to Treatment Strategy. *European Urology* **56,** 430–442 (2009).
6. Stenzl, A. *et al.* Treatment of Muscle-invasive and Metastatic Bladder Cancer: Update of the EAU Guidelines. *European Urology* **59,** 1009–1018 (2011).

7. Gu, Y. *et al.* Network analysis of genomic alteration profiles reveals co-altered functional modules and driver genes for glioblastoma. *Molecular BioSystems* **9,** 467 (2013).
8. Kolch, W. & Pitt, A. Functional proteomics to dissect tyrosine kinase signalling pathways in cancer. *Nat Rev Cancer* **10,** 618–629 (2010).
9. Bononi, A. *et al.* Protein Kinases and Phosphatases in the Control of Cell Fate. *Enzyme Res* **2011,** (2011).
10. Cheng, H.-C. *et al.* Regulation and Function of Protein Kinases and Phosphatases, Regulation and Function of Protein Kinases and Phosphatases. *Enzyme Research, Enzyme Research* **2011, 2011,** e794089 (2011).
11. Ubersax, J. A. & Ferrell Jr, J. E. Mechanisms of specificity in protein phosphorylation. *Nature Reviews Molecular Cell Biology* **8,** 530–541 (2007).
12. Petricoin, E. F. *et al.* Mapping Molecular Networks Using Proteomics: A Vision for Patient-Tailored Combination Therapy. *JCO* **23,** 3614–3621 (2005).
13. Graves, P. R. & Haystead, T. A. J. Molecular Biologist's Guide to Proteomics. *Microbiol Mol Biol Rev* **66,** 39–63 (2002).
14. Pandey, A. & Mann, M. Proteomics to study genes and genomes. *Nature* **405,** 837–846 (2000).
15. Hunter, T. Signaling—2000 and Beyond. *Cell* **100,** 113–127 (2000).
16. Paul, M. K. & Mukhopadhyay, A. K. Tyrosine kinase – Role and significance in Cancer. *Int J Med Sci* **1,** 101–115 (2004).

17. Lemmon, M. A. & Schlessinger, J. Cell signaling by receptor-tyrosine kinases. *Cell* **141,** 1117–1134 (2010).
18. Gschwind, A., Fischer, O. M. & Ullrich, A. The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat Rev Cancer* **4,** 361–370 (2004).
19. Jr, C. A. J. *et al. Immunobiology*. (Garland Science, 2001).
20. Danilova, N. The evolution of adaptive immunity. *Adv. Exp. Med. Biol.* **738,** 218–235 (2012).
21. Cantrell, D. A. T-cell antigen receptor signal transduction. *Immunology* **105,** 369–374 (2002).
22. Pasare, C. & Medzhitov, R. Toll-like receptors: linking innate and adaptive immunity. *Microbes and Infection* **6,** 1382–1387 (2004).
23. Newton, K. & Dixit, V. M. Signaling in Innate Immunity and Inflammation. *Cold Spring Harb Perspect Biol* **4,** a006049 (2012).
24. Jouhi, L. *et al.* Different Toll-Like Receptor Expression Patterns in Progression toward Cancer. *Front Immunol* **5,** (2014).
25. Basith, S., Manavalan, B., Yoo, T. H., Kim, S. G. & Choi, S. Roles of toll-like receptors in Cancer: A double-edged sword for defense and offense. *Archives of Pharmacal Research* **35,** 1297–1316 (2012).
26. Pin, E., Federici, G. & Petricoin, E. F. in *Current Protocols in Protein Science* (John Wiley & Sons, Inc., 2001).
27. Pierobon, M., Wulfkuhle, J., Liotta, L. & Petricoin, E. Application of molecular technologies for phosphoproteomic analysis of clinical samples. *Oncogene* (2014).

