Investigating the Role of MicroRNAs in the Pathogenesis of Kidney Cancer Subtypes and Their Clinical Utility as Cancer Biomarkers

by

Samantha Jane Wala

A thesis submitted in conformity with the requirements for the degree of Degree Master of Science
Laboratory Medicine and Pathobiology
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Abstract

Renal cell carcinoma (RCC) is the most common adult kidney neoplasm and constitutes a number of distinct subtypes. Papillary RCC (pRCC), a poorly characterized RCC subtype, can be subdivided into type 1 or 2. The objective of this study was to understand the pathogenesis of pRCC type 1. We identified genes and microRNAs (miRNAs) that are dysregulated in pRCC type 1. Pathway analysis showed enrichment of the focal adhesion pathway. We showed that miR-199a-3p, which is downregulated in pRCC type 1, regulates members of this pathway. Moreover, RCC subtypes may be difficult to differentiate by microscopy due to overlapping features. We demonstrated that miR-221 and -222 can discern chromophobe (chRCC) and renal oncocytoma, a benign neoplasm, from clear cell RCC (ccRCC) and pRCC. miR-126 can distinguish ccRCC from pRCC, whereas miR-15b and miR-22 had overlapping expression levels in chRCC and oncocytoma.
Acknowledgments

I would like to thank my mentor, Dr. George Makram Yousef, for his guidance and support both inside and outside the lab. I am truly grateful towards Dr. Yousef for giving me the opportunity to grow into the self-assured and tenacious student that I have become throughout my journey in graduate school. I would also like to express my gratitude towards my committee members, Dr. Jason Fish, Dr. Andrew Evans, Dr. Jason Karamchandani and Dr. Alexander Romaschin, for their expertise and thoughtful questions. These accomplished scientists have helped me further my research and prepare me as a scientist.

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Finally, I want to thank my family and close friends for all their support and patience during my graduate studies. I would also like to dedicate my thesis to my grandmother ('babcia') who passed away to pancreatic cancer in April 2010. I aspire that my research will shed new light on novel strategies to design effective treatments against kidney cancer.
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<td>CAV2</td>
<td>caveolin 2</td>
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<tr>
<td>ccRCC</td>
<td>clear cell RCC</td>
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<td>chRCC</td>
<td>chromophobe RCC</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>FFPE</td>
<td>formalin-fixed paraffin-embedded</td>
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<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
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<tr>
<td>HPRT1</td>
<td>hypoxanthine phosphoribosyltransferase 1</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>p-Akt</td>
<td>phosphorylated Akt</td>
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<td>pRCC</td>
<td>papillary RCC</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcription PCR</td>
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<td>RCC</td>
<td>renal cell carcinoma</td>
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<tr>
<td>TBP</td>
<td>TATA box binding protein</td>
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<tr>
<td>UTR</td>
<td>untranslated region</td>
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<td>VHL</td>
<td>Von-Hippel Lindau</td>
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"Medicine is a science of uncertainty and an art of probability."

- Sir William Osler
1 miRNAs

miRNAs (miRNAs) constitute a class of short, non-coding RNA molecules that are single-stranded. In 1993, Lee et al. discovered that lin-14 down-regulates the expression of LIN-14 protein in Caenorhabditis elegans by an RNA-RNA process (1). Ten years later, Drs. Andrew Fire and Craig Mello propose the use of double stranded RNA as experimental tools to knock-down the expression of specific proteins (2). Today, miRNAs are known to be evolutionarily conserved and have important roles in both physiological and pathological processes. Since their discovery, many findings have been made about miRNAs, including their biogenesis, mechanisms of gene silencing and involvement in disease. These small molecules are believed to be associated with almost every biological process that occurs within a living organism (3). In addition, miRNAs are predicted to target more than a third of human genes (4). Therefore, it is not surprising that these RNA molecules are related to many diseases, including cancer. Although miRNAs were first identified more than two decades ago, new findings about these potent regulators are continuously shedding light on the complex and important role miRNAs have in both health and disease.

1.1 Biogenesis of miRNAs

miRNAs are encoded within introns or intergenic regions (5, 6). These regulatory molecules are first transcribed in the nucleus as long primary miRNA transcripts by RNA polymerase II or III (7, 8). Primary miRNA transcripts that are transcribed by polymerase II have a 5' 7-methyl guanylate cap and 3' polyadenylated tail (8, 9). Drosha, an RNase III protein with nuclease activity, then processes these transcripts into ~70 nucleotide long stem loop structures called precursor miRNAs (10). The DiGeorge syndrome critical region gene 8, also known as Pasha, is a double stranded RNA-binding protein required for processing primary miRNA transcripts into precursor miRNAs as well (11). These RNAs are subsequently recognized by Exportin-5 and transported from the nucleus to the cytosol (12, 13). In the cytoplasm, precursor miRNAs are processed by another RNase III enzyme, Dicer, to generate an ~20 nucleotide double stranded RNA (14). One strand of this duplex, called the guide strand, mediates RNA silencing via
association with the RNA-induced silencing complex (3). The other strand, referred to as the passenger strand, is degraded (15). In addition to the miRNA guide strand, the RNA-induced silencing complex includes Argonaute 2, the catalytic subunit, which mediates slicing of the target mRNA (3). Knowledge of the machinery behind miRNA biogenesis is essential for a comprehensive understanding of the function of miRNAs and factors affecting their expression.

1.2 Mechanisms of miRNA Function

Briefly, miRNAs downregulate the expression of many gene targets at the post-transcriptional level by binding to the seed sequence located within the 3' untranslated region (UTR) (5, 6). miRNAs regulate gene expression in both plants and animals (16). In plants, these molecules preferentially exhibit complementary base-pairing to their target mRNA via the seed sequence, which encompasses nucleotides 2-8 from the 5' terminus of the miRNA (17). This leads to cleavage of its target genes (18). In animals, the seed region of miRNAs predominantly has imperfect base-pairing to the target sequence, favouring translational repression rather than mRNA cleavage (19, 20). It has been shown that the miRNA can still execute translational inhibition if its binding site is cloned into the 5' UTR sequence of a gene (21). Furthermore, these RNA molecules can repress gene expression throughout the different steps of translation, such as initiation and elongation (22). Mathonnet et al. (2007) show that let-7 regulates gene expression by repressing recognition of the 7-methyl guanylate cap by translation initiation factors (23). Another research group provides evidence that miRNAs may negatively regulate the elongation process because ribosomes on mRNAs were released more rapidly from the nascent polypeptide in cells transfected with the targeting small interfering RNA compared to the negative control (24). The RNA-induced silencing complex can also suppress protein translation by sequestering target mRNAs in processing bodies (25). Moreover, miRNAs are subject to RNA editing, a process by which adenosine deaminase acting on RNA enzymes convert an adenosine residue to inosine (8). This mechanism allows for miRNAs to expand their pool of mRNA targets (9, 10). Overall, the classical function of gene silencing by miRNAs adds another dimension to the complex regulation of gene expression in addition to transcription factors, methylation and chromatin remodeling, among others.

There are emerging research findings that propose additional roles for miRNAs other than silencing complementary mRNA targets. In fact, several researchers suggest that miRNAs can
upregulate protein translation. Place et al. (2008) show that miR-373 targets complementary promoter sites of E-cadherin and cold-shock domain-containing protein C2 to induce gene expression (26). In addition, Vasudevan et al. (2007) demonstrate that miRNAs have the potential to induce translation of target mRNAs (27). An independent research group also reveals that miR-10a can induce the expression of ribosomal proteins via a sequence in the 5' UTR (28). Therefore, miRNAs may be fine-tuning protein levels by either inducing or repressing gene expression under given conditions in order to maintain cellular homeostasis. Other mechanisms of gene regulation by miRNAs have been proposed in recent years. For instance, Hwang et al. (2007) present the possibility that miRNAs can behave as transcription factors since miRNAs can be imported back into the nucleus by a motif in their 5’ region (29). Moreover, miRNAs may act as decoys that are able to release mRNA sequences targeted for translational inhibition (30). Another study provided evidence that miRNA-dependent gene methylation occurs in *Arabidopsis* (31). In summary, these non-coding RNA molecules have a diversity of functions that may further our understanding of the different dimensions of gene and protein expression regulation.

### 1.3 miRNAs in Disease

miRNAs are endogenous RNA molecules that are fundamental for maintaining cellular physiology (32). This is supported by their important function in skeletal development, inflammation and pregnancy, among others (33-35). However, miRNAs may have aberrant expression, which can result in the initiation and propagation of disease. miR-135a is upregulated in diabetic skeletal muscle and downregulates the expression of the insulin receptor substrate 2 gene, which is linked to the etiology of type 2 diabetes (36, 37). Additionally, miRNAs may be involved in tumorigenesis by possessing either tumor suppressive or oncogenic functions. To provide support for miRNA dysregulation in cancer, Calin et al. (2004) perform a genome-wide study and map a significant number of miRNAs to chromosomal regions that are frequently altered in cancer (38). The promoters of miRNAs are also methylated in cancer in contrast to normal tissue (39-41). Extensive investigations have been conducted to establish putative roles for these regulatory molecules in cancer. Interestingly, the same miRNA can either drive or repress tumorigenesis depending on the given cancer. This indicates that the tumor microenvironment may, in part, dictate the miRNA function. For example, in breast and gastric cancer, miR-10b acts as an oncogene by silencing the expression of homeobox D10, a tumor
suppressor (42, 43). On the contrary, in neuroblastoma cells, miR-10b indirectly inhibits Myc expression, suggesting a tumor suppressive role for this miRNA (44). Furthermore, components of the miRNA biogenesis machinery, such as Dicer, can also be dysregulated in disease, resulting in aberrant miRNA expression (45, 46).

In addition to having a role in disease, these non-coding RNA molecules can also serve as biomarkers for various pathological states, including diabetes, cardiovascular disease, rheumatoid arthritis and cancer (47-50). A comprehensive discussion about miRNAs as potential biomarkers in cancer, particularly renal cell carcinoma (RCC), is found in Section 3.2.5 of Chapter 1.

2 Renal Neoplasms

RCC is the most commonly diagnosed kidney neoplasm in adults, and it is estimated that there will be 63,920 new cases of renal cancer in the United States this year (51, 52). RCC is one of the top 10 most common malignancies diagnosed amongst the Western nations, and more than half of all RCC cases are diagnosed incidentally (53, 54). This cancer is not a single disease; instead it encompasses a spectrum of histological subtypes. Clear cell RCC (ccRCC) is the most prevalent RCC subtype, constituting 80-90% of all RCC diagnoses (51). The five-year cancer-specific survival rate for patients with ccRCC is approximately 68.9% (55). The second most common RCC subtype is papillary RCC (pRCC), which comprises 10-15% of RCC cases and has a cancer-specific survival of 87.4% (51, 55). Chromophobe RCC (chRCC) is the third most frequent RCC subtype, comprising 4-5% of all RCC diagnoses and has a cancer-specific survival of 86.7% (51, 55). Finally, there exists an RCC subtype called unclassified RCC, which serves as a designated subgroup of poorly differentiated RCC tumors that do not conform to the defined features of any specific subtype. Both ccRCC and pRCC originate from the proximal convoluted tubule of the kidney, whereas chRCC and oncocytoma, a benign renal neoplasm that shares morphological features with RCC (56), stem from the distal convoluted tubule. Nevertheless, there are many more RCC subtypes, such as collecting duct RCC, Xp11 translocation RCC and clear cell papillary RCC, which may now be recognized as the fourth most common RCC subtype (57, 58). In addition, there is emerging evidence that there may be more RCC subtypes yet to be included, such as thyroid-like follicular RCC and succinate dehydrogenase B mutation-
associated RCC (57). In summary, RCC is an "umbrella" term that encompasses a number of distinct variants with different survival outcomes.

2.1 Clear Cell RCC

ccRCC, previously referred to as conventional RCC, is the most common RCC subtype and has an aggressive clinical behavior. Under the microscope, the cytoplasm of ccRCC tumor cells may appear either clear or eosinophilic (59). The name 'clear cell' is derived from the pale appearance associated with this RCC subtype due to the accumulation of glycogen and lipid in the cancerous cells (69). A hallmark of this tumor is loss of chromosome 3p, which harbors the Von-Hippel Lindau (VHL) gene (60). This gene is an important tumor suppressor in ccRCC as it promotes proteolytic degradation of angiogenic factors, such as the hypoxia inducible factor-1, in conditions of normoxia (61). In the majority of patients with ccRCC, the VHL gene is deleted, hypermethylated or mutated, resulting in a highly hypoxic and vascular tumor (62-64). In addition to loss of 3p, other common cytogenetic aberrations observed in ccRCC include loss of 14q, 8p, 4q, 9p and 6q, as well as gain of 5q and 8q (70, 71). Certain copy number alterations in ccRCC patients have been associated with a significantly worse prognosis (72). Moreover, chromosomal aberrations have been correlated with a distinct gene signature (73). A significant proportion of ccRCC patients will develop metastasis. Therefore, there is a great interest in examining prognostic markers for this RCC subtype. From Dr. Yousef's lab, galectin-1 was shown to retain its prognostic significance independent of other clinicopathologic variables for disease-free survival in ccRCC patients (p = 0.036, Hazard Ratio: 2.08) (74). Moreover, changes in chromatin remodeling and metabolic pathways have been reported to be associated with ccRCC pathogenesis (66-68). Therefore, ccRCC is a highly studied RCC subtype since it is prevalent and has a dismal prognosis.

