Role of the PI3K/Akt pathway in human renal cell carcinoma

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Biochemistry

To
The Honors College
Oakland University

In partial fulfillment of the
requirement to graduate from
The Honors College

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(May 14, 2014)

Abstract

Renal cell carcinoma (RCC) is the most common form of renal cancer. Currently, RCC accounts for 9 out of 10 cases of kidney cancer diagnosed in the United States. According to the American Cancer Society, there were approximately 65,150 new cases in 2013 and 13,680 deaths were expected from this disease. Current data clearly shows that RCC responds very poorly to current chemotherapy and radiation treatment options. This results in invasive surgery being the only viable option in many cases. Therefore, there is an urgent need for better clinical treatment options. As with other forms of cancer, there have been suggestions of altered functioning of intracellular signaling pathways responsible for the pathogenesis of RCC. Treatment with drugs that inhibit these particular pathways could lead to apoptosis of renal cancer cells and improvement in patient outcomes. Since PI3K and members of its intracellular signaling cascade are often activated in different cancers, we investigated whether this pathway was involved in RCC. Our study is the first to reveal the activation of PI3K pathway in human RCC biopsies with a comparison across all grades of cancer.

Introduction

Function and Structure of the Kidney

The kidneys are the body's major organs that remove excess fluid and metabolic waste products. The kidneys work to produce urine, a liquid by-product that consists of 95% water and 5% dissolved gases and solids, which includes nitrogenous wastes (urea, uric acid, ammonium, and creatinine), electrolytes, and pigments from drugs and food [1]. Once made, urine is delivered to the bladder via smooth muscle tubes called the ureters and eventually excreted from the body through the urethra. In addition to urine production, the kidneys regulate the acid, salt, and potassium levels in the body [2] and release hormones to stimulate the production of red blood cells, maintain calcium metabolism, and control blood pressure [3].

Anatomically, the kidneys are located below the rib cage at the lower back of the body. A cross section of the kidney reveals three primary sections: the renal medulla, cortex, and pelvis (Figure 1). The renal pelvis serves as a funnel to collect and transfer the urine into the ureters [4]. The renal medulla and cortex both contain millions of tiny, tubular structures called nephrons, the basic functional units of the kidney. Here, the blood is filtered to regulate the concentration of water and salts in the body by removing wastes and excess water and reabsorbing ions whenever they run low [5].

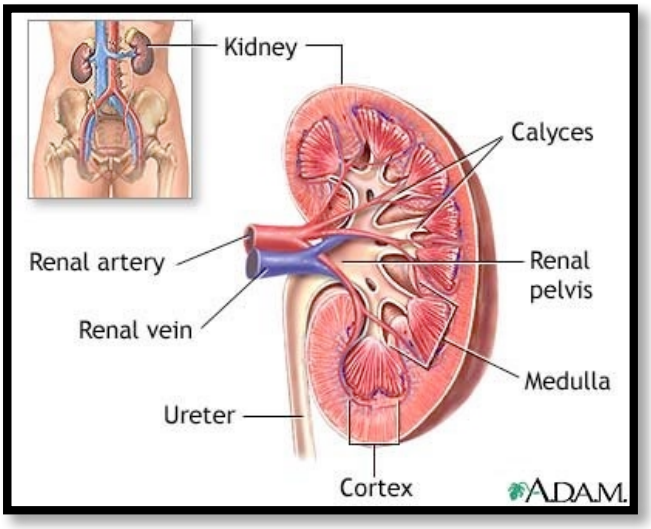

Figure 1. Cross section of the kidney showing the three main sections. Source: http://wikis.lib.ncsu.edu

The nephron consists of two primary components: the glomerulus and the renal tubule (Figure 2). The glomerulus is a capillary or tiny blood vessel that acts as a sieve to keep proteins and cells retained within the bloodstream and filter out extra fluid and wastes, which are transferred to the Bowman's capsule [2]. The Bowman's capsule is a sac-like structure that surrounds the glomerulus, serving as the start of the tubular region and initiating the first steps in filtration to form urine. The rate at which filtrate is formed by the glomerulus per minute is called the glomerular filtration rate (GFR). A high GFR (>90 mL/min/1.73 m^2) can lead to kidney damage mainly from proteinuria, a condition where an abnormally high amount of protein is found in the urine [6]. On the other hand, a low GFR (<15 mL/min/1.73 m^2) indicates kidney failure, necessitating a kidney transplant or dialysis [6], the latter of which is essentially an artificial means of filtering the blood.

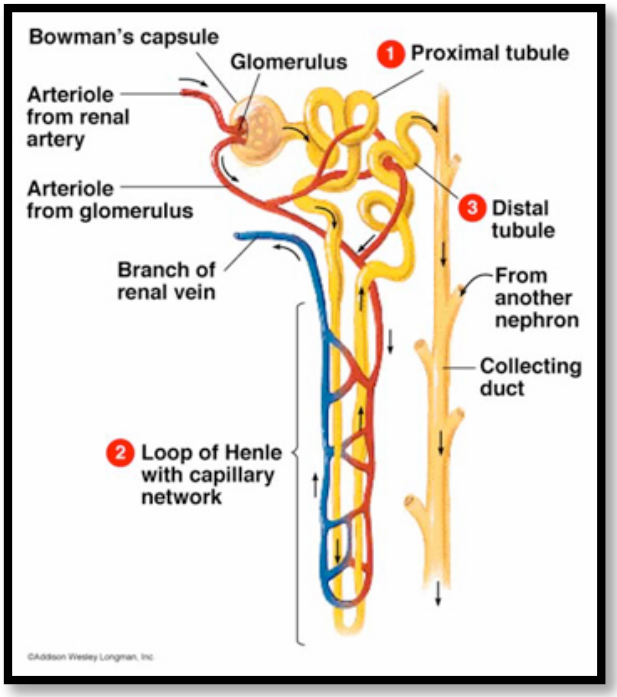

Figure 2. Diagram of the structure of the nephron. Source: www.gloucesterhs.ocdsb.ca

From the Bowman's capsule, the filtrate enters the proximal convoluted tubule. Here, all of the amino acids and glucose as well as a majority of salts (sodium and potassium) and water are reabsorbed from the filtrate [7]. In addition, bicarbonate ions in the filtrate are exchanged with secreted hydrogen ions, which helps to regulate the pH of the filtrate. Fluid in the filtrate then moves to the thin descending limb of loop of Henle. This section of the loop of Henle has a high permeability to water and a low permeability to ions and urea, causing water to be

reabsorbed passively and thereby concentrating the urine into a hypertonic state [7]. The filtrate is then transported to the thin ascending limb of loop of Henle, where the permeability of water and ions is reversed, allowing ions such as sodium and chloride to be reabsorbed by diffusion [7]. Following the thin ascending limb, the thick ascending limb of loop of Henle is also impermeable to water, but instead actively reabsorbs sodium, potassium, and chloride ions, which dilutes the urine [7].