28. Paweletz, C. P. *et al.* Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. *Oncogene* **20,** 1981–1989 (2001).
29. Rapkiewicz, A. *et al.* The needle in the haystack: Application of breast fine-needle aspirate samples to quantitative protein microarray technology. *Cancer Cytopathology* **111,** 173–184 (2007).
30. Mak, I. W., Evaniew, N. & Ghert, M. Lost in translation: animal models and clinical trials in cancer treatment. *Am J Transl Res* **6,** 114–118 (2014).
31. Schuh, J. C. L. Trials, Tribulations, and Trends in Tumor Modeling in Mice. *Toxicol Pathol* **32,** 53–66 (2004).
32. Attarwala, H. TGN1412: From Discovery to Disaster. *J Young Pharm* **2,** 332–336 (2010).
33. Suntharalingam, G. *et al.* Cytokine Storm in a Phase 1 Trial of the Anti-CD28 Monoclonal Antibody TGN1412. *New England Journal of Medicine* **355,** 1018–1028 (2006).
34. Wagner, A. *et al.* Results from a phase 2 randomized, placebo-controlled, double blind study of the hedgehog (HH) pathway antagonist IPI-926 in patients (PTS) with advanced chondrosarcoma (CS). (2013).
35. Lee, M. J. *et al.* Hedgehog pathway inhibitor saridegib (IPI-926) increases lifespan in a mouse medulloblastoma model. *Proc Natl Acad Sci U S A* **109,** 7859–7864 (2012).
36. Masters, J. R. HeLa cells 50 years on: the good, the bad and the ugly. *Nat Rev Cancer* **2,** 315–319 (2002).

37. Hughes, P., Marshall, D., Reid, Y., Parkes, H. & Gelber, C. The costs of using unauthenticated, over-passaged cell lines: how much more data do we need? *BioTechniques* **43,** 575, 577–578, 581–582 passim (2007).
38. Villasante, A. & Vunjak-Novakovic, G. Tissue-engineered models of human tumors for cancer research. *Expert Opin Drug Discov* **10,** 257–268 (2015).
39. Edmondson, R., Broglie, J. J., Adcock, A. F. & Yang, L. Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery and Cell-Based Biosensors. *Assay Drug Dev Technol* **12,** 207–218 (2014).
40. Li, H., Fan, X. & Houghton, J. Tumor microenvironment: the role of the tumor stroma in cancer. *J. Cell. Biochem.* **101,** 805–815 (2007).
41. Alberts, B. *et al.* Fibroblasts and Their Transformations: The Connective-Tissue Cell Family. (2002).
42. Hayward, S. W. *et al.* Malignant Transformation in a Nontumorigenic Human Prostatic Epithelial Cell Line. *Cancer Res* **61,** 8135–8142 (2001).
43. Kurtova, A. V. *et al.* Stromal-mediated collagen I signal in promoting bladder cancer progression. *Cancer Research* **74,** 4801–4801 (2014).
44. Valiathan, R. R., Marco, M., Leitinger, B., Kleer, C. G. & Fridman, R. Discoidin domain receptor tyrosine kinases: new players in cancer progression. *Cancer Metastasis Rev.* **31,** 295–321 (2012).
45. Olumi, A. F. *et al.* Carcinoma-associated Fibroblasts Direct Tumor Progression of Initiated Human Prostatic Epithelium. *Cancer Res* **59,** 5002–5011 (1999).
46. Espina, V. *et al.* Laser-capture microdissection. *Nat Protoc* **1,** 586–603 (2006).

47. Mueller, C. *et al.* Glioblastoma Cell Enrichment Is Critical for Analysis of Phosphorylated Drug Targets and Proteomic–Genomic Correlations. *Cancer Res* **74,** 818–828 (2014).
48. Baldelli, E. *et al.* Impact of upfront cellular enrichment by laser capture microdissection on protein and phosphoprotein drug target signaling activation measurements in human lung cancer: Implications for personalized medicine. *Proteomics Clin Appl* **9,** 928–937 (2015).
49. Seddon, A. M., Curnow, P. & Booth, P. J. Membrane proteins, lipids and detergents: not just a soap opera. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1666,** 105–117 (2004).
50. Signore, M. & Reeder, K. A. Antibody validation by Western blotting. *Methods Mol. Biol.* **823,** 139–155 (2012).
51. Baldelli, E. *et al.* Functional signaling pathway analysis of lung adenocarcinomas identifies novel therapeutic targets for KRAS mutant tumors. *Oncotarget* **6,** 32368–32379 (2015).
52. Riella, L. V., Paterson, A. M., Sharpe, A. H. & Chandraker, A. Role of the PD-1 pathway in the immune response. *Am. J. Transplant.* **12,** 2575–2587 (2012).
53. He, J., Hu, Y., Hu, M. & Li, B. Development of PD-1/PD-L1 Pathway in Tumor Immune Microenvironment and Treatment for Non-Small Cell Lung Cancer. *Sci Rep* **5,** (2015).