2.2 Papillary RCC

An in-depth overview of pRCC is presented in Section 4 of Chapter 1.

2.3 Chromophobe RCC

chRCC was first described as a distinct entity in 1985 (65). This rare cancer has several characteristic features that allow for discrimination from the other RCC subtypes, as well as renal oncocytoma, a benign renal neoplasm. Under the light microscope, the tumor cells have
abundant cytoplasm and defining cell borders (77). In addition, there are three variants of chRCC: 1) typical, 2) eosinophilic and 3) mixed. The first is characterized by pale cytoplasm, whereas the second has a granular cytoplasm. Loss of chromosomes 1, 2, 6, 10, 13, 17, and 21 was reported in the majority of chRCC cases using comparative genomic hybridization (66). Another distinctive feature for chRCC diagnosis is positive and diffuse staining with Hale's colloidal iron (67). Moreover, chRCC displays characteristic ultrastructural features. For instance, the presence of microvesicles is indicative of this RCC subtype, although microvesicles can also be observed in oncocytoma and the eosinophilic variant of ccRCC (68). In addition, the mitochondria found in the tumor cells of chRCC differ in morphology from the mitochondria seen in the eosinophilic variant of ccRCC and oncocytoma (68). The distinct immunostaining pattern of this RCC subtype has also been examined. In particular, there are many investigators searching for a marker to differentiate between chRCC and oncocytoma (69-71). One group proposes that positive immunohistochemical staining for cytokeratin 7 and CD117, and negative staining for the paired box 2 protein can differentiate chRCC from ccRCC, pRCC and oncocytoma (72). Finally, although chRCC generally has an indolent clinical course, a study using 25 chRCC patients demonstrated that individuals with both chRCC and pRCC in the same kidney had lung metastasis (73). However, it is not clear which RCC subtype metastasized. In summary, chRCC is a rare RCC subtype and may be difficult to differentiate from oncocytoma.

2.4 Unclassified RCC

Unclassified RCC is a rare and aggressive subtype of RCC with a reportedly higher mortality rate than ccRCC (74, 75). Treatment options for unclassified RCC are limited. One research group showed from a study with 31 unclassified RCC patients that nephrectomy and interleukin-2 based immunotherapy significantly improved patient survival ($p < 0.05$) (76). However, patients with ccRCC had a more drastic improvement in survival than those with unclassified RCC (74). Currently, unclassified RCC is a poorly understood renal neoplasm at the molecular level. The genetic alterations and molecular mechanisms underlining this rare subtype have yet to be elucidated.

2.5 Renal Oncocytoma

Oncocytoma is not a malignant renal parenchyma, however it shares overlapping features with the eosinophilic variant of chRCC (77). This benign tumor is characterized by a highly granular
cytoplasm (59). There are less chromosomal aberrations observed in oncocytoma compared to RCC (78). However, loss of chromosomes 1 and Y are frequently observed in oncocytoma cases (79, 80). Additionally, it was shown by comparing oncocytoma to matched normal kidney tissue that there is alteration in the mitochondrial DNA as well, which mapped to the mitochondrial cytochrome c oxidase subunit I (81). Furthermore, a retrospective study using 138 patients from the Mayo Clinic revealed that 14 cases had renal oncocytoma co-occurring with RCC (82). Patients with co-existent oncocytoma and RCC had a disease-free survival of 100% with no reported metastatic disease (82). Moreover, investigators previously have characterized the molecular profile of oncocytoma, particularly markers that can discern oncocytoma from chRCC (83). Molecular features of this renal tumor include include weak expression for vimentin and RCC antigen (84, 85). Nevertheless, there may be significant overlap in morphological features between chRCC and renal oncocytoma (86). In light of such significant overlap, there is a report that suggests that chRCC and oncocytoma represent a spectrum of the same disease (87). Another group suggests that chRCC and oncocytoma may originate from a common progenitor lesion (88). More investigations are required in order to verify the biological relation between chRCC and oncocytoma. In summary, oncocytoma is a benign renal neoplasm, but poses a challenge for pathologists in discerning this tumor from malignant kidney cancers.

3 Molecular Classification of RCC: History, Opportunities and Challenges

The classification of RCC, a heterogeneous disease, has been subject to many advances in its history due to our evolving knowledge and understanding of this cancer type. In the late 1980's, it was proposed that all renal neoplasms, including adenomas and carcinomas, be classified on the basis of the tumor cell type, growth pattern and cytological grading (89). Subsequently, it was determined that the different renal tumors have distinct karyotypic features (90). Therefore, Kovacs et al. (1997) put forward the Heidelberg classification system, which combined both distinct histological and genetic features of an RCC tumor (80). The designation of the different RCC subtypes according to the guidelines set forth by the Heidelberg classification is still commonly referred to and prognostically informative (91). Today, the knowledge that different chromosomal changes results in specific gene and protein expression profiles has driven many research efforts to discover robust biomarkers and classification schemes for RCC. However, microscopy persists as an important tool for differentiating between the distinct subtypes,
although there are limitations. Therefore, a molecular marker that can help classify difficult RCC cases is warranted.

### 3.1 The Clinical Significance of Accurately Diagnosing RCC Subtypes

Misdiagnosis of ccRCC occurs in approximately 1-20% of all cases (92). It is imperative for the pathologist to correctly diagnose the RCC subtype, as histology itself is a prognostic marker and will help the oncologist determine the appropriate course for patient care management (93, 94). For instance, watchful waiting is commonly restricted to patients with confirmed oncocytoma (51). Additionally, the different RCC subtypes have varying responses to targeted therapy for metastatic disease. Currently, patients diagnosed with ccRCC have the most favorable response to targeted therapy as opposed to patients with non-clear cell RCC (95, 96). More specifically, it has been shown that a greater percentage of patients with chRCC had a more favorable response to sunitinib or sorafenib, which are both tyrosine kinase inhibitors, compared to patients with pRCC (97). Aside from characteristic morphological features, there are molecular markers used for distinguishing RCC subtypes. For example, carbonic anhydrase 9 has higher expression in patients with ccRCC compared to patients with other RCC subtypes, such as pRCC, chRCC, unclassified RCC and Xp11.2 translocation RCC (98). However, the ability to accurately classify the different RCC subtypes still remains an important challenge.

### 3.2 Potential Molecular Biomarkers for RCC

A landmark achievement for molecular research was the completion of the Human Genome Project (99). Now, there are many comprehensive projects being conducted in order to investigate the genomic, transcriptomic and proteomic changes associated with disease. With today's advancing technology, high-throughput assays, such as microarray analysis and whole genome sequencing, allow for the simultaneous screening of an assortment of molecular markers. Such biomarkers hold the promise of revolutionizing patient care management towards personalized medicine by: 1) distinguishing healthy individuals from non-healthy, 2) distinguishing between distinct subtypes or variants of a disease, 3) identifying patients who are likely to respond to a specific treatment ('predictive marker') and 4) subclassifying patients according to the natural course of their disease ('prognostic marker') (100). In this report, the
ability of molecular markers to distinguish the most common RCC subtypes, namely ccRCC, pRCC and chRCC, as well as oncotyoma, was examined.

### 3.2.1 Copy Number Alterations

The different RCC subtypes display unique chromosomal aberrations. In general, loss of chromosome 3p is restricted to ccRCC cases (101). Other chromosomal aberrations that are specific to ccRCC include gain of 5q and loss of 8p, 9, 14 and 18 (102). For patients with pRCC, gain of chromosomes 7, 17, 12, 16 and 20, and loss of chromosome Y are commonly observed (90). Using 26 ccRCC, 13 pRCC and 11 chRCC cases, loss of heterozygosity at 1p, 6p, 10p, 13q and 21q were more frequently observed in chRCC compared to ccRCC and pRCC (103). Unlike RCC, oncocytoma cases can have a normal karyotype. However, investigators have previously reported the loss of chromosomes 1, 14 and Y in this benign renal neoplasm (79, 102, 104). Finally, the genes encoded within the chromosomal alterations observed in the different RCC subtypes have been previously correlated to various biological pathways (105).

### 3.2.2 mRNA

In addition to chromosomal copy number changes, RCC subtyping can also be based on differential mRNA expression. Different studies using high-throughput microarray analysis have shown that distinct RCC subtypes can cluster separately from one another based on cDNA expression (106). Similarly, Takahashi et al. (2003) found different gene signatures associated with the different kidney cancers using microarray analysis (107). The differential expression of several candidate genes was re-evaluated in an independent cohort by immunohistochemistry (107). Glutathione S-transferase alpha expression was specific to ccRCC, alpha-methylacyl-CoA was preferentially expressed in pRCC and carbonic anhydrase 2 had the strongest staining in chRCC and oncocytoma (107). Another study analyzed the expression levels of various matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases from 30 RCC cases from different histological subtypes (108). By quantitative reverse transcription PCR (qRT-PCR), they found that the mRNA expression levels of different matrix metalloproteinases are significantly different between ccRCC and pRCC (108). Furthermore, a distinguishing characteristic of ccRCC and pRCC is the level of vascularity and angiogenesis observed in these two neoplasms (109). Therefore, a research group performed qRT-PCR and determined that the mRNA expression levels of endothelin 1 and endothelin receptor type A, which are involved in the
endothelin axis, are higher in ccRCC than pRCC (110). Thus, differential mRNA expression levels between the different RCC subtypes may reflect distinct macroscopic features associated with each variant.

### 3.2.3 Protein

Proteins are the functional products of genes and therefore can provide a more accurate understanding of the biological impact arising from differential gene expression in RCC. Moreover, proteins can undergo post-translational modifications, which can determine whether the protein is in an active or inactive state. Technologies such as mass spectrometry have facilitated the ability to analyze for many differentially expressed proteins in cancer as candidate biomarkers (111). Novel high-throughput technologies, such as reverse phase protein arrays, will further drive proteomic studies in RCC and other cancers (112). Independent research groups have reported differential protein expression levels in the RCC subtypes. Dr. Yousef's lab previously identified dysregulated proteins in ccRCC compared to normal kidney tissue samples by performing mass spectrometry (113). However, there has not been extensive effort to use this high-put technology to identify a robust proteomic signature for the different RCC subtypes.

### 3.2.4 Methylation

Methylation patterns have been shown to serve as potential signatures for different cancers (114). There is evidence that ccRCC and pRCC can be distinguished from renal oncocytoma based on the methylation level at the promoter for the Ras association domain family member 1 gene (115). A more recent study identified distinct methylation patterns that can distinguish between chRCC and renal oncocytoma (101). Therefore, this form of epigenetic regulation may serve as a potential fingerprint for the unique RCC subtypes.

### 3.2.5 miRNA

Independent research groups have shown miRNAs to be differentially expressed between the most common RCC subtypes. One of the first studies to investigate differential miRNA expression in RCC subtypes was conducted by Petillo et al. (2009). In that study, investigators used tumor tissues and determined that there are 27 significantly differentially expressed miRNAs between ccRCC and pRCC, including upregulation of miR-126 and -143, and downregulation of miR-31 in the former compared to the latter (116). Moreover, 5 miRNAs were
identified to be significantly different in their expression levels between chRCC and oncocytoma, including miR-200b (116). The expression levels of a few of these miRNAs were validated by qRT-PCR. In addition, Fridman et al. (2010) used 71 formalin-fixed paraffin-embedded (FFPE) tissues and identified relative ratios between two distinct miRNAs by microarray analysis to differentiate ccRCC, pRCC, chRCC and oncocytoma using a two-step classification scheme (117). A ratio > 9.86 of miR-221 relative to miR-210 (miR-221/miR-210) was indicative of chRCC or oncocytoma, whereas a ratio < 9.86 of miR-221/miR-210 was suggestive of ccRCC and pRCC (117). Subsequently, a tumor with a ratio > 33.1 of miR-200c/miR-139-5p would be classified as chRCC instead of oncocytoma (117). Finally, in making the decision between ccRCC and pRCC, if the ratio for miR-126/miR-31 is > 2.32 then it is indicative of a ccRCC diagnosis rather than pRCC, with the opposite holding true (117). Using 5 FFPE tissues of each ccRCC, pRCC, chRCC and oncocytoma, Powers et al. (2011) identified miR-142-3p, -20a and -21 to be upregulated in ccRCC and pRCC compared to chRCC and oncocytoma, whereas miR-221 and -222 had greater expression in chRCC and oncocytoma relative to ccRCC and pRCC (118). Moreover, they identified miR-126, -126* and 143 to be highly expressed in ccRCC compared to pRCC, and the expression levels of miR-124, -200b, -429 and -629* are elevated in chRCC relative to oncocytoma (118). This study also showed that the majority of the differentially expressed miRNAs did not correlate with chromosomal imbalances in the RCC cases (118). However, a weakness of this study was that the miRNA expression levels were measured only by microarray analysis and not independently confirmed by qRT-PCR. In 2011, Dr. Yousef's lab published a miRNA-based multistep decision tree for differentiating between the most common RCC subtypes. The training cohort included microarray analysis on 50 fresh frozen tissues from ccRCC, pRCC, chRCC and oncocytoma patients (119). From this analysis, a panel of 65 different miRNAs was identified to subclassify RCC and oncocytoma with high accuracy (119). It has also been reported that there is differential miRNA expression between ccRCC, pRCC and clear cell papillary RCC, which shares overlapping features with ccRCC and pRCC (120).