After the loop of Henle, the filtrate is moved to the distal convoluted tubule (DCT). Like the proximal convoluted tubule, the DCT also regulates the pH of the filtrate by secreting hydrogen ions and absorbing bicarbonate. Potassium is secreted and sodium is actively reabsorbed, the latter process mediated by the steroid hormone aldosterone [8]. Although the DCT is fairly impermeable to water, the presence of antidiuretic hormone (ADH) can increase its permeability, causing the urine to become more concentrated [7]. From the DCT, the filtrate is transported to the collecting ducts, a series of tubules that connect the nephron to the ureters. The collecting duct system is composed of two types of cells: principal cells and intercalated cells [7]. The principal cells are involved in secreting potassium and reabsorbing sodium, a process regulated by aldosterone, as well as reabsorbing water, which is regulated by ADH [8]. The intercalated cells play a role in acid-base homeostasis by secreting hydrogen ions and reabsorbing potassium [7]. At this stage, the urine has an optimal balance of ions and water and is taken via the ureters to the bladder to be stored and eventually excreted from the body through the urethra.

Background on Cancer

Cancer is defined as the uncontrollable growth and division of abnormal cells. These

rapidly dividing cells tend to cluster together to form a growth of tissue called a tumor. Tumors can be classified as benign, meaning that they are noncancerous and do not spread, or malignant, meaning that they are cancerous and spread throughout the body destroying neighboring tissues. Those that are malignant can be locally invasive or metastatic. Locally invasive tumors invade neighboring tissues while metastatic tumors migrate from one region of the body to another, primarily through blood and/or lymphatic vessels [9, 10]. There are currently more than 100 known types of cancer, each generally named for the organ of origin [10]. Each type of cancer can be grouped into one of five primary categories (Figure 3).

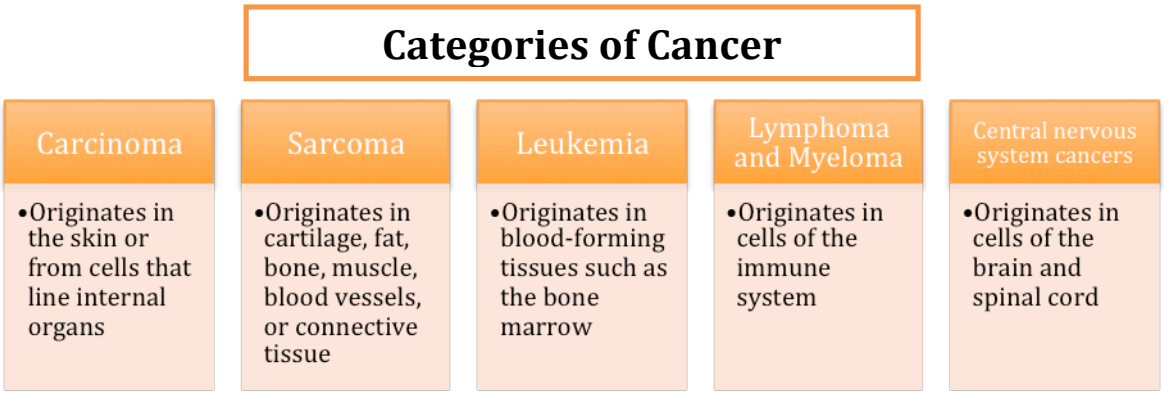

Figure 3. Five primary categories of cancer.

Cancer can develop from a number of factors, both at the genetic and environmental level as well as from the interaction between the two. It is known that greater than 90 percent of all cancers involve some underlying genetic alteration, which can be inherited or induced by environmental agents [9]. Some genetic disorders, such as Beckwith-Wiedemann and Wiskott-Aldrich syndrome, alter the immune system and are linked with an increased risk of developing cancer [9]. In many types of cancers, there are three primary types of genes that can become mutated and affect cell growth. Oncogenes regulate the normal growth of cells. In the case of cancer, certain oncogenes are mutated and are unable to control cellular growth and proliferation. In contrast to oncogenes, tumor suppressor genes recognize and interrupt abnormal cell growth.

However, if mutated, these genes will allow cells to grow indefinitely and form tumors. Finally, mismatch-repair genes, as their name suggests, work to repair mismatches that arise in the nucleotides of DNA during replication [9]. If these genes cease to function properly because of mutations, the DNA transmitted to new cells will contain errors, which can then damage the cells or alter normal cell growth patterns [10].

In addition to genetics, there are several lifestyle and environmental factors that may play a role in causing cancer. For example, smoking is known to greatly increase the risk of developing lung cancer. In fact, it contributes to more than 80 percent of all lung cancer deaths, with men 23 times more likely and women 13 times more likely to develop the disease compared to non-smokers [11]. Contact with viruses like the Epstein-Barr virus (causes mononucleosis) and HIV (causes AIDS) are risk factors for developing certain lymphomas [9]. Exposure to toxic chemicals such as asbestos in building insulation and formaldehyde in embalming fluid are also known to cause certain types of cancer.

In terms of treatments, there are four main ways in which cancer can be controlled or even eradicated: surgery, radiation, chemotherapy, and biological therapies. Surgery can be an effective option to use if the cancer is localized or contained within a specific area of the body. Cancerous tissue or tumors can be removed and, in the cases of certain cancers such as breast cancer, the surgeon can also remove underarm lymph nodes to prevent the cancer from spreading [12]. Similar to surgery, radiation is used to treat localized cancers. In radiation therapy, the body is exposed to ionizing radiation (high-energy particles that can remove an electron from an atom or molecule) to damage the DNA of cancer cells and thereby cause cellular death [13]. It can be administered externally, similar to having an x-ray taken, or internally. Called brachytherapy, small containers or “seeds” are implanted into the body next to a tumor and continually release

small doses of radiation [14]. This form of radiation can be more effective as it provides a higher overall dosage of radiation to a smaller area over a shorter period of time [14].

