

## Current Status of Biomarker Discovery in Human clear Cell Renal Cell Carcinoma

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### Abstract

The incidence of clear cell renal cell carcinoma (ccRCC) continues to increase while very few treatment options are available for therapy. Development of metastatic disease dramatically decreases patient survival; therefore detection at an early stage while still localized to the renal capsule is imperative for a favorable prognosis. With recent advances in molecular technologies and subsequent understanding of the underlying pathology of disease, molecular markers have been defined that are predictive of tumor stage, metastatic potential and patient prognosis. Analysis of these biomarkers along with current staging techniques already used in the clinic may allow for implementation of personalized post-surgical treatment plans as well provide further molecular pathways for targeted therapeutic interventions. In this review we will highlight the potential ccRCC biomarkers that have been identified through a variety of molecular techniques to date, and provide insight into research models for biomarker discovery.

**Keywords:** Clear Cell Renal Cell Carcinoma (ccRCC); Proteomics; Biomarkers; Gene Profiling; microRNA

### Introduction

Renal cell carcinoma (RCC) is the third most prevalent urological cancer, the 10th most common cause of cancer death in men and the 9th most common cause in women. Within this year more than 54,000 individuals will be diagnosed with RCC and around 12,000 will die from this disease [1]. Commonly asymptomatic, a third of RCCs are diagnosed when they are already metastatic resulting in a 95% mortality rate [2].

There are four major subtypes of RCC, papillary, chromophobe, collecting duct and clear cell, all of which originate from the renal tubular epithelium. Clear cell renal cell carcinoma (ccRCC) accounts for approximately 80% of all renal cancers and is therefore the most common subtype. Standard of care for the treatment of patients presenting with localized ccRCC is radical or partial nephrectomy, however ~30% of these patients develop metastatic ccRCC even after surgery [3]. Metastatic tumors are usually identified in subsequent follow-up visits, post-surgery using imaging techniques that detect the mass of the established metastatic tumor. Traditionally metastatic ccRCC is chemo- and radiation-resistant providing literally no treatment options (cytokine therapy is beneficial in <5% patients) other than surgical [4]. The development of metastatic ccRCC greatly reduces the 5-year survival of ccRCC patients to less than 10% [5]. Therefore, early detection of ccRCC is of utmost importance for treatment and patient survival [2].

With the more common use of imaging procedures in the clinic, ccRCC and other masses within the kidney are being more readily identified and at an earlier stage. However, as these images cannot determine tumor stage, the best predictor of stage progression still remains tumor staging of postoperative tissue. As this staging process requires the patient to undergo surgery, the need for the identification of biomarkers capable of determining RCC type, tumor stage and metastatic potential that are obtainable from non-invasive or minimally invasive techniques / biological fluids (i.e. serum or urine)

are desirable. However, biomarkers for early detection of ccRCC or metastatic disease potential do not currently exist resulting in late diagnosis and subsequent poor prognosis for patients with metastatic disease [6]. This is in spite of the relatively high availability of tissue, blood and urine samples compared to other human cancers that could be used to identify diagnostic and prognostic biomarkers of ccRCC.

The NIH defines a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological response to a therapeutic intervention.” [7]. This review will focus on potential biomarkers in clear cell renal cell carcinoma that classify stage of disease, metastatic potential and patient outcomes as identified through immunohistochemistry, gene profiling, proteomics, microRNA and cytogenetic signatures. These biological markers of disease also have the potential to be novel therapeutic targets for drug development in a disease with few treatment options.

### von Hippel-Lindau (VHL) as a biomarker of ccRCC

As cancer is a multifaceted disease leading to multiple changes in the genetic make up of patients, extensive research has been undertaken to identify underlying mutations and those genes that are epigenetically silenced in ccRCC patient tissue. The most documented mutation in ccRCC is in the tumor suppressor gene, von Hippel-Lindau (VHL) which has been reported to occur in ~50% of all sporadic ccRCCs

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[8]. Further silencing of the promoter of the VHL promoter through epigenetic changes has been reported in a further 10-20% of ccRCC [9].