The ability of these non-coding RNA molecules to differentiate between the different subtypes of the same cancer is not restricted to RCC. Additionally, miRNAs are strong candidates for biomarker discovery due to the fact that these molecules can remain stable outside the cell by being encapsulated by exosomes or complexed with Argonaute 2 (121, 122). Therefore, miRNAs
can be isolated from a number of biospecimen, including urine, plasma and sputum (123-125). Recently, miR-210 was detected in the serum of ccRCC patients and may be a promising non-invasive test for this renal neoplasm (126, 127). Therefore, miRNAs have the potential to serve as non-invasive biomarkers for pathological states due to their stability in biological fluids. In addition, miRNAs can also be extracted from FFPE tissues due to their short length (136). miRNAs are also more robust biomarkers than mRNA molecules because of their ability to accurately classify undifferentiated tumors, whereas mRNA could not perform the same classification (128). This is possibly due to the tissue-specific nature of miRNAs (129). Moreover, miRNA expression can be evaluated in needle core biopsies. A group has shown that the expression levels of miR-141 and -200b in fine-needle aspirations can differentiate RCC from normal kidney with high specificity and sensitivity (130). Finally, the use of miRNAs as biomarkers is a growing field due to the recent research efforts to establish technologies to directly quantitate miRNA expression (131, 132).

3.3 Current Challenges of RCC Subtyping

There are impediments to accurately subclassifying the different RCC subtypes, which is of great importance since these unique entities have varying clinical courses and responses to therapy. A major challenge to subclassifying RCC with high accuracy is that different subtypes can share similar cytological elements. For instance, the granular variant of chRCC may be difficult to discern from oncocytoma, a benign renal neoplasm (86). Therefore, there are many research efforts towards the discovery of robust biomarkers for RCC stratification.

There are challenges and limitations associated with identifying biomarkers that have high sensitivity and specificity in subclassifying RCC subtypes. For instance, there is heterogeneity amongst tumors from the same RCC subtype (‘intratumor heterogeneity’). Several research groups have proposed that ccRCC may be separated into two distinct subgroups based on different clinical behavior, gene expression pattern and signaling pathway activation (133-136). Therefore, there is strong evidence for heterogeneity at the molecular-level within an RCC subtype. Another challenge for accurately stratifying the RCC subtypes is the occurrence of neoplasms that display elements observed from two separate subtypes. For example, there are tumors identified as a hybrid between chRCC and oncocytoma (137, 138). The hybrid oncocytic chromophobe tumor is currently classified under chRCC (57).
In addition, there are technical aspects that challenge the identification of robust biomarkers in RCC. Collaborations between various research institutions in different regions of the world are necessary to account for patient heterogeneity when proposing and validating a potential RCC biomarker (139). Moreover, consistency between laboratories with regards to specimen storage and handling is necessary to avoid artifacts that may skew results (140). There are proposed guidelines to help address this issue and thereby, achieve reproducible results between independent research groups (141). In summary, there are many challenges facing the ability to accurately discern between the different RCC subtypes using robust molecular biomarkers.

4 Papillary RCC

4.1 Clinical Overview

The term 'papillary' is not exclusive to pRCC since papillae structures can also be seen in other RCC subtypes, including ccRCC (59). Foamy macrophages and psammoma bodies are typically seen in pRCC (142). There is a significantly high incidence of pRCC in patients with end-stage renal disease in comparison to the general population (143). A recent study provides further evidence for a relationship between renal injury and the occurrence of pRCC (144). There are genetic hallmarks associated with this RCC variant that allow for differential diagnosis from the other RCC subtypes. Trisomy for chromosomes 7 and 17, and loss of chromosome Y are commonly observed in pRCC patients (145). The MET proto-oncogene has been reported in both hereditary and sporadic pRCC (146). In addition, this growth factor receptor has also been reported to be over-expressed in the hereditary and sporadic variants (147, 148). The promoter region of the serine peptidase inhibitor Kunitz type 2 gene, which negatively regulates Met, has also been shown to be hypermethylated in 40% of sporadic pRCC cases (149). Furthermore, it was recently reported that Met expression is significantly greater in pRCC relative to ccRCC (150). Another marker for pRCC is strong expression for alpha-methylacyl-CoA in comparison to the other RCC subtypes (151). Although many researchers have studied the molecular mechanisms underlining pRCC, this RCC subtype still remains a poorly understood malignancy in comparison to the other RCC subtypes, such as ccRCC.

Patients with non-metastatic pRCC demonstrate therapeutic benefit from cytoreductive therapy (152). In terms of systemic therapy, there is currently none available in the clinic for patients with metastatic pRCC. However, one study documented that a subset of patients were treated
with rapamycin-like mTOR inhibitors for one year (153). Roos et al. (2011) provide evidence supporting the up-regulation of hypoxia inducible factor-1α expression in pRCC (154).

However, pRCC is not a highly vascular tumor and in general, has a low response to sorafenib and sunitinib, which are small molecule multi-tyrosine kinase inhibitors that target receptors, such as the vascular endothelial growth factor receptor (155). A major challenge for improving the therapeutic strategy for patients with metastatic pRCC is the rarity of this disease. Therefore, only through coordinated research studies between different hospital centers can there be major advancements in instating effective treatment options for this cancer. There is a growing interest in investigating the pathogenesis of pRCC. This may be in part due to the fact that metastatic pRCC was shown to have the worst survival compared to other common non-clear cell RCC subtypes (156). Moreover, one study provides evidence that patients with pRCC or ccRCC had similar disease recurrence frequency (4.1% and 4.7%, respectively) and cancer-specific survival (143.8 and 147.8 months, respectively) during a follow-up period that averaged more than 5 years (157). Another study also demonstrates that the 5-year cancer-specific survival of non-metastatic ccRCC (84%) and pRCC (90%) are not significantly different (158). Nonetheless, there is currently no standard of care with regards to targeted therapy for patients with pRCC.

pRCC is a heterogeneous disease as it can be further subdivided into type 1 or type 2 (159, 160). The World Health Organization acknowledges the designation of pRCC into type 1 and type 2 (59). Type 1 and type 2 pRCC demonstrate unique characteristics at both the chromosomal and molecular level, including differential miRNA expression (161-163). Moreover, pRCC type 1 and type 2 differ in their clinical behaviors. Through multivariate analysis, it has been shown that the type of pRCC retains prognostic significance independent of other variables, such as tumor size and grade, for both overall survival and disease-free survival (164). Therefore, future research studies on pRCC should distinguish between the two distinct types.

4.2 Papillary RCC Subtypes

4.2.1 Papillary RCC Type 1

Under the microscope, small cells with pale cytoplasm surrounding papillae structures are indicative of pRCC type 1 (159). The presence and extent of tumor necrosis are prognostically significant in pRCC type 1 and not in pRCC type 2 (165). Another feature that is characteristic of pRCC type 1 is gain of chromosome 17 (162, 166). There are also molecular differences
between the two pRCC variants. For instance, there is greater expression of cytokeratin 7 in pRCC type 1 than pRCC type 2 (167). Moreover, it has been reported that the mRNA expression of MET, encoded on chromosomal locus 7q31, is overexpressed in sporadic pRCC type 1 cases (168). Pathway analysis performed by Yang et al. (2005) have shown that genes dysregulated in this tumor type are significantly enriched in the G1-S checkpoint regulation \( (p = 0.018) \) (169).

Aside from these findings, there is little known about the biological pathways underlining pRCC type 1. Knowledge about such oncogenic processes may help in the design and development of effective targeted therapy for this RCC subtype. Although pRCC type 1 has an indolent clinical course, its metastatic form was previously associated with shorter survival than metastatic pRCC type 2 (170). Currently, there is no study that has investigated the response of pRCC patients to systemic therapy according to tumor type.

### 4.2.2 Papillary RCC Type 2

Large cells with a granular cytoplasm are typical of pRCC type 2 (167). From a study using 22 pRCC type 1 and 35 pRCC type 2 tissue samples, karyotypic analysis revealed that type 2 has a significantly greater number of chromosomal alterations than type 1 \( (p = 0.018) \) (162). In addition, loss of chromosomal arms 1p and 3p, and gain of 5q were restricted to pRCC type 2 and not observed in pRCC type 1 (162). The immunohistochemical profile of pRCC type 2 has also been evaluated. Both the vascular endothelial growth factor receptor 2 and 3 have significantly higher expression in pRCC type 2 compared to type 1 (162). Hereditary pRCC type 2 is characterized by mutations in the housekeeping gene, fumarate hydratase (171). Fumarate hydratase is a member of the Krebs cycle and converts fumarate to L-malate. Therefore, mutated fumarate hydratase results in increased levels of fumarate. This aberrant accumulation has downstream effects, including activation of a stress response pathway (172). Sporadic pRCC type 2 has been demonstrated to have a gene signature that is associated with loss of functional fumarate hydratase (173). Additionally, overexpression of Myc and activation of its signaling pathway were associated with high-grade pRCC type 2 (174). With regards to clinical behavior, pRCC type 2 is associated with a worse prognosis compared to pRCC type 1 (164). Therefore, although this is an aggressive variant of RCC, there is currently no effective systemic therapy available for patients with metastasis.
4.2.3 Papillary RCC Not Otherwise Specified

Investigators have reported the occurrence of pRCC tumors displaying mixed features of both type 1 and 2, which are referred to as not otherwise specified (175). This type of pRCC has similar age of incidence as pRCC type 1 and 2 (176). Pignot et al. (2007) reported 3 out of 133 (~2%) pRCC cases with mixed features (175). These tumors were classified as type 2 because they predominantly had type 2 features. Another study that distinguished pRCC type 1 and 2 identified cases of mixed pRCC, which more closely resembled pRCC type 2 and were classified as such (177). The existence of a mixed pRCC type may support the hypothesis that pRCC type 2 tumors originate from pRCC type 1 (178). A study evaluating whether there are molecular similarities shared between pRCC not otherwise specified, and pRCC type 1 and type 2 is yet to be conducted.

4.3 The Molecular Pathways Underlining Papillary RCC

4.3.1 HGF/MET Signaling Pathway

Met is a 190-kDa transmembrane tyrosine kinase receptor encoded by the MET proto-oncogene (179). It is expressed in various cell types, including hepatocytes and epithelial cells (180). In addition, this protein interacts with its ligand, the hepatocyte growth factor (Hgf), to stimulate various cellular processes, such as proliferation, cell migration and invasion (181-183). Overexpression of Met has been reported in different cancer types, including non-small cell lung cancer, breast cancer and thyroid cancer (184-186). Additionally, the Met receptor can be constitutively activated by mutation in cancer (187). Met is a heterodimer and consists of both an extracellular α-chain and intracellular β-chain, which harbors the kinase activity (188). Upon binding to Hgf, Met becomes activated through autophosphorylation of specific tyrosine residues located within its intracellular domain (189, 190). Subsequently, the receptor can promote activation of downstream targets (191-193). Moreover, it has been demonstrated that Met can be activated through an Hgf-independent manner by interacting with the fibronectin receptor, α5β1 integrin (194). Met activates a number of downstream pathways, including the focal adhesion and Akt signaling pathway to regulate various cellular processes (195, 196). Since Met has been implicated in the etiology of various malignancies, small molecule inhibitors against this receptor have been designed as therapeutic strategies for cancer treatment (197, 198).
4.3.2 MTOR Signaling Pathway

The mammalian target of rapamycin (mTOR) was first identified in 1994 (199). Today, this protein is highly investigated in different physiological and pathological states due to its pleiotropic functions (200, 201). mTOR is a serine-threonine kinase and member of the phosphoinositide 3-kinase-related kinase family (202). This protein can participate in the formation of two distinct complexes: 1) mTOR complex 1 and 2) mTOR complex 2. Both complexes consist of mTOR, mammalian lethal with Sec13 protein 8 and DEP-domain-containing mTOR-interacting protein (202). mTORC complex 1 is known to promote cell growth and proliferation, whereas the processes regulated by mTOR complex 2 are poorly understood (202). However, the mTOR complex 2 has been previously shown to phosphorylate Akt at Ser\textsuperscript{473} (203, 204).