Unlike surgery and radiation, chemotherapy is used to treat invasive cancers. It can prevent the cancer from spreading further, slow down the cancer's growth, or even kill the cancer cells of metastatic tumors. Chemotherapy comprises a regimen of potent drugs, administered either orally or intravenously, that travel through the bloodstream to target and kill rapidly dividing cancerous cells [14]. Also called immunotherapy, biological therapy works on the principle of using the body's immune system to fight off cancer. Treatments involve the use of biological response modifiers (BRMs), agents that can strengthen a weak immune system and direct it to destroy cancerous cells [15]. BRMs can include interferons and interleukins, which serve as stimulators to activate certain immune cells that can, in turn, kill cancer cells [15]. Monoclonal antibodies and cytokines, on the other hand, function as markers that bind to and flag cancer cells, providing a target for immune cells as well as drugs [15].

Information on Renal Cell Carcinoma

Renal cell carcinoma (RCC) is a cancer that originates in the epithelium of the proximal convoluted tubule of the nephron. It comprises numerous subtypes of diseases that possess distinct histological features and genetic characteristics [16]. RCC is the most common form of renal cancer in adults, accounting for greater than 90% of all cases [17]. There are about 13 incidents of RCC per 100,000 cases each year, and in 2009, there were over 50,000 cases of malignant renal tumors diagnosed and about 13,000 deaths [17]. With regards to demographics, RCC is more common in individuals of Northern European ancestry and has a higher incidence in men than women (1.6:1) [17].

In terms of genetics, the cancer can occur in both nonhereditary and hereditary fashions with both forms involving alterations to chromosome 3 [18]. Certain tumor suppressors (TSC, VHL) and oncogenes (MET) have been linked with the formation of tumors in various subtypes of renal cancer [19, 20]. As for environmental and lifestyle factors, cigarette smoking doubles the risk of developing RCC and accounts for as many as one third of all reported cases [17]. In addition, obesity has been shown to have a positive linear relationship with risk [17].

It is known that RCC tends to display little to no warning signs. If signs are present, they tend to include fever, weight loss, night sweats, and malaise, symptoms that can be attributed to many other diseases [17]. Thus, it is no surprise that approximately one-third of patients are presenting with metastatic disease at the time of diagnosis. Metastatic RCC is often treated initially with surgery, with patients undergoing either radical or partial nephrectomies [21]. Unfortunately, 40-50% of patients initially treated with surgical intervention tend to develop recurrent metastatic disease, resulting in significant mortality.

In addition to few early warning signs, RCC responds extremely poorly to all currently available chemotherapy agents as well as radiation therapy [22]. There is, therefore, interest in developing better treatment options for RCC. Through clinical trials in recent years, there have been a number of new drugs that have shown promise as front-line agents. Three such drugs (sunitinib, bevacizumab, and sorafenib) work by inhibiting vascular endothelial growth factor (VEGF), a signal protein produced by cells to stimulate angiogenesis, the formation of new blood vessels [23]. In the case of RCC, renal tumors overexpress VEGF in order to support the growing cancer cells with a sufficient amount of blood supply. If VEGF is inhibited, the tumors cannot form new blood vessels and will not be able to grow beyond a certain point [24].

Similar to VEGF, another approach to treating RCC is through the mammalian target of

rapamycin (mTOR). mTOR is a serine/threonine protein kinase, which means it is an enzyme that phosphorylates (attaches a phosphate group to) a serine or threonine (two types of amino acids) residue found in its substrate in order to activate or deactivate the substrate. mTOR has been known to regulate cell growth, proliferation, and survival. Thus, mTOR-targeted agents such as the drug temsirolimus inhibit the growth of renal tumors and can improve the overall rates of survival among patients [24].

JAK/STAT Pathway

Although there are several drugs available to treat RCC, one potential mediator of the molecular mechanisms of the disease may be the Janus Kinases (JAKs). The JAK proteins are a family of intracellular non-receptor tyrosine kinases. A tyrosine kinase phosphorylates a specific protein by transferring a phosphate group from ATP to a tyrosine residue on the protein. Four mammalian JAKs have been identified – JAK1, JAK2, JAK3 and TYK2. Of these, JAK2 is expressed ubiquitously.

The JAK/STAT pathway is involved in cell growth, differentiation, and death [25]. The JAKs function by first binding to cell surface cytokine receptors as well as G-protein coupled receptors (GPCRs). A ligand specific to its receptor binds to it, activating the JAKs (Figure 4). The activated JAKs, in

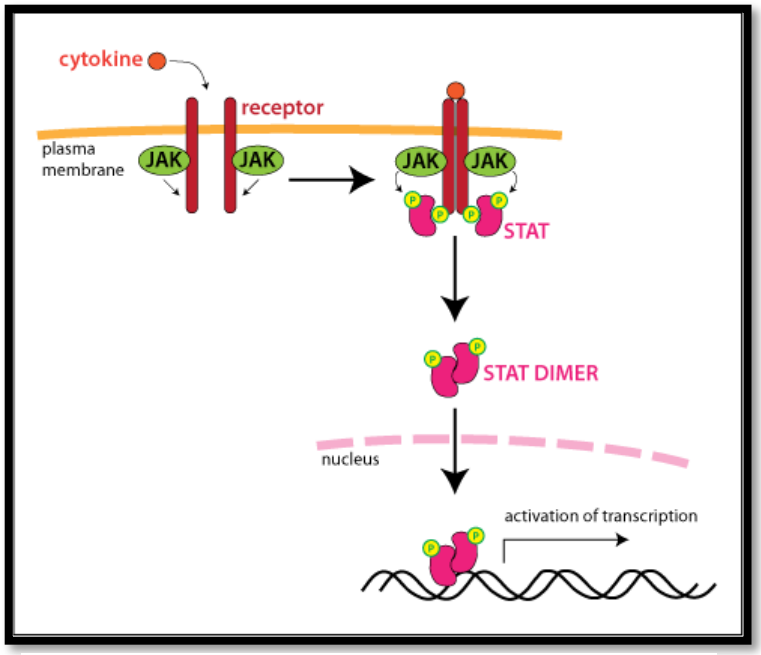

Figure 4. JAK/STAT signaling pathway. Source: http://courses.washington.edu/conj/bess/jakstat/jakstat.htm

turn, phosphorylate the receptor to create a docking site for proteins containing SH2 domains such as STAT3 [26]. The STAT proteins (Signal Transducer and Activator of Transcription) are transcription factors, which means they regulate the production of mRNA from the DNA sequence of a gene. Currently, seven STAT isoforms are known (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6). JAK activation stimulates the phosphorylation of the STATs which then homo or hetero-dimerize and migrate to the nucleus. After entering the nucleus, the dimerized STATS bind to their regulatory sequences in the DNA and alter gene expression by activating or repressing the transcription of their target genes [25].