The protein product of the VHL gene interacts with hypoxia-inducible factors (HIFs) leading to HIFs subsequent ubiquitination and proteosomal degradation. Loss of VHL and subsequent HIFa accumulation leads to expression of transforming growth factor alpha causing activation of the signaling cascade epidermal growth factor receptor (EGFR), PI3 kinase, AKT, I $\kappa$ B $\alpha$  to NF- $\kappa$ B [10]. Downstream targets of the HIF signaling pathways are involved in cell survival, proliferation and angiogenesis [11]. The identification of these signaling cascades has brought about a number of targeted therapies that are approved for the treatment of RCC. Sunitinib, Sorafenib and Pazopanib which target receptor tyrosine kinases including VEGFR and Temsirolimus targets the mammalian target of rapamycin (mTOR). However these new targeted therapies are not able to cure ccRCC and their benefits to patient survival is only a number of months [12].

Studies examining VHL mutations as a predictor of prognosis for ccRCC have been inconsistent. Two large studies have identified that somatic VHL gene alterations lead to improved survival especially in early stage (stage1-2) disease [13,14] but others state that mutations that lead to loss of function of VHL are associated with a poorer prognosis [15] and decreased overall survival [16].

## Gene Expression Profiling

Gene profiling techniques have been used extensively in distinguishing transcriptional changes in patients with ccRCC compared to normal kidney tissue. Gene expression profiles have been created to distinguish RCC subtypes, gene signatures for early stage ccRCC and to define progression and metastatic potential of tumors. Studies comparing ccRCC, chromophobe and oncocytoma subtypes of RCC identified expression profiles for each of these subtypes of RCC that correlated strongly with histopathological classification. In these studies vimentin expression was identified to be a sensitive and specific marker for ccRCC [17]. Later studies confirmed that vimentin expression clearly differentiated ccRCC from chromophobe RCC and the added loss of CD9 expression in ccRCC as another identifying gene marker verified by immunohistochemical staining of patient tissue [18].

Studies from our laboratory have previously identified that an early event in ccRCC is loss of expression of the type III TGF- $\beta$  receptor (T $\beta$ RIII) and that re-expression of T $\beta$ RIII leads to decreased ccRCC cell growth and induction of apoptosis [19,20]. We have also identified that the loss of T $\beta$ RIII is due to epigenetic silencing of the transcription factor GATA3, a positive regulator of T $\beta$ RIII expression [21]. Loss of T $\beta$ RIII and later loss of T $\beta$ RII between stage 2 and stage 3 ccRCC identifies that the inability of cells to respond to TGF- $\beta$  signaling is an important event in ccRCC initiation and progression [19]. Both T $\beta$ RIII and GATA3 are being further investigated as candidate biomarkers for ccRCC as well as potential targets for therapy. Recent gene expression profiling of ccRCC has identified that high expression of cell cycle related genes aurora kinase B (AURKB), cyclin dependant kinase 1 (CDK1) and polo-like kinase 1 (PLK1) are associated with advanced ccRCC and decreased patient survival [22]. Inhibition of PLK1 in tumor bearing mice inhibited tumor growth suggesting PLK1 as a potential therapeutic target in ccRCC patients.

Analysis of gene expression signatures from early stage ccRCC have shed light on pathways that are modulated in early tumorigenesis when compared to matched patient normal renal tissue [23]. Three pathways

were identified which are involved with immune activation, down-regulation of metabolism and loss of normal renal function. Along with these pathway changes, the transcription factors GATA3, TFAP2L1, TFAP2B and DMRT2, all of which are important for normal renal development, were identified to be down-regulated in these early stage ccRCC tumors. The down-regulation of metabolism and loss of normal renal function were verified by a separate group who also identified up-regulation of many signal transduction genes in ccRCC [24].

Genes have further been identified which are differentially expressed in ccRCC patients with relatively non-aggressive disease compared to those with aggressive disease [25,26]. In studies by Takahashi *et al.* ceruloplasmin had the highest increase in expression when compared to normal renal tissue while vascular endothelial growth factor (VEGF) was highly expressed in 96% of 29 patient ccRCC samples. These studies also identify groups of genes whose up-regulation in ccRCC support favorable outcome, versus another group of genes that when lost in ccRCC are associated with a poorer outcome. Later investigations by Kosari *et al.* identified a panel of 34 genes that were predictive for the determination of aggressive, non-aggressive and non-neoplastic samples [26]. Both of these studies identify potential biomarkers for disease that can be measured from primary site tumors and might be predictive of tumor aggressiveness and metastatic potential.