Activation of Akt is dependent upon phosphorylation at Ser\textsuperscript{473} and Thr\textsuperscript{308} (205, 206). Downstream targets and effectors of phosphorylated Akt (p-Akt) include p27, endothelial nitric oxide synthase and the BCL2-associated agonist of cell death (207-209). Phosphorylation of Akt (Ser\textsuperscript{473}) may be associated with tumor progression since it has potential prognostic significance in different cancers (210, 211). Activation of Akt promotes a cascade of molecular changes, from modulation of mRNA translation to promotion of cell survival (212, 213).

mTOR and its signaling pathway have a significant role in carcinogenesis. Therefore, several drugs have been developed to inhibit this protein, such as temsirolimus (CCI-779) and everolimus (RAD001) (214). These small molecules are derivatives of rapamycin and able to form an mTOR-inhibiting complex with the FK506 binding protein 12 (215, 216). Two multicentre studies demonstrated the benefit of temsirolimus over interferon-α treatment in patients with metastatic RCC (217, 218). A phase III clinical trial showed that everolimus has a therapeutic advantage in patients who have metastatic RCC and are non-responsive to other targeted treatments used for this cancer type (219). However, blocking mTOR can induce expression of insulin receptor substrate 1, which can circumvent the inhibitory effects of rapamycin and activate Akt in breast and prostate cancer cells (220). Tumors treated with rapamycin-like mTOR inhibitors are at risk of developing resistance (221). Therefore combination therapy may be necessary in order to target potent downstream effectors of mTOR signaling.
4.3.3  Focal Adhesion and Extracellular Matrix Pathway

The focal adhesion and extracellular matrix (ECM) pathway is a dynamic process that is integral in the maintenance of cellular physiology. Members of this pathway have been previously shown to be dysregulated in different cancer types and have a functional consequence. Knockdown of talin-1 expression in an osteosarcoma cell line led to decreased cell adhesion and migration, suggesting an oncogenic role for this protein (222). The overexpression of another player in the focal adhesion and ECM pathway, laminin γ1, may promote cellular invasion and migration in prostate cancer (223). Moreover, miRNAs have been shown to regulate this central pathway within various cancers (224-226). miR-199a-3p has been demonstrated to target caveolin 2 (CAV2) in endothelial and breast cancer cells (227). Furthermore, mTOR and Met have been shown to intersect with members of the focal adhesion and ECM pathway. Met can activate focal adhesion kinase by phosphorylating tyrosine 194 and causing conformational changes (228). Moreover, resistance to mTOR inhibitors has been associated with a change in the integrin cellular localization and expression profile (229). Therefore, this pathway has important consequences on cancer as it can regulate cellular phenotypes associated with tumorigenesis and regulate other biological pathways that are frequently targeted by drug inhibitors.

4.3.3.1  Integrins

Integrins facilitate coordination between the ECM and intracellular cytoskeleton and cell-cell adhesion (230). Integrins accomplish these roles by assembling into heterodimers, which consist of α and β subunits, and thereby can act as receptors that may bind to components of the ECM or counter-receptors on adjacent cells (231). These adhesion molecules regulate signal transduction, which influence various cellular processes, including cell motility and survival (232, 233). From 25 ccRCC with matched normal, Sültmann et al. (2005) reported that ITGB8 has higher expression in cancer tissue (234). However, the role of aberrant ITGB8 expression in RCC is yet to be elucidated. Furthermore, ITGB8 can be post-transcriptionally regulated by miRNAs. This gene has been shown to be a target of miR-93 by luciferase reporter assay in U343 cells (235).

4.3.3.2  Caveolins

Caveolins are the core proteins found in the membranes of caveolae (235, 236). Caveolae are important in that they are able to mediate endocytosis and transcytosis of macromolecules (237).
There are a total of three caveolins currently reported in the literature: 1) caveolin 1, 2) CAV2 and 3) caveolin 3 (237). Caveolin 1 has been investigated in various cancers (238-240). Unlike caveolin 1, there is little known with regards to the role of CAV2 in carcinogenesis. However, a research group reported that modulating the expression level of CAV2 with no change in caveolin 1 expression resulted in different effects on cell proliferation depending on the cancerous cell line (241). In addition, it was also recently shown that knock-down of Cav2 expression by small interfering RNA in A498 and 786-O cells led to significantly reduced proliferation, wound healing and invasion compared to negative controls (242).

5 Rationale

A single miRNA can regulate the expression of hundreds of genes (243). Lim et al. (2005) transfected miR-124, which is highly expressed in the brain, into the HeLa cancer cell line and microarray analysis was performed on RNA isolated from these cells (244). It was determined that miR-124 transfection led the HeLa cells to take on a gene expression profile similar to that of the brain (244). This phenomenon was not restricted to miR-124, but was also observed when miR-1, which is a hallmark for heart and skeletal muscle, was transfected into HeLa cells and led to cells expressing a muscle-like gene profile (244). In addition, a miRNA and its gene targets can be enriched in a single biological pathway. Investigators transfected let-7b in two different cancer cell lines and observed a dysregulation in the expression of genes involved in the cell cycling pathway (245). Our research group has previously demonstrated that various miRNAs are dysregulated in RCC and have a role in its initiation and progression (246-248). With this knowledge, there is strong incentive for identification of a dysregulated biological pathway enriched with aberrantly expressed miRNAs and their predicted gene targets in a poorly understood RCC subtype. An understanding about the underlining biological mechanisms driving a kidney cancer can promote the use or design of effective targeted therapies.

In addition to elucidating cancer-promoting pathways, miRNAs can also serve as strong candidates for biomarker discovery in RCC subtyping. Gottardo et al. (2007) provided the first evidence that this class of non-coding RNA molecules can differentiate RCC from normal kidney tissue (249). From this early study, an interest in distinguishing RCC subtypes from one another based on differential miRNA expression arose within the research community (116-118, 249). The popularity of miRNAs as diagnostic markers for the different kidney cancer subtypes
may be due to their tissue-specificity and stability (129, 250). For instance, miRNAs can be reliably extracted from FFPE tissues (251). Furthermore, small amount of RNA in the picogram range are sufficient to precisely quantify miRNA expression by qRT-PCR (252). Therefore, miRNA isolation can be performed on small tissue samples, such as fine-needle aspirates (253). In summary, miRNAs hold great promise in serving as biomarkers for distinguishing ccRCC, pRCC, chRCC and oncocytoma.

6 Hypothesis

Aberrantly expressed miRNAs and their predicted gene targets may elucidate pathways involved in the pathogenesis of pRCC type 1. In addition, a small number of miRNAs may distinguish between the most common RCC subtypes, namely ccRCC, pRCC and chRCC, and oncocytoma with high accuracy.

7 Objectives

7.1 To investigate a role for dysregulated miRNAs and genes in pRCC type 1

7.2 To validate a classification scheme with a limited number of miRNAs that can differentiate ccRCC, pRCC, chRCC and oncocytoma
Chapter 2
The Focal Adhesion and Extracellular Matrix Pathways Are Dysregulated in Papillary RCC Type 1

1 Introduction

pRCC is the second most common RCC subtype and can be further subdivided into type 1 and 2 (159). pRCC type 1 and 2 share several characteristics, but these two entities also have a number of distinctions at various levels. With respect to morphology, small basophilic cells are a feature of pRCC type 1 whereas large eosinophilic cells are commonly observed in type 2 (159). The two pRCC types also exhibit different molecular aberrations and clinical behaviors. Specifically, pRCC type 1 is characterized by trisomies 7 and 17, and loss of chromosome Y (254). Overexpression of MET has been previously reported in sporadic pRCC type 1, therefore it may have an integral role in the pathogenesis of this kidney cancer subtype (148). Currently, there is a lack in our understanding of the biological pathways involved in the initiation and progression of pRCC type 1. Addressing this issue may lead to the design and use of specific and effective drug treatments for pRCC, which are not currently available in the clinic (150).

Short, non-coding RNA molecules, called miRNAs, downregulate the expression of target genes at the posttranscriptional level. It has been reported that miRNAs may regulate the expression of more than 50% of mRNAs in mammals (255). Therefore, it is not surprising that miRNAs have been implicated in many physiological processes. Moreover, we have previously reported miRNAs to be differentially expressed in RCC and that their aberrant expression plays a crucial role in cancer initiation and progression (256). To our knowledge, the biological effect of aberrantly expressed miRNAs has not yet been studied in pRCC type 1.

Members of the focal adhesion and ECM pathways are integral in maintaining normal cell function. Therefore, dysregulation of these components may lead to adverse consequences, such as cancer initiation and propagation. The focal adhesion and ECM pathways have been investigated in various cancer types, such as prostate cancer (257). Aberrant expression of these components may be reflected in unregulated cell migration, proliferation and cell survival. The genes MET and MTOR have been linked to both biological pathways. Met can activate the focal adhesion kinase and mTOR can regulate the expression of various matrix metalloproteinases (228, 258, 259). Moreover, several members of focal adhesion and ECM, such as secreted
phosphoprotein 1 and vascular cell adhesion molecule 1, have been previously reported to be differentially expressed in pRCC (260, 261). However, whether this pathway is affected in pRCC type 1 is yet to be elucidated.

In this study, we examined differential miRNA and gene expression in pRCC type 1 compared to normal kidney. Using this data, we performed pathway analysis and the predicted targets of significantly differentially expressed miRNAs in pRCC type 1 were found to be enriched in the focal adhesion and ECM pathways. We provide experimental evidence that a number of the members involved in this biological pathway have dysregulated expression in pRCC type 1 compared to normal kidney tissue. Furthermore, we validated the downregulation of miR-199a-3p in pRCC type 1. We show that miR-199a-3p targets members of the focal adhesion and ECM pathways, including CAV2, ITGB8, MET and MTOR. We finally provide evidence that miR-199a-3p regulates proliferation in a kidney cancer cell line. To our knowledge, this is the first study to propose biological mechanisms contributing to the pathogenesis of pRCC type 1.

2 Materials and Methods

2.1 Patient Sample Collection

A total of 11 fresh frozen pRCC type 1 tissues with matched normal kidney from the cortex were collected from St. Michael's Hospital and the Ontario Tumor Bank in Toronto, Ontario with research ethics board approval. A pathologist confirmed that the cancer tissue was pRCC type 1. In addition, previously published microarray analysis on miRNA expression in pRCC type 1 and normal kidney was used in this study (262). We also investigated the gene expression profile of pRCC type 1 and normal kidney tissue using publically available microarray data sets from Gene Expression Omnibus, specifically GSE7023 and GSE26574.

2.2 Cell Culture and Transient Transfection

In vitro experiments were performed using the 786-O cell line, which was obtained from the American Type Culture Collection (ATCC, Manassas, VA). 786-O cells, which are derived from primary ccRCC and are defective for the VHL gene, were used for in vitro experiments because there is currently no cell line commercially available for pRCC. We did not use ACHN or CAKI-1 cells because these cell lines are derived from metastatic sites of ccRCC, and pRCC type 1 has
a low rate for metastasis. Cells were grown in RPMI media with 10% fetal bovine serum and stored at 37°C with 5% CO₂.

When performing transfection, cells were first seeded in a cell culture plate and incubated overnight. On the following day, 786-O cells were given fresh media and then transfected with 30 nM of *mirVana™* miR-199a-3p mimic (Applied Biosystems, Foster City, CA) or *mirVana™* miRNA negative control #1 (Applied Biosystems) using siPORT™ *NeoFX™* Transfection Agent (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol. Mimics were diluted with Opti-MEM® Reduced Serum Media (Invitrogen). Each transfection experiment was conducted three times in order to obtain a biological triplicate.

### 2.3 RNA Extraction

When extracting RNA from 786-O cells, cells were lysed with QIAzol Lysis Reagent (Qiagen, Mississauga, ON). RNA was recovered from the cell lysates using the miRNeasy Mini Kit (Qiagen). The 260/280 and 260/230 ratios of the RNA extracts were measured using the NanoDrop 2000c spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE). RNA was stored at -80°C until use.

### 2.4 Quantitative Reverse Transcription PCR (qRT-PCR)

The expression level of miR-199a-3p and RNU44, which served as a reference gene, were measured by performing qRT-PCR with 5 ng of RNA and TaqMan® miRNA assays (Applied Biosystems) (252). For the reverse transcription reaction, the following cycling conditions were used: 16°C for 30 mins, 42°C for 30 mins and 85°C for 5 mins. Subsequently, we measured for the cDNA expression level of miR-199a-3p by performing real-time PCR with the StepOnePlus™ System (Applied Biosystems).