54. Hochwald, S. N., Bloom, D., Golubovskaya, V. & Cance, W. G. in *Surgery* (eds. FACS, J. A. N. M. et al.) 1933–1945 (Springer New York, 2008). doi:10.1007/978-0-387-68113-9_93

55. Foley, E. J. Antigenic Properties of Methylcholanthrene-induced Tumors in Mice of the Strain of Origin. *Cancer Res* **13,** 835–837 (1953).

56. Gross, L. Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. *Cancer Research* **3,** 326–333 (1943).

57. Gubin, M. M., Artyomov, M. N., Mardis, E. R. & Schreiber, R. D. Tumor neoantigens: building a framework for personalized cancer immunotherapy. *J Clin Invest* **125,** 3413–3421 (2015).

58. Greenman, C. *et al.* Patterns of somatic mutation in human cancer genomes. *Nature* **446,** 153–158 (2007).

59. Riley, J. L. PD-1 signaling in primary T cells. *Immunol Rev* **229,** 114–125 (2009).

60. Taube, J. M. *et al.* Colocalization of Inflammatory Response with B7-H1 Expression in Human Melanocytic Lesions Supports an Adaptive Resistance Mechanism of Immune Escape. *Sci Transl Med* **4,** 127ra37 (2012).

61. Chen, J., Jiang, C. C., Jin, L. & Zhang, X. D. Regulation of PD-L1: a novel role of pro-survival signalling in cancer. *Ann Oncol* **27,** 409–416 (2016).

62. Ratner, M. Genentech's PD-L1 agent approved for bladder cancer. *Nat Biotech* **34,** 789–790 (2016).

63. Spranger, S. *et al.* Up-Regulation of PD-L1, IDO, and Tregs in the Melanoma Tumor Microenvironment Is Driven by CD8+ T Cells. *Sci Transl Med* **5,** 200ra116 (2013).

64. Bernstein, M. B. *et al.* Radiation-Induced Modulation of Costimulatory and Coinhibitory T-Cell Signaling Molecules on Human Prostate Carcinoma Cells Promotes Productive Antitumor Immune Interactions. *Cancer Biother Radiopharm* **29,** 153–161 (2014).

65. Thompson, R. H. *et al.* Costimulatory molecule B7-H1 in primary and metastatic clear cell renal cell carcinoma. *Cancer* **104,** 2084–2091 (2005).

66. Patel, S. P. & Kurzrock, R. PD-L1 Expression as a Predictive Biomarker in Cancer Immunotherapy. *Mol Cancer Ther* **14,** 847–856 (2015).

67. Rosenberg, J. E. *et al.* Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. *Lancet* **387,** 1909–1920 (2016).

68. Dong, Z. *et al.* Differential regulation of matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2 expression in co-cultures of prostate cancer and stromal cells. *Int. J. Cancer* **93,** 507–515 (2001).

69. Jiang, X., Zhou, J., Giobbie-Hurder, A., Wargo, J. & Hodi, F. S. The Activation of MAPK in Melanoma Cells Resistant to BRAF Inhibition Promotes PD-L1 Expression That Is Reversible by MEK and PI3K Inhibition. *Clin Cancer Res* **19,** 598–609 (2013).

70. Sumimoto, H., Imabayashi, F., Iwata, T. & Kawakami, Y. The BRAF–MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. *J Exp Med* **203,** 1651–1656 (2006).

71. He, X. *et al.* Differentiation of a Highly Tumorigenic Basal Cell Compartment in Urothelial Carcinoma. *Stem Cells* **27,** 1487–1495 (2009).

72. Liu, A. Y. *et al.* Bladder expression of CD cell surface antigens and cell-type-specific transcriptomes. *Cell Tissue Res* **348,** 589–600 (2012).

73. Sjödahl, G. *et al.* Infiltration of CD3+ and CD68+ cells in bladder cancer is subtype specific and affects the outcome of patients with muscle-invasive tumors1. *Urologic Oncology: Seminars and Original Investigations* **32,** 791–797 (2014).

74. O'Hurley, G. *et al.* Garbage in, garbage out: A critical evaluation of strategies used for validation of immunohistochemical biomarkers. *Molecular Oncology* **8,** 783–798 (2014).

75. Gown, A. M. Current issues in ER and HER2 testing by IHC in breast cancer. *Mod Pathol* **21,** S8–S15 (2008).

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