The JAK/STAT pathway is negatively regulated at numerous points. Certain protein tyrosine phosphatases remove the phosphate group from cytokine receptors and activated STATs, thereby preventing the receptor docking sites from forming and deactivating the STATS, respectively [27]. The suppressors of cytokine signaling (SOCS) family of proteins can inhibit signaling by inactivating the N-terminal of JAKs, by blocking the docking sites of STATs, or by targeting and flagging bound proteins for proteasomal degradation [28]. In the nucleus, protein inhibitors of activated STAT (PIAS) are proteins, like STATS, that regulate transcription. Certain PIASs function by blocking the DNA-binding sites of STATS or by recruiting co-repressors of transcription [29].

In regards to renal physiology, activation of the JAK/STAT signaling promotes inflammation and apoptosis in healthy kidney tubule cells. On the other hand, this signaling stimulates invasiveness and cell survival in RCC cell lines *in vitro* [30, 31]. Currently, there are a number of JAK family inhibitors in clinical trials for a variety of cancers, including Ruxolitinib, a JAK2 inhibitor for the treatment of myelofibrosis. However, none of the JAK family inhibitors have been tested for RCC [32].

PI3K/Akt/mTOR Pathway

Phosphatidylinositol-3 kinases (PI3Ks) are a lipid kinase family of proteins that phosphorylate inositol phospholipids to create the second messenger (intracellular signaling molecule that is released in response to extracellular signals to regulate a particular pathway) phosphatidylinositol-3, 4, 5-trisphosphate (PIP_3) [33]. This second messenger interacts with Akt, a crucial serine/threonine protein kinase that plays multiple roles in cellular processes, and causes it to translocate to the inner side of the plasma membrane [34]. Here, Akt is activated by phosphorylation from phosphoinositide-dependent kinase (PDK) 1 and PDK2. Activated Akt can then serve as a modulator to a number of substrates involved in cell proliferation, growth, and survival such as mTOR. It is known that the PI3K/Akt/mTOR signaling pathway is overactive in many human cancers, resulting in reduced apoptosis and increased proliferation [33].

In terms of negative regulation, there are many proteins involved but perhaps only two of which are significant to our study. Phosphatase and tensin homolog (PTEN) is a protein that acts as a tumor suppressor by regulating the cell cycle [35]. Specifically, PTEN is a phosphatidylinositol-3, 4, 5-trisphosphate 3-phosphatase, meaning that it deactivates PIP_3, which, in turn, leads to the down regulation of Akt [36]. In many primary and metastatic human cancers, mutations in the *PTEN* gene result in a loss of protein activity, which then leads to a positive, unchecked regulation of the PI3K/Akt/mTOR pathway [37].

In addition to PTEN, Src homology region 2 domain-containing phosphatase-1 (SHP-1) is another negative regulator of the PI3K/Akt/mTOR pathway. SHP-1 is a member of the protein tyrosine phosphatase family. Overexpression of SHP-1 has been found to prevent the phosphorylation of Gα-interacting vesicle-associated protein (GIV), an enhancer of

PI3K/Akt/mTOR signals, by catalyzing the dephosphorylation of GIV, resulting in the depletion of phospho-GIV-PI3K complexes and the suppression of Akt [38]. More interestingly though, SHP-1 is also believed to be a negative regulator of the JAK/STAT pathway, as SHP-1 expression in myeloma cells correlates with a reduction in STAT3 phosphorylation [39, 40]. Thus, SHP-1 may serve as a connection point between the two major pathways.

Finally, the PI3K/Akt/mTOR pathway has been associated with the uncontrollable growth and survival seen in many human cancers. We, therefore, hypothesize that PI3K/Akt/mTOR signaling is altered in RCC. Our results indicate that positive regulators of this pathway such as PI3K subunits and Akt were activated in primary RCC tumors.

Methods

All studies were performed on existing samples (n=70) stored at the William Beaumont BioBank. The RCC tumor samples were obtained from patients under Beaumont Hospital approved IRB (HIC 2012-151) and were classified as clear cell renal carcinomas by a Beaumont Hospital pathologist. The tumor samples were then divided into histological Fuhrman grades: Grade 1 (n=3), Grade 2 (n=11), Grade 3 (n=14), and Grade 4 (n=3). We focused on the analysis of Grade 1 and Grade 2 versus Grade 3 and Grade 4 RCC tumors. In addition to these analyses, we also compared Grade 1 and Grade 4 tumors. The specimen preparation and analysis was carried out at the Beaumont Hospital BioBank, while data analysis was done at Oakland University. All required CITI training has been completed.

Western Blotting

The tumor tissue samples were minced and the cells were lysed to prepare protein extracts. The lysis buffer consisted of 50 mM Tris-HCL (pH 8), 150 mM sodium chloride, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 0.1% protease inhibitor cocktail (phosphatase inhibitor cocktails 1-3). The lysates were centrifuged as 14,000 rpm at 4°C for 15 minutes to remove debris. The supernatants were saved, and protein concentrations were determined using a Bio-Rad assay kit. Approximately 20-50 μg of the protein supernatants were mixed with loading dye and lysis buffer. Samples were then boiled for 5 minutes at 95°C and separated on SDS-polyacrylamide gels (SDS-PAGE; 7.5% polyacrylamide). The proteins from the SDS PAGE were transferred onto nitrocellulose membrane. Membranes were then blocked for 1 hour in 5% BSA tris buffered saline with tween (0.01%; TBS-T) solution followed by washing (twice with TBS-T rinse, 5 minute TBS).