For gene quantification, reverse transcription was performed on 500 ng of extracted RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The following cycling conditions were used: 25°C for 25 mins, 37°C for 120 mins and 85°C for 5 mins. The resulting cDNA was diluted to 100 ng and quantified by real-time (RT) PCR using the Fast SYBR Green Master Mix (Applied Biosystems) and primers designed with Primer3 (v. 0.4.0) software (Table 1). The TATA box binding protein (*TBP*) and hypoxanthine
phosphoribosyltransferase 1 (HPRT1) genes were used as endogenous controls for gene quantification. The real-time PCR reaction was performed on the Viia™ 7 Real-Time PCR System (Applied Biosystems) with these cycling conditions: 25°C for 10 min, 37°C for 120 min and 85°C for 5 mins.

Each experiment represents a summary of 3 technical replicates. The expression levels of both miRNAs and genes were quantified by the comparative Ct method using the following formula:

$$\Delta Ct = Ct(\text{target}) - Ct(\text{reference})$$

$$Fold - change = 2^{-\Delta Ct}$$
Table 2.1. List of Forward and Reverse Primer Sequences Used for Measuring mRNA Expression by Quantitative Reverse Transcription PCR (qRT-PCR)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Direction</th>
<th>Sequence (5’ → 3’)</th>
</tr>
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<tbody>
<tr>
<td>CAV2</td>
<td>Forward</td>
<td>GGCTCAACTCGCATCTCAAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGATTTCAAAGAGGGCATGG</td>
</tr>
<tr>
<td>ITGB8</td>
<td>Forward</td>
<td>TTCTCCCGTGACCTTCGTCT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTGTCAAAGACACAGCATGGA</td>
</tr>
<tr>
<td>MET</td>
<td>Forward</td>
<td>ATACCGTCCTATGGCTGGTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTTTGGGCTGGGGGTATAACA</td>
</tr>
<tr>
<td>MTOR</td>
<td>Forward</td>
<td>TTCCGTTCATCTCTGGTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAGGCCTCATTGACATCTGG</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Forward</td>
<td>TTGCTGACCTGCTGGATTAC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCTCCACCAATTACTTCTCC</td>
</tr>
<tr>
<td>TBP</td>
<td>Forward</td>
<td>TTCGGAGAGTTCTGGGATTGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGGACTGTTCCTTCACTTCTG</td>
</tr>
</tbody>
</table>

2.5 PCR Array

Total RNA was extracted from fresh frozen tissues samples of 5 pRCC type 1 and 5 normal kidney cases, and was separately pooled. Each pool was subject to reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Subsequently, a total of 50 ng of RNA from either the cancer or normal tissue pool was added to each well of the TaqMan® Array Human Extracellular Matrix & Adhesion Molecules 96-well plate, Fast (Applied Biosystems). Real-time PCR was performed on the StepOnePlus™ System (Applied Biosystems) as per the manufacturer’s instructions.
2.6 Protein Extraction and Western Blot Analysis

786-O cells were transfected for 72 hrs with 30 nM of mirVana™ miR-199a-3p mimic (Applied Biosystems, Foster City, CA) or mirVana™ miRNA negative control #1 (Applied Biosystems). Cells were lysed using the Lysis Buffer 11 (R&D Systems, Minneapolis, MN), which was spiked with protease inhibitor mixture (Roche, Laval, Quebec) and Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich, Oakville, Ontario). Cell lysates were subsequently centrifuged at 12x10^3 x g for 10 mins at 4°C, and the resulting supernatants were collected and stored at -80°C until later use. Protein concentration was quantified using the Micro BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol.

Protein extracts were diluted with sodium dodecyl sulfate buffer containing beta-mercaptoethanol, and denatured at 95°C for 5.5 mins. To each well of a 10% polyacrylamide gel, 25 µg of total protein was added. After running the gel, proteins were transferred onto a blot. Following transfer, the blot was blocked with 5% skim milk. The blot was then stained with Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb (Cell Signaling Technology, Danvers, MA) and mTOR (7C10) Rabbit mAb (Cell Signaling Technology). Blots were incubated with diluted antibody overnight at 4°C. Following primary staining, anti-Rabbit IgG horseradish peroxidase conjugate (Promega, Madison, WI) was added to each blot. After 1 hr incubation, chemiluminescence signal was enhanced using the Pierce™ ECL Western Blotting Substrate (Thermo Scientific). The blots were then stripped of protein using Antibody Stripping Buffer (Gene Bio-Application L.T.D, Israel) for 20-30 mins. Blots were then stained with anti-β actin antibody (Cell Signaling Technology), the loading control, and secondary staining was performed with anti-Mouse IgG horseradish peroxidase conjugate (Promega) for 1 hr. Detection of the signal was carried out as previously described. Experiments were performed as biological triplicate.

2.7 Luciferase Reporter Assay

786-O cells were plated on a 96-well plate with a white and opaque bottom. After overnight incubation, 0.1 µl of DharmaFECT Duo Transfection Reagent (Thermo Scientific) was added to each well to allow for co-transfection of pMirTarget vector encoding for either the 3’ UTR of MET or MTOR (Origene, Rockville, MD) and 50 nM of mirVana™ miR-199a-3p mimic or
$\text{mirVana}^{\text{TM}}$ miRNA negative control #1 (Applied Biosystems) as indicated in the manufacturer’s protocol. Cells were transfected for 24 hrs before britelite$^{\text{TM}}$ plus (PerkinElmer, Netherlands) was added to measure resulting luminescence signal. A total of 3 biological and 3 technical replicates were performed for each experiment.

2.8 Proliferation Assay

786-O cells were seeded on a 96-well plate and after overnight incubation, transfected with 30 nM of $\text{mirVana}^{\text{TM}}$ miR-199a-3p mimic or $\text{mirVana}^{\text{TM}}$ miRNA negative control #1 (Applied Biosystems) using siPORT$^{\text{TM}}$ NeoFX$^{\text{TM}}$ Transfection Agent (Applied Biosystems). Cells were incubated at 37°C in 5% CO$_2$ for 24 hrs. Subsequently, Cell Proliferation Reagent WST-1 (Roche, Germany) was added to each cell culture well. After 30 minutes, absorbance from the formazan product was measured at 440 nm. Each experiment represents a summary of 3 biological and 3 technical replicates.

2.9 Bioinformatic Analysis

We analyzed miRNA microarray data in pRCC type 1 and normal kidney by performing hierarchical clustering using SparseHierarchicalClustering from GenePattern (http://www.broadinstitute.org/cancer/software/genepattern/) (263). In addition, we conducted supervised clustering with another algorithm from GenePattern called ClassNeighbors (264). We obtained a list of statistically significantly ($p < 0.05$) up- or down-regulated genes in pRCC type 1 using GeneSpring software. Furthermore, we performed miRNA target prediction analysis using the following softwares: PicTar (http://pictar.mdc-berlin.de/) and TargetScan (http://www.targetscan.org/vert_50/). We also looked at the enrichment of predicted gene targets for dysregulated miRNAs in pRCC type 1 in biological pathways using DIANA - mirPath software (http://diana.cslab.ece.ntua.gr/pathways/). We also used the Database for Annotation, Visualization and Integrated Discovery (DAVID) to investigate which pathways show enrichment for the differentially expressed genes in pRCC type 1 (http://david.abcc.ncifcrf.gov/). We also conducted Gene Ontology analysis to investigate pathways enriched with up- or down-regulated genes in pRCC type 1 (http://www.pantherdb.org/).
3 Results

3.1 Genes and miRNAs Are Dysregulated in pRCC Type 1 and Are Enriched in the Focal Adhesion and ECM Pathways

Using publically available microarray data for pRCC type 1 and normal kidney tissue, unsupervised clustering was performed and separation between the two groups based on differential gene expression was observed (Figure 2.1). In addition, a similar distinction was drawn between pRCC type 1 and normal kidney tissue using previously published miRNA microarray data (Data not shown). Supervised clustering was conducted to determine significantly dysregulated genes and miRNAs in pRCC type 1 and normal kidney tissue. Table 2.2 provides a list of the top most significantly dysregulated miRNAs in pRCC type 1 ($P < 0.05$). We validated our results to an independent data set (GSE41282). With both lists of significantly differentially expressed miRNAs and genes in pRCC type 1, pathway analysis was performed. A common biological pathway observed using significantly dysregulated genes and predicted targets of differentially expressed miRNAs was the focal adhesion pathway. Figure 2.2 depicts the predicted interactions between the top 20 up- or downregulated miRNAs in pRCC type 1 listed in Table 2.2 with members of the focal adhesion pathway. Additionally, pathway analysis was performed for significantly dysregulated genes in pRCC type 1 and different biological pathways were identified (Table 2.3). Moreover, we show that a member of each pathway has been previously reported to be dysregulated in pRCC type 1. In addition, these biological pathways have been investigated in other cancer types. Furthermore, we performed Gene Ontology analysis using genes that have significantly increased or decreased expression in pRCC type 1 compared to normal kidney (Figure 2.3).

To validate the in silico findings, the mRNA expression level of 92 different members of the focal adhesion and ECM pathways was measured in pRCC type 1 and normal kidney tissue samples by qRT-PCR. Table 2.4 shows a partial list of the most significantly down- or upregulated genes in pRCC type 1 relative to normal kidney.

Additionally, the mRNA expression levels of other genes involved in the focal adhesion and ECM pathways were also measured by qRT-PCR. It was determined that $CAV2$, $ITGB8$ and $MET$ have decreased mRNA expression in pRCC type 1 relative to normal kidney tissue (Figure
2.4). The mRNA expression of *MTOR* was not decreased in our patient cohort (Data not shown).
Figure 2.1. Hierarchical Clustering of Gene Microarray Data from pRCC Type 1 and Normal Kidney Tissues. Gene expression data for pRCC type 1 and normal kidney tissues was collected from publically available datasets. Unsupervised clustering was performed with SparseHierarchicalClustering from GenePattern, which used the 5000 genes with the highest variance. A) GSE26574 and B) GSE7023. The pRCC type 1 and normal kidney samples are shown in the columns, and genes are listed in the rows. Red is representative for overexpression, whereas blue is indicative of underexpression.
Figure 2.2. Dysregulated miRNAs in pRCC Type 1 Are Predicted to Target Genes in the Focal Adhesion Pathway. Using microarray expression data, a list of the top 20 significantly up- or down-regulated miRNAs in pRCC type 1 was generated using ClassNeighbors. The potential interaction between the significantly dysregulated miRNAs and their target genes within the focal adhesion pathway was determined using the TargetScan miRNA prediction software. The red boxes indicate predicted gene targets of the dysregulated miRNAs. miR-199a-3p is highlighted.
Table 2.2. A Partial List of the Most Significantly Up and Down-Regulated miRNAs in pRCC Type 1

<table>
<thead>
<tr>
<th>Up-regulated</th>
<th>Score</th>
<th>Down-regulated</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-197</td>
<td>5.993</td>
<td>hsa-miR-200c</td>
<td>12.129</td>
</tr>
<tr>
<td>hsa-miR-23b*</td>
<td>5.368</td>
<td>hsa-miR-199a-5p</td>
<td>9.233</td>
</tr>
<tr>
<td>hsa-miR-380</td>
<td>4.576</td>
<td>hsa-miR-199a-3p</td>
<td>9.147</td>
</tr>
<tr>
<td>hsa-miR-1229</td>
<td>4.477</td>
<td>hsa-miR-139-5p</td>
<td>8.521</td>
</tr>
<tr>
<td>hsa-miR-548l</td>
<td>4.231</td>
<td>hsa-miR-100</td>
<td>8.312</td>
</tr>
<tr>
<td>hsa-miR-92b</td>
<td>4.003</td>
<td>hsa-miR-652</td>
<td>7.748</td>
</tr>
<tr>
<td>hsa-miR-367*</td>
<td>3.974</td>
<td>hsa-miR-660</td>
<td>7.473</td>
</tr>
<tr>
<td>hsa-miR-346</td>
<td>3.941</td>
<td>hsa-miR-502-3p</td>
<td>7.349</td>
</tr>
<tr>
<td>hsa-miR-411*</td>
<td>3.726</td>
<td>hsa-miR-532-5p</td>
<td>7.252</td>
</tr>
<tr>
<td>hsa-miR-519b-3p</td>
<td>3.683</td>
<td>hsa-miR-145</td>
<td>7.170</td>
</tr>
<tr>
<td>hsa-miR-92a</td>
<td>3.670</td>
<td>hsa-miR-126</td>
<td>6.580</td>
</tr>
<tr>
<td>hsa-miR-1197</td>
<td>3.655</td>
<td>hsa-miR-532-3p</td>
<td>6.366</td>
</tr>
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<td>hsa-miR-1260</td>
<td>3.507</td>
<td>hsa-miR-500*</td>
<td>6.337</td>
</tr>
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<td>hsa-miR-299-5p</td>
<td>3.466</td>
<td>hsa-miR-214</td>
<td>6.070</td>
</tr>
<tr>
<td>hsa-miR-101*</td>
<td>3.401</td>
<td>hsa-miR-215</td>
<td>6.020</td>
</tr>
<tr>
<td>hsa-miR-25</td>
<td>3.391</td>
<td>hsa-miR-363</td>
<td>6.003</td>
</tr>
<tr>
<td>hsa-miR-520d-5p</td>
<td>3.336</td>
<td>hsa-miR-378</td>
<td>5.993</td>
</tr>
<tr>
<td>miRNA</td>
<td>Value</td>
<td>Partner miRNA</td>
<td>Value</td>
</tr>
<tr>
<td>------------------</td>
<td>-------</td>
<td>---------------</td>
<td>----------</td>
</tr>
<tr>
<td>hsa-miR-28-3p</td>
<td>3.321</td>
<td>hsa-miR-187</td>
<td>5.686</td>
</tr>
<tr>
<td>hsa-miR-520f</td>
<td>3.320</td>
<td>hsa-miR-127-3p</td>
<td>5.684</td>
</tr>
<tr>
<td>hsa-miR-24-2*</td>
<td>3.316</td>
<td>hsa-miR-501-5p</td>
<td>5.629</td>
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</table>
Table 2.3. A Partial List of the Biological Pathways Enriched with Dysregulated Genes in pRCC Type 1