## Protein markers of ccRCC in tissue

Immunohistochemical evaluation of tissue samples derived from patients who have undergone nephrectomy have allowed for validation of findings observed in gene array analysis and for the identification of potentially novel prognostic protein biomarkers for tumor recurrence, aggressiveness and patient survival. Along with other factors such as tumor size, stage and nuclear grade, these biomarkers could compliment and assist in determining survival or treatment options available to those patients showing greatest risk for recurrence and metastasis. The use of PAX-8, PAX-2, human kidney injury molecule-1 (hKIM-1), renal cell carcinoma monoclonal antibody (RCCma), CD10 and hepatocyte nuclear factor-1- $\beta$  as immunohistochemical biomarkers for the diagnosis of metastatic ccRCC have recently been reviewed and therefore will not be discussed within this article [27]. However, these markers have the potential for increased sensitivity and specificity for the diagnosis of metastatic ccRCC.

A number of studies have identified protein biomarkers whose expression analysis could assist with post-operative decisions as to treatment options for ccRCC patients by distinguishing those with higher risk for reoccurrence of disease or metastasis. A number of factors encompassing tumor size, tumor stage, Eastern Cooperative Oncology Group performance status, and the expression of Ki67, EpCAM and p53 have all been identified to be significantly associated with recurrence in ccRCC [28]. Other molecular markers such as the expression in primary ccRCC tumors of the calcium-dependent phospholipid-binding protein annexin II is associated with higher tumor stage and is an independent predictor of metastasis [29]. High levels of plasminogen activator inhibitor-1 expression in ccRCC is associated with high nuclear grade, advanced stage, distant metastatic sites and/or decreased disease-free survival [30]. On the flip side, low tumor expression of the Type II TGF- $\beta$  receptor (T $\beta$ RII) has been associated with a less aggressive tumor phenotype and a better overall cancer-specific survival compared to patients with higher expression levels of T $\beta$ RII [31]. Decreased expression of TIMP3 is observed in 95% of ccRCC tumors and significantly correlates with decreased expression of T $\beta$ RII [32]. Analysis of patient tumor molecular signatures consisting

of the expression of Ki-67, p53, VEGFR-1, epithelial VEGFR-1 and epithelial VEGF-D are capable of predicting disease free survival for localized ccRCC after nephrectomy [33].

Molecular signatures have also been utilized for the prediction of disease prognosis and patient survival. One predictive model for survival takes into account metastasis status, tumor stage, Eastern Cooperative Oncology Group performance status, CAIX, p53 and vimentin [34]. The expression of survivin in ccRCC has been identified as an independent predictor of ccRCC progression and cancer specific death [35], while expression of B7-H1 in ccRCC tumors has been associated with rapid cancer progression and accelerated cancer specific mortality [36]. Together, survivin and B7-H1 are collaborative predictors of ccRCC patient survival [37]. Tissue arrays from 150 patients with metastatic ccRCC identified that increased expression of p53, gelsolin and Ki-67 and decreased expression of carbonic anhydrase 9 and 12 correlated with an unfavorable prognosis [38]. Previous studies had already shown a correlation of carbonic anhydrase 9 (CAIX) expression, alone or in combination with Ki-67, as a predictor of patient survival [39,40]. Further studies examining CAIX stated that although loss of CAIX was associated with increased risk of ccRCC related death, it could not be classified as an independent marker and that its expression in other extra-renal organs questions its ability to be a prognostic marker or therapeutic target of ccRCC [41]. However, high expression of CAIX in ccRCC and RCC has been shown to predict responsiveness to interleukin 2 therapy [42].

Data from the analysis of grade 2 ccRCC tumors suggest a different role for gelsolin than those seen in the metastatic tumor studies above as in grade 2 tumors, decreased expression of gelsolin along with an increased expression of Ki-67 is suggestive of poor cancer-specific survival [43]. Along with high expression of Ki-67 and p53, the high expression of VEGF and caveolin are associated with decreased patient survival while high expression levels of bcl-2 and cyclin D1 are associated with increased survival. Of these biomarkers, the expressions of p53 and bcl-2 have been identified as individual predictors of disease specific survival [44].