Incubation was done overnight at 4°C using appropriate antibodies specific for either phosphorylated or total forms of the proteins being investigated. Blots were washed again once with TBS-T for 20 minutes and twice with TBS for 10 minutes. Membranes were incubated for 1 hour at room temperature using either mouse or rabbit anti-IgG horseradish peroxidase as the secondary antibody. Membranes were developed with enhanced chemiluminescence (ECL) in a darkroom using autoradiography film to visualize labeled bands. After developing with phospho-specific antibodies, membranes were incubated in stripping reagent and analyzed so that the total protein and phosphorylated protein levels could be compared. Blots were also probed for β-actin to ensure equal protein loading in all wells. Data were analyzed with ImageJ software to quantitate the pixel density of the bands.

Results

Previously, a detailed microarray-based study utilizing primary RCC tumors was carried out in my mentor's laboratory. In this study, it was found that RCC tumors had elevated levels of mRNAs for Lipopolysaccharide Binding Protein (LBP) and Microtubule Associated Protein 7 (MAP7D2). LBP is well known to activate cytokine receptors that in turn signal via the JAK/STAT pathway, leading to altered transcription of STAT target genes that potentially include MAP7D2. MAP7D2 is known to be associated with chemoresistance in other types of cancers (particularly resistance to cisplatin in liver cancer cells) but has not been investigated in RCC [41]. Additionally, the microarray analysis revealed decreased levels of NKCC2 transporter mRNA in the cancerous samples compared to the benign and normal. These data are not surprising, as the NKCC2 is only expressed in kidney epithelial cells, and its expression is often lost as the kidney cells transition to cancerous phenotypes. This study also revealed an increase in the mRNA levels of the p110beta subunit of PI3K. However, no increase was noted in the levels of mRNAs for Akt, PI3K subunits p110delta, p85alpha, p85beta, p85gamma, or any of the regulatory proteins of this pathway (unpublished and not shown). Since the level of mRNA is not always indicative of protein expression or activity, a further study was undertaken to determine whether activation of any of these PI3K/Akt/mTOR pathway members were activated in RCC tumors.

To test this hypothesis, Western blot analyses of the proteins derived from RCC tumors were carried out. The Western blots were first analyzed for expression of phosphorylated proteins followed by the levels of total respective proteins or cytoplasmic beta-actin as a loading control. The signals in the autoradiograms were quantitated by densitometry, and data were plotted as histograms, essentially as detailed in Methods.

As shown in Figure 5, increased expression of the p85 regulatory subunit of PI3K was noted in all RCC tumor grades when compared with its expression in normal kidney tissues. We next analyzed expression of the p110beta catalytic subunit of PI3K in these samples. The data revealed an increased expression of p110beta catalytic subunit in higher grade tumors (Figure 6). It should be noted that as RCC tumor grade increased, the expression of both subunits also increased. Intriguingly, the levels of the p85 and p110beta subunits were also elevated significantly in benign tumors, which is puzzling and unexplained at this time.

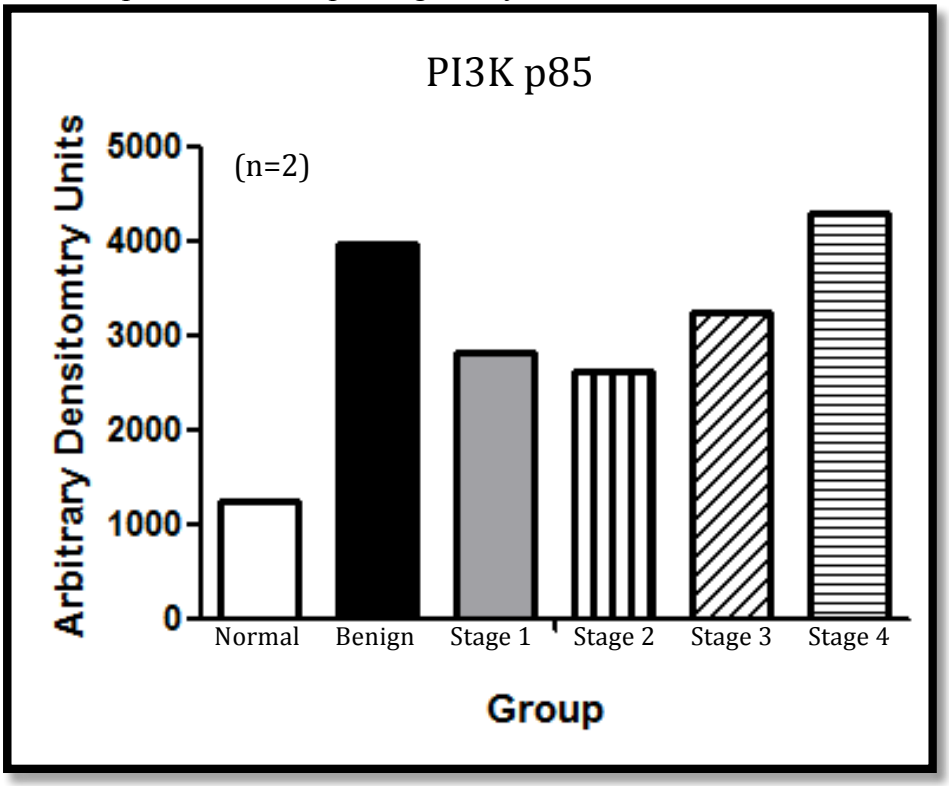

Figure 5. Expression of PI3K p85 subunit in normal, benign, and various stages of RCC tumors.