<table>
<thead>
<tr>
<th>Biological Pathway</th>
<th>$p$-value</th>
<th>Member of Pathway Dysregulated in PRCC Type 1</th>
<th>Pathway Reported in Other Cancer Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte transendothelial migration</td>
<td>0.0030</td>
<td>CLDN7 (169)</td>
<td>Breast cancer (265)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Melanoma (266)</td>
</tr>
<tr>
<td>Complement and coagulation cascades</td>
<td>0.0067</td>
<td>FHL1 (169)</td>
<td>Breast cancer (267)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thyroid cancer (268)</td>
</tr>
<tr>
<td>Cell adhesion molecules</td>
<td>0.0074</td>
<td>CLDN7 (169)</td>
<td>Ovarian cancer (269)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Endometrial cancer (270)</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>0.0348</td>
<td>MET (271)</td>
<td>Ovarian cancer (272)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prostate cancer (257)</td>
</tr>
</tbody>
</table>
Figure 2.3. Gene Ontology Pie Charts. A) The significantly up-regulated genes in pRCC type 1 ($p < 0.05$) compared to normal kidney tissue were identified using publically available datasets GSE7023 and GSE26574. Using gene list analysis software, the products of the over-expressed genes in pRCC type 1 were mapped to different biological processes defined by Gene Ontology. B) Using GSE7023 and GSE26574, genes that were significantly down-regulated in pRCC type 1 ($p < 0.05$) relative to normal kidney from both datasets were identified. These genes were then correlated to biological processes designated by Gene Ontology.
Table 2.4. A Partial List of Up- or Down-Regulated Members of the Focal Adhesion and ECM Pathways in pRCC Type 1 Relative to Normal Kidney Tissue

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Unigene</th>
<th>Gene Name</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>Hs.153304</td>
<td>CD44 molecule</td>
<td>9.5029</td>
</tr>
<tr>
<td>CLEC3B</td>
<td>Hs.162844</td>
<td>C-type lectin domain family 3, member B</td>
<td>-13.4228</td>
</tr>
<tr>
<td>CNTN1</td>
<td>Hs.355024</td>
<td>Contactin 1</td>
<td>-13.2626</td>
</tr>
<tr>
<td>COL11A1</td>
<td>Hs.266273</td>
<td>Collagen, type XI, alpha 1</td>
<td>38.0566</td>
</tr>
<tr>
<td>COL1A1</td>
<td>Hs.164004</td>
<td>Collagen, type 1, alpha 1</td>
<td>19.1859</td>
</tr>
<tr>
<td>FN1</td>
<td>Hs.1549976</td>
<td>Fibronectin 1</td>
<td>9.5115</td>
</tr>
<tr>
<td>ITGA8</td>
<td>Hs.233321</td>
<td>Integrin alpha 8</td>
<td>-13.5501</td>
</tr>
<tr>
<td>ITGAM</td>
<td>Hs.355885</td>
<td>Integrin alpha M</td>
<td>9.4734</td>
</tr>
<tr>
<td>ITGB2</td>
<td>Hs.164957</td>
<td>Integrin beta 2</td>
<td>9.3786</td>
</tr>
<tr>
<td>KAL1</td>
<td>Hs.608006</td>
<td>Kallmann syndrome 1 sequence</td>
<td>9.4786</td>
</tr>
<tr>
<td>LAMA3</td>
<td>Hs.165042</td>
<td>Laminin, alpha 3</td>
<td>9.3994</td>
</tr>
<tr>
<td>MMP12</td>
<td>Hs.899662</td>
<td>Matrix metalloproteinase 12</td>
<td>19.1859</td>
</tr>
<tr>
<td>MMP2</td>
<td>Hs.1548727</td>
<td>Matrix metalloproteinase 2</td>
<td>9.5293</td>
</tr>
<tr>
<td>MMP3</td>
<td>Hs.968305</td>
<td>Matrix metalloproteinase 3</td>
<td>174.9229</td>
</tr>
<tr>
<td>MMP8</td>
<td>Hs.1029057</td>
<td>Matrix metalloproteinase 8</td>
<td>37.5978</td>
</tr>
<tr>
<td>MMP9</td>
<td>Hs.957555</td>
<td>Matrix metalloproteinase 9</td>
<td>76.8927</td>
</tr>
<tr>
<td>SPP1</td>
<td>Hs.959010</td>
<td>Secreted phosphoprotein 1</td>
<td>9.5056</td>
</tr>
<tr>
<td>Gene</td>
<td>Accession</td>
<td>Description</td>
<td>Expression</td>
</tr>
<tr>
<td>--------</td>
<td>-----------</td>
<td>-----------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>THBS2</td>
<td>Hs.1568063</td>
<td>Thrombospondin 2</td>
<td>9.3423</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Hs.99999139</td>
<td>TIMP metalloproteinase inhibitor</td>
<td>9.436</td>
</tr>
<tr>
<td>VCAM1</td>
<td>Hs.103372</td>
<td>Vascular cell adhesion molecule 1</td>
<td>9.0579</td>
</tr>
<tr>
<td>VCAN</td>
<td>Hs.171642</td>
<td>Versican</td>
<td>37.6786</td>
</tr>
</tbody>
</table>

Figure 2.4. mRNA Expression of *CAV2, ITGB8* and *MET* Is Higher in pRCC Type 1 Relative to Normal Kidney Tissue. The expression levels of *CAV2, ITGB8* and *MET* were measured from RNA extracted from 11 fresh frozen pRCC type 1 tissues with matched normal kidney tissue by qRT-PCR. The reference gene used was *TBP*. The statistical significance of the results for *CAV2* and *ITGB8* was determined using the unpaired t-test with Welch's correction, and the Mann-Whitney test was used for *MET*. *p < 0.05, ****p < 0.0001.

One of the top miRNAs that had decreased expression in pRCC type 1 compared to normal kidney tissue was miR-199a-3p. The decreased expression of miR-199a-3p was validated in fresh frozen samples of pRCC type 1 tissue by qRT-PCR (Figure 2.5).
Figure 2.5. miR-199a-3p Expression Is Down-Regulated in PRCC Type 1 Compared to Normal Kidney Tissue. A) Amplification plot of miR-199a-3p and RNU44 expression in a pRCC type 1 and normal kidney tissue sample. B) Using 11 fresh frozen pRCC type 1 tissues with matched normal kidney tissue, miR-199a-3p was relatively quantified by qRT-PCR. The endogenous control that was used for normalization was RNU44.
3.2 miR-199a-3p Targets Members of the Focal Adhesion and ECM Pathways

Pathway analysis revealed that the predicted gene targets of miR-199a-3p are enriched in many different biological pathways, in particular the focal adhesion and ECM-receptor interactions pathway (Table 2.5). *In vitro* experiments were performed to validate the ability of this miRNA to regulate the expression of genes and proteins associated with the focal adhesion and ECM pathways. Cells that were transfected with a mimic for miR-199a-3p had a significantly lower mRNA expression level for *CAV2* (*p* = 0.0321), *ITGB8* (*p* = 0.0168) and *MET* (*p* = 0.0282) compared to cells transfected with a non-targeting miRNA mimic control (Figure 2.6). At the protein level, mTOR and phosphorylated Akt had higher expression level in the negative control relative to 786-O cells transfected with the miR-199a-3p mimic (Figure 2.7). However, the differences in MTOR and phosphorylated Akt expression between the experimental and negative control groups did not reach statistical significance. Furthermore, luciferase reporter assay was conducted to evaluate the interaction between miR-199a-3p and the 3’ UTR of both *MET* and *MTOR*. 786-O cells co-transfected with the miR-199a-3p mimic and plasmid encoding for the 3’ UTR of *MET* or *MTOR* had a significantly lower luminescence signal than the negative experimental control (Figure 2.8).
Table 2.5. Pathways Significantly Enriched for Predicted Gene Targets of miR-199a-3p

<table>
<thead>
<tr>
<th>Biological Pathway</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM-receptor interaction</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Regulation of actin cytoskeleton</td>
<td>0.001</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>0.002</td>
</tr>
<tr>
<td>ErbB signaling pathway</td>
<td>0.004</td>
</tr>
<tr>
<td>Glioma</td>
<td>0.004</td>
</tr>
<tr>
<td>mTOR signaling pathway</td>
<td>0.008</td>
</tr>
<tr>
<td>MAPK signaling pathway</td>
<td>0.008</td>
</tr>
<tr>
<td>Melanoma</td>
<td>0.010</td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>0.014</td>
</tr>
<tr>
<td>Small cell lung cancer</td>
<td>0.029</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>0.036</td>
</tr>
</tbody>
</table>
Figure 2.6. miR-199a-3p Down-Regulates mRNA Expression of CAV2, ITGB8, MET and MTOR. 786-O cells were transfected with a mimic for miR-199a-3p or non-targeting miRNA mimic for 24 hours. Total RNA, including miRNAs, was isolated from cell lysates. Reverse transcription using random primers was performed, followed by cDNA amplification with specific primers against candidate target genes. Relative quantification was performed using the Comparative C_T method with HPRT1 as the reference gene. The experiment was carried out in triplicate. Statistical significance was measured using the t-test with Welch's correction. *p < 0.05, **p < 0.01. A) CAV2, B) ITGB8, C) MET and D) MTOR.
Figure 2.7. miR-199a-3p May Target Protein Expression Levels of mTOR and p-Akt. 786-O cells were transfected with a miR-199a-3p and non-targeting miRNA mimic by forward transfection for 72 hrs. Cells were subsequently lysed on ice and extracted lysates were centrifuged at 4°C for 10 mins at 12,000 g. The supernatant was isolated and stored at -80°C until use. Total protein was run on a 10% separating gel, followed by transfer onto a blot. Proteins of interest were stained with antibodies against mTOR and p-Akt. Actin was used as the loading control. Experiments were performed in triplicate. Western blot images of A) mTOR and B) p-Akt. C) Relative expression of mTOR and p-Akt in 786-O transfected with miR-199a-3p compared to cells treated with non-targeting miRNA mimic. Results did not reach statistical significance.
Figure 2.8. miR-199a-3p Interacts with the 3’ UTR of MET and MTOR. 786-O cells were co-transfected with pMirTarget vector encoding the 3’ UTR of MET or MTOR and a mimic for miR-199a-3p or non-targeting miRNA control for 24 hrs. Following transfection, cells were lysed and treated with luciferase substrate to allow generation of light via the luciferase enzymatic reaction. After 5 minutes of incubation, luminescence was quantified for both cells treated with the miR-199a-3p or non-targeting miRNA mimic using a multi-well spectrophotometer. The statistical significance between the experimental and negative control results was measured by the Mann-Whitney test. **p < 0.01, ***p < 0.001.
3.3 miR-199a-3p Has a Negative Effect on Cellular Proliferation

To investigate for a biological impact of miR-199a-3p downregulation in pRCC type 1, 786-O cells were transfected with a mimic for miR-199a-3p to determine its effect on cellular proliferation. Cells transfected with a non-targeting miRNA mimic had significantly greater absorbance for formazan than cells treated with miR-199a-3p mimic ($p = 0.013$) (Figure 2.9), indicating a higher number of viable cells from the negative control group after 24 hrs of transfection.

![Graph showing relative formazan absorbance](image)

**Figure 2.9. A Role for miR-199a-3p in PRCC Type 1.** 786-O cells were seeded in a white, opaque 96-well plate and transfected with a miR-199a-3p or non-targeting miRNA mimic for 24 hrs. After transfection, cells were treated with a tetrazolium salt for 0.5 hrs before measuring for absorbance at 440 nm with a multi-well spectrophotometer. The statistical significance between the differences in formazan absorbance from cells treated with miR-199a-3p or non-targeting miRNA mimic was quantified using the Mann-Whitney test. $p < 0.05$. 
4 Discussion

pRCC type 1 is a poorly understood kidney cancer, for which there is currently no effective targeted therapy. In this study, we provide evidence that genes are differentially expressed in pRCC type 1 compared to normal kidney tissue (Figure 2.1). In addition, miRNAs are also aberrantly expressed in pRCC type 1, including miR-199a-3p (Table 2.2). Down-regulation of miR-199a-3p expression in this RCC subtype was validated by qRT-PCR (Figure 2.5). Our lab previously showed that miR-199a-3p is also downregulated in ccRCC (273). Moreover, the focal adhesion pathway was significantly associated with both the dysregulated miRNAs and genes identified in pRCC type 1, as shown in Figure 2.2 and Table 2.3, respectively.