Two studies have identified that the p65/NF- $\kappa$ B signaling pathway may be a potential therapeutic target for ccRCC because of its correlation of expression with VEGF and osteopontin (OPN). Dordevic *et al.* identified that a higher, inhomogeneous expression of VEGF was associated with p65/NF- $\kappa$ B activity [45], while further research from members of the same group suggest that p65/NF- $\kappa$ B signaling may be involved in OPN-mediated ccRCC progression [46]. As VEGF is an important factor in angiogenesis, a process known to be involved in tumor progression and metastasis, and osteopontin has been identified to play a role in tumorigenesis and metastasis [47], targeting these proteins and the members of the transcription factor family NF- $\kappa$ B could be important therapeutic options for the treatment of ccRCC.

We have recently analyzed over 75 individual ccRCC normal-tumor tissue protein lysate pairs by standard proteomic methods. These samples can also be subdivided into four different pathological stages, Stage 1 to Stage 4. The proteomic analysis of a pooled subset of these samples was done for each of the 4 normal-tumor ccRCC pooled sample pairs (n=5 from each stage grouping). As shown in Table 1, select proteins based on our previous studies [23] and other studies described in the preceding paragraphs are provided. Averaged spectral count values for each pool and condition are listed. The enolase over-expression in the tumor samples and lack of aldolase B expression is consistent with other previous reports [27], and data from the Copland laboratory [23]. As highlighted by aldolase B and using the same spectral counting criteria, there are an additional 80 enzymes involved

Protein Name	Stage 1		Stage 2		Stage 3		Stage 4	
	N	T	N	T	N	T	N	T
Aldolase B	53	ND	45	ND	85	ND	79	39
Gamma Enolase	ND	63	ND	12	ND	46	ND	29
Adipophilin	ND	63	ND	12	ND	46	ND	29
TGF-beta induced	23	107	14	12	12	36	11	20
Vimentin	107	168	60	62	96	189	201	271
Isocitrate DH	11	3	11	2	11	ND	8	3
Gelsolin	11	13	12	2	8	7	8	12
Ceruloplasmin	9	10	9	6	3	8	12	16

**Table 1: Selected expression of putative ccRCC protein biomarkers in pooled normal (N) and tumor (T) tissues representative of Stage 1-4 cancers.** Individual renal tissues (10-15 mg) from matched tumor and normal pairs were processed by trifluoroethanol (TFE) solubilization and sonication as adapted from Wang *et al.* [104]. Following trypsin digestion, the resulting peptides were separated by MudPIT and analyzed for protein identification as described in the legend to Table 2. Results are listed as spectral counts for each indicated protein.

in basic metabolic processes that were at least 2-fold or greater under-expressed in the tumor tissues, across all four stages. Thirty five of these enzymes are involved in lipid and coenzyme A metabolism (data not shown). Conversely, in addition to gamma-enolase and its expression trend of tumor over-expression, three other enzymes upstream and downstream of enolase were similarly over-expressed: pyruvate kinase M1/M2, 6-phosphofructokinase and ATP citrate synthase. These dramatic changes in metabolic enzyme levels are consistent with the Warburg effect and the transcriptomic data previously reported [23].

### Fluid based biomarkers: Serum

Fluid based biomarkers for detection, stage, progression or regression of disease with available targeted treatment options are attractive due to their ease of acquisition and for ease of follow-up monitoring of disease during subsequent patient checkups. On a more global scale, the identification of fluid based biomarkers could allow for the earlier detection of a number of primary and metastatic growths within the patient [48]. Over the past decade, there have been extensive biomarker discovery efforts using emerging mass spectrometry-based methods that include profiling of low molecular weight serum proteins [49,50] and analysis of post-translational modifications like glycosylation [51]. Once discovered and validated, detection of serum biomarkers is routinely done with automated ELISA formats, the most common example being detection of serum prostate specific antigen for prostate cancers [51]. Multiplexed immunological assays incorporating bead-based approaches are also being developed [52]. For ccRCC, a number a number of proteins identified to be over-expressed at the protein level in resected patient tissues have also been identified to be elevated in ccRCC patient serum. Notably, elevated levels of VEGF [53] have been identified as has elevated expression of osteopontin [54]. Elevated levels of osteopontin were associated with poor survival of patients and as a potential marker for distant metastasis [54]. Other serum markers of note include: elevated levels of B7x, a protein that inhibits cell-cycle progression, proliferation, and cytokine production of both CD4+ and CD8+ T cells which is elevated in ccRCC patient serum and is associated with advanced stage disease [55]; ceruloplasmin, the major copper carrying blood protein has also been identified to be secreted by ccRCC tumors and elevated levels have been observed in patient serum [56,57]; and significant elevation of TGF- $\beta$ 1 has been recorded in plasma from patients with ccRCC [58]. Analysis of soluble B7-H1 in patient sera agreed with findings from IHC studies in that higher expression of this protein in preoperative samples was associated with large tumors, advanced grade and poor patient survival [59].