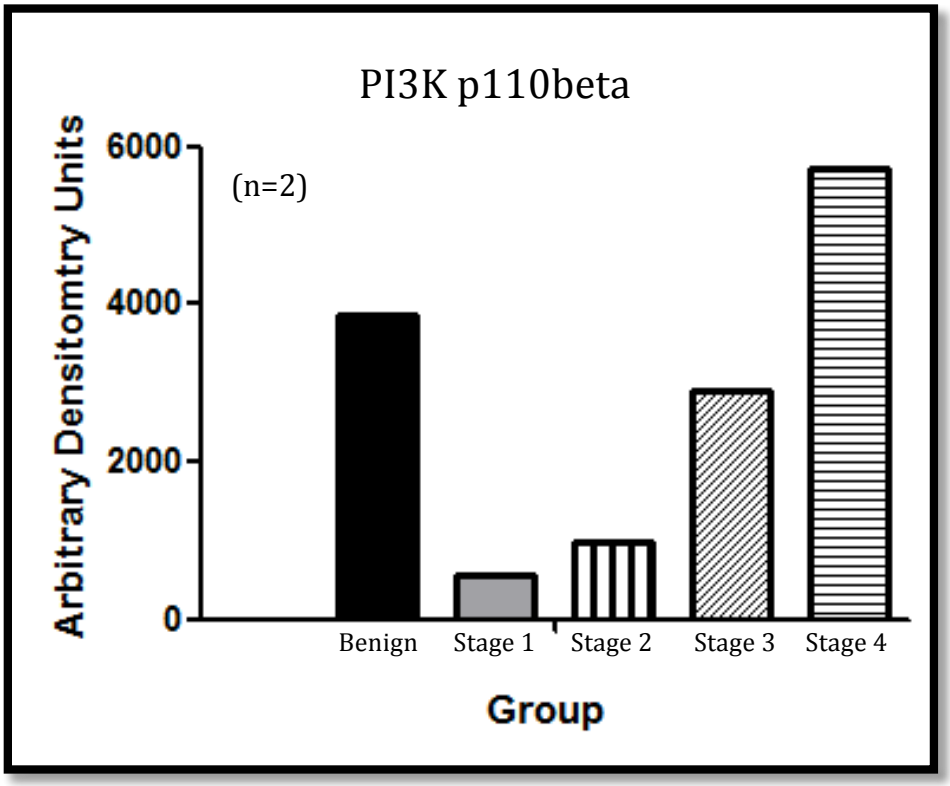

Figure 6. Expression of PI3K p110beta subunit in benign and various stages of RCC tumors.

Since the levels of a regulatory and catalytic subunit

of PI3K were elevated in higher grade tumors, we further analyzed whether additional subunits of this kinase were also similarly elevated in RCC tumors. We performed Western blot experiments analogous to those for Figures 5 and 6 and determined the expression of the p110delta catalytic subunit in RCC tumors. Again, the data revealed an elevated expression of PI3K p110delta in RCC tumors (Figure 7). As expected, stage 1 tumors had lower levels of p110delta when compared with stage 4 tumors that had the highest levels of this protein. Moreover, in contrast to previously noted increased expression of p85 and p110beta subunits in benign tissues (see Figures 5 and 6), p110delta levels were significantly lower when compared with stage 1 to 4 RCC tumors.

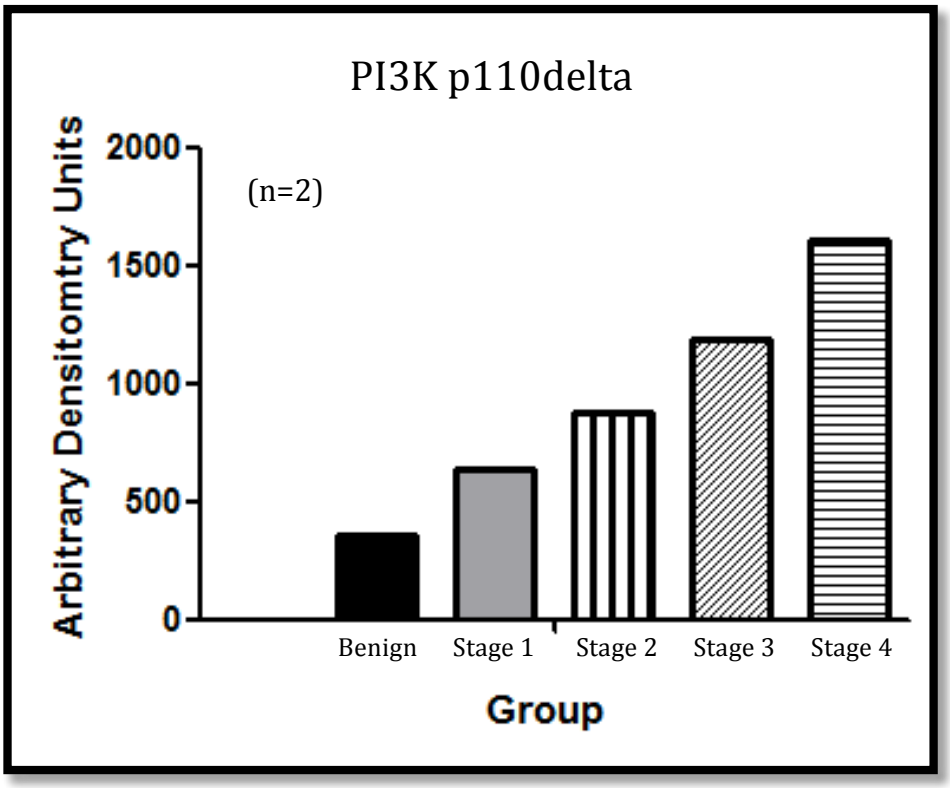

Figure 7. Expression of PI3K p110delta subunit in benign and various stages of RCC tumors.

Since PI3K phosphorylates inositol phospholipids and is often itself phosphorylated [33], we wished to determine whether, in addition to an increase in the expression of catalytic as well as regulatory subunits of PI3K in RCC tumors, a concomitant phosphorylation-dependent activation of this kinase also occurs in RCC tumors. To test this possibility, a Western blot experiment similar to that conducted for Figures 5-7 was carried out in conjunction with antibodies specific for the phosphorylated form of PI3K p85/p55 subunits (Anti-Phospho-PI3K

p85 pTyr458+p55 pTyr199 Antibody). Consistent with a previously noted increased expression of the p85 subunit in RCC tumors (see Figure 5), the activation of PI3K was also elevated in RCC tumors (Figure 8). Of note is that the PI3K activation in RCC tumors mimicked the expression of the p85 subunit in that the benign tumors had higher levels and activation of the p85 subunit when compared with the stage 1 tumors. The activation and expression of the p85 subunit was consistently and significantly elevated in stage 4 tumors.