The aberrant expression levels of different components of the focal adhesion and ECM pathway were confirmed by performing a PCR array (Table 2.4). Two of the genes, secreted phosphoprotein 1 and vascular cell adhesion molecule 1, have been previously reported to be upregulated in pRCC, which is in agreement with our findings (260, 261). Moreover, trisomy of chromosome 17 is characteristic of pRCC type 1. We showed that COL1A1, ITGB3 and ITGB4, which are encoded on chromosome 17, have increased expression in pRCC type 1 compared to normal kidney tissue. Resistance to mTOR inhibitors has been linked to aberrant integrin regulation (229). Therefore, dysregulation of integrins in pRCC type 1 may play a role in the dismal responses of patients to rapamycin-like mTOR inhibitor treatment. Moreover, we provide evidence that matrix metalloproteinases may be aberrantly expressed in pRCC type 1. We also measured the mRNA expression of additional members of the focal adhesion and ECM pathways. CAV2 and MET were significantly upregulated in pRCC type 1 compared to normal kidney (\( p < 0.05 \)), whereas upregulation of ITGB8 did not reach statistical significance (Figure 2.4).

Our research group has previously identified miR-199a-3p to be downregulated in RCC (273). Dysregulation of miR-199a-3p in this cancer has been corroborated by an independent group of researchers (274). The predicted gene targets of miR-199a-3p are significantly enriched in the ECM-receptor interaction and focal adhesion pathways (\( p < 0.05 \)) (Table 2.5). In addition, we provide evidence that miR-199a-3p regulates the expression of components of the focal adhesion and ECM pathways. The mRNA expression levels of CAV2, ITGB8, MET and MTOR were significantly downregulated in 786-O cells transfected with a miR-199a-3p mimic compared to...
the negative control group (Figure 2.6). miR-199a-3p transfection also led to decreased mTOR and p-Akt protein expression levels relative to 786-O cells treated with a non-targeting miRNA mimic (Figure 2.7). Therefore, down-regulation of p-Akt in 786-O cells transfected by miR-199a-3p may be due to suppressed mTOR expression by miR-199a-3p since p-Akt is not a predicted target of miR-199a-3p. We also show that this miRNA directly regulates the expression of MET and MTOR using a luciferase reporter assay (Figure 2.8). Furthermore, we propose that miR-199a-3p may have a tumor suppressive function in pRCC type 1 by inhibiting cellular proliferation (Figure 2.9). This effect by miR-199a-3p has also been observed in other kidney cancer cell lines (274). Additionally, miR-199a-3p can inhibit the cellular proliferation of hepatocellular carcinoma cell lines (275).

The miR-199a stem-loop is encoded on chromosomes 1 and 19 and encodes for both miR-199a-3p and miR-199a-5p. miR-199a-3p has been previously reported to have decreased expression in a number of cancer types, including hepatocellular carcinoma and osteosarcoma (276, 277). The biological impact of the aberrant expression of this miRNA has also been investigated. For instance, it was demonstrated to have tumor suppressive functions in hepatocellular carcinoma by inhibiting cell proliferation and cell cycle progression, and promoting apoptosis (278). In osteosarcoma, miR-199a-3p was shown to also inhibit cell cycle progression, proliferation, and migration (279). Moreover, miR-199a-3p has been experimentally validated to target genes, including MET, MTOR, CD44, CAV2 and the Brm subunit of the SWI/SNF chromatin remodeling complex (280-284). Therefore, the dysregulated expression of miR-199a-3p may serve as an important event in cancer initiation and progression via the focal adhesion and ECM pathway.

The clinical benefit of rapamycin-like mTOR inhibitors for metastatic pRCC has been previously studied. One retrospective study that included 14 patients with metastatic pRCC reported that 3 of these patients were administered rapamycin-like mTOR inhibitors for more than one year (285). Therefore, a predictive marker for rapamycin-like mTOR inhibitor treatment is warranted in advanced pRCC. The interaction between miR-199a-3p and mTOR may highlight a mechanism by which this therapeutically targetable protein has aberrant expression in pRCC type 1. We believe it would be warranted to study whether miR-199a-3p has a predictive value for rapamycin-like mTOR inhibitor treatment in pRCC cases.
To our knowledge, we are the first to propose that the focal adhesion and ECM pathways are dysregulated in pRCC type 1 through analysis of both gene and miRNA expression levels. More specifically, we provide evidence that miR-199a-3p is downregulated in this RCC subtype and it regulates members of the focal adhesion and ECM pathways. In addition, we suggest that miR-199a-3p may contribute to pRCC type 1 by inhibiting cellular proliferation via downregulation of mTOR. The new information we provide concerning a molecular mechanism underlining pRCC type 1 may contribute to the design of effective targeted therapy for patients with this RCC subtype.

5 Conclusions

There is differential gene and miRNA expression in pRCC type 1 compared to normal kidney tissue. Both dysregulated genes and miRNAs were shown to be enriched in the focal adhesion and ECM pathways by in silico analysis. Many members of the focal adhesion and ECM pathways, including matrix metalloproteinases, CAV2 and MET, have up- or down-regulated expression in pRCC type 1 compared to normal kidney tissues. Moreover, miR-199a-3p has decreased expression in pRCC type 1 relative to normal kidney, and may target members of the focal adhesion and ECM pathways, including CAV2, ITGB8, MET and MTOR. miR-199a-3p may serve as a tumor suppressor in pRCC type 1 by inhibiting cellular proliferation and targeting putative oncogenes, such as MET and MTOR.

6 Future Experiments

There are some limitations associated with this study that need to be addressed when conducting future research on pRCC type 1. For instance, there were a small number of pRCC type 1 samples from patients available for conducting experiments. This RCC subtype is a rare renal neoplasm; therefore it is warranted that future investigators collaborate with other institutions in order to collect a high number of pRCC type 1 cases for experimental testing. In addition, we performed our in vitro experiments in one cell line. It would have been beneficial to use other cell lines as well to ensure our results are not restricted to the 786-O cell line. Future in vitro experiments should be conducted on a pRCC cell line generated from a patient sample in order to gain more accurate insight on the role of miR-199a-3p in pRCC type 1.
To confirm the importance of miR-199a-3p in pRCC type 1, there should be an initiative to investigate mechanisms that downregulate this miRNA in pRCC type 1. Bioinformatic analysis accompanied by experimental validation should be carried out to answer the question as to whether chromosomal loss, mutations, and/or methylation decrease miR-199a-3p expression in this RCC subtype using patient samples. In addition, the transcription factors that regulate the expression of miR-199a-3p via its promoter should be determined using bioinformatic analysis and chromatin immunoprecipitation assay. Finally, the expression level of the identified transcription factors should be quantified in pRCC type 1 samples relative to normal kidney tissue. Twist-1 and p53 have been previously identified to drive miR-199a-3p expression (286, 287). Moreover, there may be other processes affecting expression of this miRNA, for instance the unfolded protein response inhibits miR-199a-3p expression in HepG2 cells (288). An understanding of the mechanisms driving downregulation of miR-199a-3p may elucidate whether loss of this miRNA is an early or late event in pRCC type 1.

There are future experiments that need to be performed in order to further validate that miR-199a-3p has a tumor suppressive role in pRCC type 1 via the focal adhesion and ECM pathways. For instance, it should be further investigated whether miR-199a-3p has an impact on apoptosis and the cell cycle process in this RCC subtype. Furthermore, commercially available Met and mTOR inhibitors should be added to cells in culture to compare their negative effect on tumor cell growth and motility compared to over-expressing miR-199a-3p in the same cell line. In addition, *in vivo* experiments need to be conducted in order to gain more insight into the role of miR-199a-3p on tumor characteristics, such as tumor growth and metastatic potential. This miRNA should be stably transfected into a cell line, and the cells should then be injected into a mouse model. Moreover, components of the focal adhesion and ECM pathways, such as the matrix metalloproteinases, should be knocked-down using small interfering RNA technology to determine their significance in the pathogenesis of pRCC type 1. Finally, we would like to examine whether miR-199a-3p is dysregulated in other cancers with papillary architecture in addition to pRCC type 1, such as papillary thyroid carcinoma, papillary serous ovarian carcinoma and papillary urothelial carcinoma.
Chapter 3
miRNAs Can Subclassify Renal Cell Carcinoma Subtypes with Accuracy

1 Introduction
RCC encompasses a broad spectrum of subtypes, with the most common being ccRCC, pRCC and chRCC. These RCC subtypes have different survival outcomes and responses to systemic therapy (55, 156, 289). Therefore, accurate classification of the distinct kidney neoplasm is of clinical importance. However, studies have shown that there is inter-observer variability amongst pathologists for subtyping RCC (290). This may be due to the fact that distinct RCC tumors can share overlapping morphological features. For instance, the eosinophilic variant of chRCC and renal oncocytoma, a benign neoplasm, exhibit similar cytological elements (77). Moreover, there is an RCC subtype known as unclassified RCC. This subgroup consists of RCC tumors that do not conform to one specific subtype. Overall, RCC constitutes an array of distinct tumors that may be difficult to discriminate from one another.

miRNAs are non-coding RNAs, which are dysregulated in a number of different cancer types. Dr. Yousef’s lab previously identified miRNAs as potential biomarkers for accurate subclassification of RCC (119). This classification uses the expression of 65 different miRNAs in order to distinguish normal kidney, ccRCC, pRCC, chRCC and renal oncocytoma (119). Previous reports have also shown that miRNAs are differentially expressed between the RCC subtypes (116-118). For this study, we proposed a two-step classification system for differentiating the most common subtypes, namely ccRCC, pRCC and chRCC, as well as oncocytoma using a limited panel of miRNAs. These stable molecules may complement microscopy in order to distinguish RCC cases with inconspicuous features.

2 Material and Methods

2.1 Patient Samples
The discovery set included a total of 40 fresh, frozen primary cancer tissues from 40 patients with RCC (20 ccRCC, 10 pRCC and 10 chRCC) and 10 patients diagnosed with the benign kidney neoplasm, oncocytoma. The validation set included FFPE tissues from 30 ccRCC, 28...
pRCC, 20 chRCC and 12 oncocytoma cases. In addition, 4 unclassified RCC cases were collected. All specimen were collected from St. Michael's Hospital from Toronto, Ontario with research ethics board approval.

2.2 RNA Extraction

Sections of the FFPE tissue conforming to the standard morphology of the designated subtype were used for RNA extraction. Total RNA was extracted from FFPE tissues using the miRNeasy FFPE Kit (Qiagen, Mississauga, Ontario) as described by the manufacturer's protocol. The quality of RNA was determined spectrophotometrically by measuring the 260/280 and 260/230 ratios with the NanoDrop 2000c spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE). RNA samples were stored at -80°C.

2.3 Absolute miRNA Quantification

RNA extracted from FFPE tissues was subject to qRT-PCR using the TaqMan® miRNA assays (Applied Biosystems, Foster City, CA, USA) (252). A standard curve for each miRNA of interest was generated using mirVana™ mimics (Applied Biosystems). Quantification of cDNA for both the standards and unknowns was conducted on the Viia™ 7 Real-Time PCR System (Applied Biosystems) and represent a summary of 3 technical replicates. The unknown absolute expression level of each miRNA was interpolated from a semi-log curve.

3 Results

3.1 A Classification Scheme for RCC Subtypes Using 6 miRNAs

miRNA microarray data for ccRCC, pRCC, chRCC and oncocytoma was used to generate a decision tree for tumor classification, as shown in Figure 3.1. From this discovery cohort, we propose in the first decision to distinguish ccRCC and pRCC from chRCC and oncocytoma based on miR-221 and -222 expression levels. Subsequently, high miR-126 and low miR-498 expression levels will favour a diagnosis of ccRCC instead of pRCC. Finally, low miR-22 and high miR-15b will be indicative of chRCC rather than oncocytoma.
miRNA microarray analysis on RNA isolated from fresh frozen tissues of 20 ccRCC, 10 pRCC, 10 chRCC and 10 oncocyto ma cases was used to develop a decision-tree model. The classification scheme is based on the differential expression levels of miR-126, -15b, -22, -221, -222 and -498.

3.2 miR-221 and -222 Can Differentiate ccRCC and pRCC from chRCC and Oncocytoma

The absolute copy number of miR-221 and -222 was quantified for ccRCC, pRCC, chRCC and oncocyto ma using FFPE tissue samples by qRT-PCR. Figure 3.2 shows a representation of a standard curve generated for miR-221 quantification. In Figures 3.3 and 3.4, there is a separation observed between ccRCC and pRCC from chRCC and oncocyto ma based on the absolute expression levels of miR-221 and -222, respectively. With respect to miR-221, the expression level ranged between 16,503 (25th percentile) - 162,011 (75th percentile) copy numbers in ccRCC and pRCC compared to 374,907 (25th percentile) - 1,617,646 (75th percentile) in chRCC and oncocyto ma. In ccRCC and pRCC, the copy number for miR-222 was between
36,209 (25\textsuperscript{th} percentile) - 847,070 (75\textsuperscript{th} percentile), whereas the range was 1,128,772 (25\textsuperscript{th} percentile) - 7,198,912 (75\textsuperscript{th} percentile) in cHCC and oncocytoma.

**Figure 3.2. miR-221 Standard Curve.** A commercially available miR-221 mimic was subject to qRT-PCR using a TaqMan\textsuperscript{®} miRNA assay. Serial dilutions of known concentrations of the miRNA mimic were amplified using the Viia\textsuperscript{TM} 7 Real-Time PCR System. The $C_T$ values associated with the expression level of miR-221 in a pRCC and oncocytoma sample are plotted with the former circled in purple and the latter circled in orange. The experiment was performed in triplicate.
Figure 3.3. Absolute Quantification of miR-221 in ccRCC, pRCC, chRCC and oncocytoma. miRNAs were extracted from FFPE tissue samples of ccRCC, pRCC, chRCC and oncocytoma. Absolute quantification of miR-221 was performed by qRT-PCR using TaqMan® miRNA assays for miR-221. A standard curve was generated using a commercially available mimic for miR-221. Real-time PCR reactions were performed in triplicate for both the standards and FFPE tissues.
Figure 3.4. Absolute Expression of miR-222 in ccRCC, pRCC, chRCC and oncocytoma.

Total RNA, including microRNAs, were isolated from FFPE tissues of ccRCC, pRCC, chRCC and oncocytoma. The absolute expression level of miR-222 was determined using TaqMan® miRNA assays and by performing qRT-PCR. Using a mimic for miR-222, a standard curve was generated. Experiments were performed in triplicate.
3.3 miR-126 Can Distinguish Between ccRCC and pRCC

The expression level of miR-126 was absolutely quantified in ccRCC and pRCC by qRT-PCR. Majority of the ccRCC cases had much greater expression level for miR-126 compared to the pRCC samples, as shown in Figure 3.5. The miR-126 copy number ranged from 955,648 (25\textsuperscript{th} percentile) - 8,163,110 (75\textsuperscript{th} percentile) in the ccRCC cohort. In pRCC, miR-126 absolute expression level was 81,736 (25\textsuperscript{th} percentile) - 408,136 (75\textsuperscript{th} percentile) copy numbers. The expression level of miR-498 was low in both ccRCC and pRCC (C\textsubscript{T} value > 34) (Data not shown). Therefore, this miRNA could not be reliably quantitated by qRT-PCR, and the decision was made to exclude this miRNA from the RCC subclassification model.

![Figure 3.5](image)

**Figure 3.5. Absolute Quantification of miR-126 in ccRCC and pRCC.** The absolute expression level of miR-126 was measured in FFPE samples of ccRCC and pRCC by qRT-PCR with TaqMan\textsuperscript{®} miRNA assays. Absolute miR-126 copy number for each sample was interpolated from a standard curve generated with a commercially available miRNA mimic. Real-time PCR reactions were performed in triplicate.
3.4 There Is Overlap in miR-15b and -22 Expression Between chRCC and Oncocytoma

The expression levels of miR-15b and -22 were quantified in FFPE tissues of chRCC and oncocytoma. Overall, chRCC had higher miR-15b expression relative to oncocytoma (Figure 3.6). miR-15b expression level in chRCC ranged between 443,078 (25th percentile) - 862,147 (75th percentile) copy numbers, whereas miR-15b expression spanned between 230,965 (25th percentile) - 514,668 (75th percentile) molecules in oncocytoma. There was significant overlap in the expression levels of miR-22 between chRCC and oncocytoma, as shown in Figure 3.7. miR-22 expression ranged between 561,625 (25th percentile) - 865,876 (75th percentile) copy numbers in chRCC and varied between 367,657 (25th percentile) - 1,733,284 (75th percentile) copy numbers in oncocytoma.

![Figure 3.6. Expression Level of miR-15b in chRCC and Oncocytoma.](image-url)

RNA was isolated from FFPE tissues of chRCC and oncocytoma, and used for measuring miR-15b expression by TaqMan® miRNA assays and qRT-PCR. A standard curve for miR-15b was established using a mimic for this miRNA. Experiments were performed in triplicate.
Figure 3.7. Absolute Expression of miR-22 in chRCC and Renal Oncocytoma. The expression level of miR-22 was measured in FFPE tissues of both chRCC and oncocytoma by qRT-PCR using TaqMan® miRNA assays. A standard curve for miR-22 was generated using a mimic for this miRNA. miR-22 expression was quantified in triplicate for both the standards and tissue samples.
3.5 Expression of miR-221, -222 and -126 in Unclassified RCC

The expression levels of miR-221, -222 and -126, which were included in the classification scheme for the most common RCC subtypes and oncocytoma (Figure 3.1), were quantified in 4 cases of unclassified RCC. In Figure 3.8, unclassified RCC sample B had the greatest miR-221 expression compared to the other unclassified RCC cases. In addition, samples A and C had miR-221 expression levels similar to those observed in chRCC and renal oncocytoma, whereas miR-221 expression in sample D more closely overlapped with that of ccRCC and pRCC cases. From Figure 3.9, unclassified RCC sample B had the highest level of miR-222 expression. Samples A and D had overlapping expression levels of miR-222 observed in ccRCC and pRCC, and miR-222 expression from sample C was more similar to chRCC and oncocytoma. Furthermore, sample C had the greatest miR-222 expression from the other unclassified RCC cases. As shown in Figure 2.10, all unclassified RCC samples had similar miR-126 expression levels as those seen in the ccRCC cohort.
Figure 3.8. Expression Level of miR-221 in ccRCC, pRCC, chRCC, Oncocytoma and Unclassified RCC. miR-221 was quantified from 4 FFPE tissues of unclassified RCC by qRT-PCR using TaqMan® miRNA assays. A standard curve was generated for absolute quantification of miR-221 using a mimic for this miRNA. Experiments were performed in triplicate.
Figure 3.9. Level of miR-222 Expression in ccRCC, pRCC, chRCC, Oncocytoma and Unclassified RCC. Using 4 FFPE tissues of unclassified RCC, miR-222 expression was measured by qRT-PCR with TaqMan® miRNA assays. A standard curve was constructed using known amounts of a miR-222 mimic. Real-time PCR reactions were performed in triplicate.
Figure 3.10. Expression Level of miR-126 in Unclassified RCC Samples Compared to ccRCC and pRCC. Total RNA, including miRNAs, was extracted from 4 unclassified RCC samples. The expression level of miR-126 was quantified by qRT-PCR using a standard curve for this miRNA. Real-time PCR reactions were conducted in triplicate.

4 Discussion

RCC is a heterogeneous disease encompassing a spectrum of distinct subtypes. The subtypes of this cancer may be difficult to accurately differentiate from one another due to overlapping morphologies. Therefore, due to the high tissue specificity and stability of miRNAs, these non-coding RNA molecules have been proposed to subclassify RCC (142, 291). In this study, we validated a classification scheme based on the expression levels of 5 different miRNAs (Figure 3.1). From our validation cohort, we observed miR-221 and -222 to distinguish chRCC and oncocytoma from ccRCC and pRCC (Figure 3.3 and 3.4, respectively). miR-221 and -222 are
encoded less than 1 kilobases apart on chromosome X. Both miRNAs have been implicated in promoting epithelial-mesenchymal transition (291). miR-221 and -222 also have common targets, such as p27 and B-cell chronic lymphocytic leukemia/lymphoma 2 binding component 3 (292). Moreover, these miRNAs have been previously reported to correspond to RCC tumors originating from the convoluted tubule, which is in agreement with our results (118).

We evaluated the expression of miRNAs that can discern between ccRCC and pRCC, and chRCC and oncocytoma. In Figure 3.5, we demonstrated that the absolute expression level of miR-126 can differentiate between ccRCC and pRCC. This miRNA is commonly associated with angiogenesis (293). However, there are emerging findings that suggest an important role for miR-126 in regulating innate immune function (294-296). Dr. Yousef’s lab has previously reported up-regulation of miR-126 in ccRCC tissue relative to matched normal kidney (248). Unlike ccRCC, pRCC is a highly hypovascular tumor (109, 248) Thus, it is not surprising that miR-126 expression is significantly elevated in ccRCC relative to pRCC. In addition, miR-15b and -22 were candidates for distinguishing chRCC from renal oncocytoma. However, both miRNAs did not draw a distinct separation between chRCC and this benign renal neoplasm as shown in Figures 3.6 and 3.7. These two tumors have been shown to share similar molecular profiles. For instance, chRCC and oncocytoma displayed similar immunostaining for the receptor tyrosine kinase Ron, which was distinct from the staining pattern observed in the other RCC subtypes (297). Another research group showed a high level of similarity between chRCC and renal oncocytoma in the expression of 1,550 different transcripts from microarray analysis (106). Therefore, measuring the expression levels of multiple miRNAs and other molecular biomarkers simultaneously may provide accurate classification of chRCC and oncocytoma.

From this analysis, miR-126, -221 and -222 were the most powerful biomarkers for subclassifying ccRCC, pRCC, chRCC and oncocytoma. Unclassified RCC is a rare RCC subtype for which we have little understanding in terms of its molecular changes. We quantified miR-221, -222 and -126 in 4 unclassified RCC subtypes and correlated their expression levels with those observed in the most common RCC subtypes, as well as oncocytoma. We observed that unclassified RCC sample B had the greatest miR-221 and -222 expression levels, as shown in Figures 3.8 and 3.9, respectively, suggesting that this tumor may originate from the distal tubules. Furthermore, from Figure 3.10, all unclassified RCC cases had similar miR-126 expression levels to those from ccRCC. Therefore, the elevated miR-126 expression observed in
unclassified RCC may be a result of high vascularity in the tumors. Therefore, these cases may receive therapeutic benefit from sorafenib and sunitinib.

In summary, we were able to demonstrate that a small number of miRNAs can subclassify the most common RCC subtypes and oncocytoma. Interestingly, one of the cases included in our validation set was initially diagnosed as ccRCC, but upon second evaluation the diagnosis was changed to chRCC. The miR-222 expression level of this particular case overlapped with that observed in the ccRCC and pRCC cohort. Therefore, our classification scheme may serve as a complementary tool to current ones used for subclassifying difficult cases of RCC.

5 Conclusions

The expression levels of miR-221 and -222 can distinguish between ccRCC and pRCC from chRCC and oncocytoma. miR-126 can discern ccRCC from pRCC, which both originate from the proximal tubules. In addition, there was overlap observed between chRCC and oncocytoma, which arise from the distal tubules, in their expression levels of miR-15b and -22. However, miR-15b was a stronger biomarker than miR-22 for discerning chRCC from the benign neoplasm. To our knowledge, we are the first to measure the expression levels of miR-126, -221 and -222 in unclassified RCC. We provide evidence that this rare RCC subtype may have similar miRNA expression levels to those observed in the most common RCC subtypes and oncocytoma. In summary, we demonstrated that a limited panel of miRNAs can sub-classify ccRCC, pRCC, chRCC and oncocytoma.

6 Future Experiments

Our RCC classification scheme needs to be validated using an independent and larger number of ccRCC, pRCC, chRCC and renal oncocytoma samples. The expression level of our candidate miRNA biomarkers should also be measured in RCC subtypes that share morphological features with other subtypes and hybrid tumors. Moreover, future studies should include RCC samples with sarcomatoid differentiation, which is an independent marker for worse prognosis in RCC, to verify if classification of the tumor subtype by miRNA expression is affected by sarcomatoid change (298). Moreover, the clinical application of in situ hybridization against miR-126, -15b, -22, -221 and -222 should be investigated. In particular, in situ hybridization may shed light on
whether pRCC samples with similar miR-126 expression to that of ccRCC cases is due to high vascularization since miR-126 is a hallmark for angiogenesis (299).

In addition to measuring the level of miRNA expression by in situ hybridization, whether miR-126, -15b, -22, -221 and -222 can be quantified in blood or urine warrants investigation and if there is differential expression of these miRNAs between ccRCC, pRCC, chRCC and oncocytoma. The expression levels of miR-221 and -222 have been previously quantitated in the plasma of RCC patients (300). Such a biomarker would allow for the RCC subtype to be diagnosed without a need for surgery, thereby preventing unnecessary complications that may be a result of biopsy, such as significant blood loss or infection (301).
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