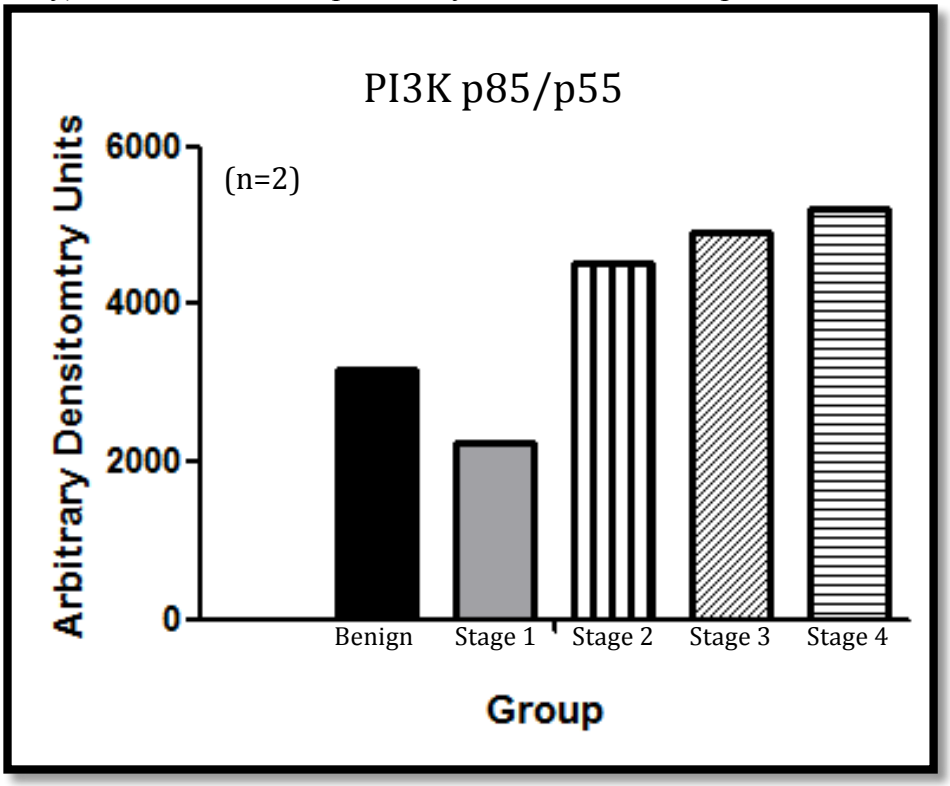

Figure 8. Activation (phosphorylation) of PI3K in benign and various stages of RCC tumors.

To further determine whether activation of PI3K contributed to oncogenic signaling pathways in RCC tumors, an additional Western blot experiment similar to that conducted for Figure 8 was performed to determine activation of the downstream target Akt. Since the activation of the PI3K/Akt/mTOR pathway is often noted in many cancers [33], activation of Akt in the RCC tumors would support a possibility of an increased oncogenic signaling in these tumors. Indeed, the data revealed significant activation of Akt in stage 4 RCC tumors when compared with the benign or stage 1-3 tumors (Figure 9). Although PI3K activation was higher in all the RCC tumors of stages 2-4 when compared with benign tumors, Akt activation, on the other hand, was similar in benign and stages 1-3 RCC tumors while stage 4 tumors had

significantly higher activation of Akt. Taken together, the data in Figures 8 and 9 would suggest that an oncogenic PI3K/Akt/mTOR pathway is likely activated in stage 4 tumors.

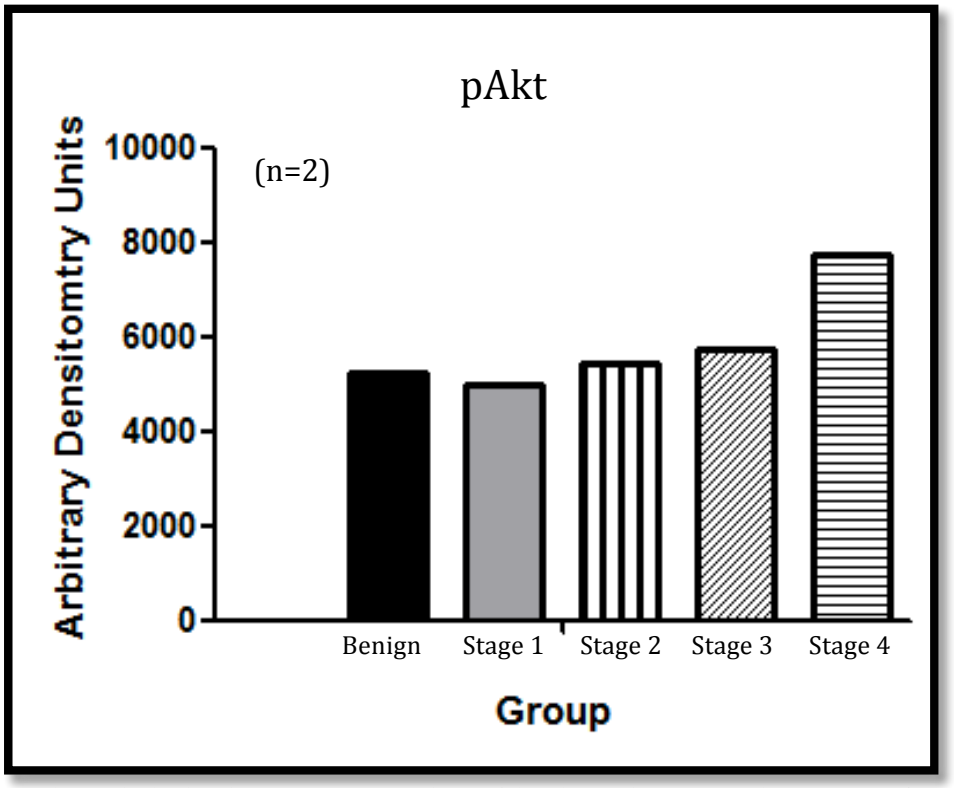

Figure 9. Activation (phosphorylation) of Akt in benign and various stages of RCC tumors.

Discussion

In this study, we conducted a microarray-based analysis that revealed altered expression of a number of cell growth and proliferation controlling genes. Among these genes, the microarray data indicated an increase in the mRNA levels of the p110beta subunit of PI3K in RCC tumors. Although the signaling by PI3K involves the formation of a heteromeric complex consisting of its catalytic and regulatory subunits, the microarray experiment did not show increased mRNA levels of any other subunits of this oncogenic kinase or the downstream transducer Akt. We conducted additional Western blot experiments and found that not only the expression of p85 and p110beta and delta subunits of PI3K was elevated, the p85/p55 regulatory subunits were also activated in RCC tumors. The expression as well as activation of PI3K was nevertheless higher in high grade tumors when compared with their benign counterparts. A similar trend was noted for the downstream signaling mediator Akt.

These data indicating activation of an oncogenic signaling pathway would be consistent with the notion that such pathways are often deregulated or hyperactivated in many cancers. Since the majority of our experiments involved a limited set (n=2) of RCC tumor samples, an appropriate statistical analysis could not be carried out. To further validate these conclusions, it would, therefore, be necessary to increase the number of specimens in each of the RCC tumor grades in subsequent experiments.

One of the emergent conclusions from our current analysis is that activation of a signaling pathway does not necessarily have to occur at the transcriptional level. In fact, the levels or activation of signaling proteins can be altered by transcriptional, posttranscriptional, and/or posttranslational mechanisms to regulate the pathway (Figure 10). The transcriptional processes often necessitate a significant number of steps that entail complexity as well as cellular

energy. The posttranscriptional processes although do not require gene transcription, but are,

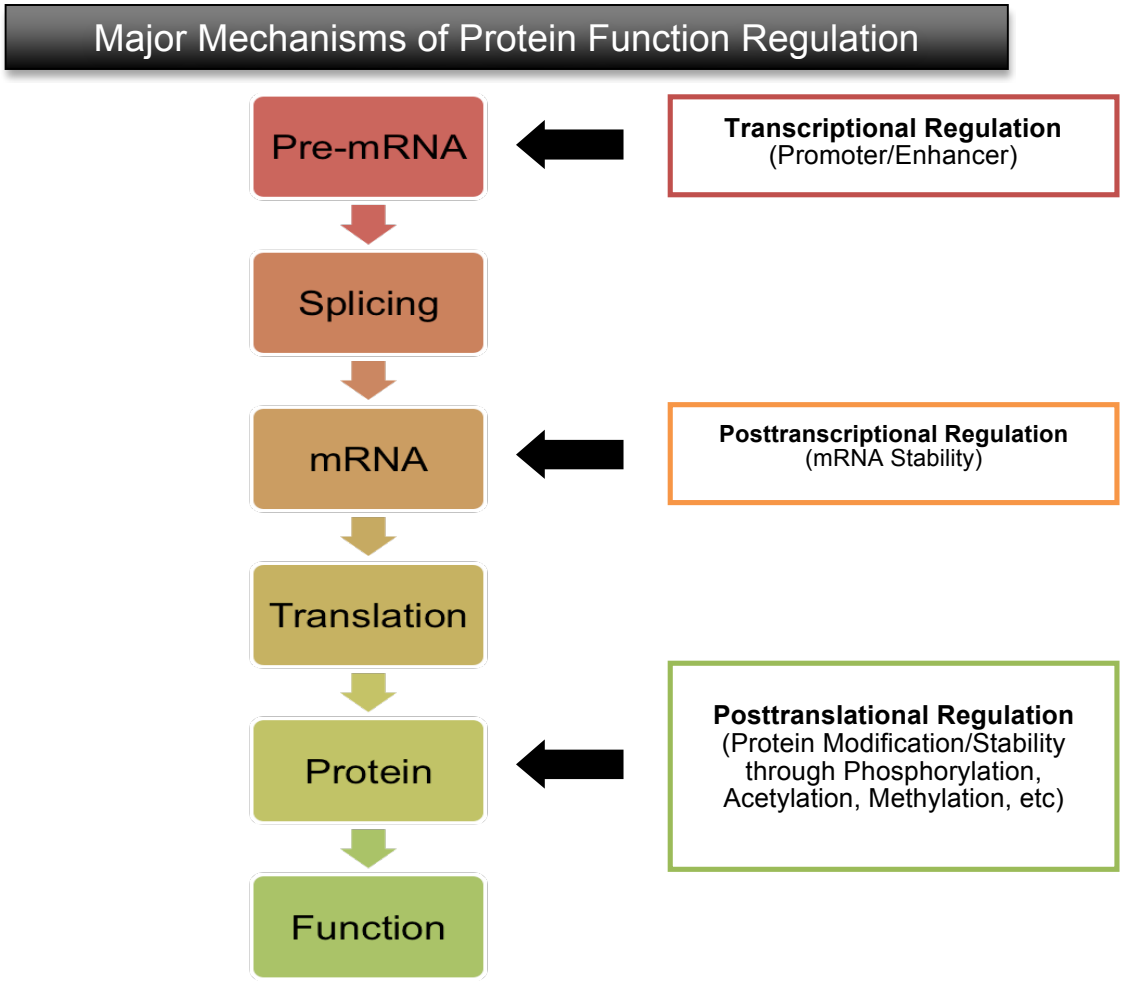

Figure 10. Three major cellular mechanisms of protein function regulation.

nonetheless, complex and equally dependent on cellular energy. The processes of posttranslational regulation, on the other hand, are independent of gene transcription, mRNA splicing, and translation. These processes involve modifications of the existing cellular proteins and are often most efficient and are activated as the first line of cellular response to alterations in its intracellular or extracellular environment. Commonly encountered changes include phosphorylation, acetylation, methylation, and ubiquitination of cellular proteins to transduce signals. The fact that our studies failed to show changes in the mRNA levels of PI3K subunits with the exception of p110beta suggests that neither gene transcription nor mRNA processing or

stabilization were likely involved. Since the expression of many subunits was elevated in RCC tumors, it would support the possibility that hyperactivation of this pathway in RCC tumors occurs predominantly at the posttranslational levels. It would be of interest to determine whether any of the oncogenic upstream regulators of PI3K activation such as growth factor receptors of the EGFR family are involved. Additionally, it would be desirable to discern the extent downstream signaling such as MAPKs and mTOR are similarly activated in higher grade RCC tumors.

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