## Urine biomarkers

Urine as a medium for biomarker analysis has a number of advantages over serum including ease of collection, lack of interfering proteins and its molecular activity changes little after sampling [60]. Examination of RCC patient urine utilizing methylation specific PCR techniques to identify promoter hypermethylation of six tumor suppressor genes discovered that hypermethylation of VHL was only found in ccRCC urine samples. Hypermethylation of p14ARF, APC and RASSF1A were more frequent in non-ccRCC samples and none of these genes were methylated in normal patient urine controls [61].

Studies undertaken in breast, bladder and prostate cancers have identified proteins expressed in the urine that correlate with each of these disease states [62,63]. Investigations in RCC have identified two novel urine biomarkers, aquaporin 1 and adipophilin, that are highly expressed in kidney cancers that originate from the proximal tubules (ccRCC and papillary) [64]. Another group has identified several components of the urine that allows for recognition of patients with ccRCC in comparison to healthy volunteer samples [65] with another using three urine specific signals to achieve the same outcome [66]. These three signals showed 100% sensitivity for stage 1 ccRCC.

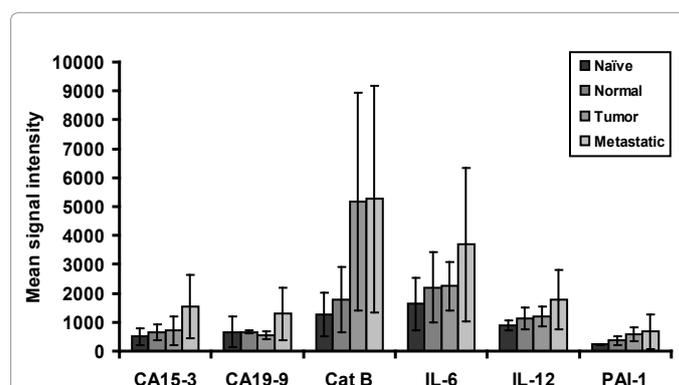
## Animal models for identification of potential biomarkers

As early as 1979, the ability of implanted patient tumors in immuno-suppressed mouse models to produce secreted proteins had been observed [67]. In these studies, a number of freshly resected solid human tumors were subcutaneously implanted into female athymic nude mice of the BALB/c background and plasma obtained at the time of serial transplantation. Each of these plasma samples were then tested for the expression of 20 different protein markers. In the case of the ccRCC tumor implants, plasma from both implanted mice expressed human albumin,  $\alpha$ 1-antitrypsin and transferrin. These studies identify that humanized mouse tumor models have the potential to identify serum soluble biomarkers of disease.

We have recently used proteomic identification techniques along with validation by real-time PCR to identify and validate serum soluble proteins that are secreted into the serum of humanized mouse tumor models in the search of ccRCC specific biomarkers. Fresh, de-identified patient ccRCC tumor tissue along with distant site normal kidney tissue were obtained from Mayo Clinic Florida (with Mayo Clinic Institutional Review Board (IRB) approval), cut into 1cm<sup>3</sup> sections and subcutaneously implanted into female nude mice in accordance with Mayo Clinic Institutional Animal Care and Use Committee (IACUC) and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 80-23, revised 1996). Less than 10% of the fresh, transplanted tumor tissues seeded within these investigations. Serum was collected via terminal heart bleeds prior to tumor harvest and subsequent passage.

Serums from these animals were analyzed by commercial serum biomarker and cytokine antibody arrays and mass spectrometry-based protein identification methods, summarized as follows. Use of a commercial antibody array allowed rapid screening of serum obtained from individual explanted mice carrying normal kidney, non-metastatic tumor and metastatic tumor tissues. As shown in Figure 1, the antibody array was effective at identifying potential candidates related to progression to metastatic disease. These include cathepsin B, CA19-9, CA15-3, PAI-1, IL-12 and IL-6. Further analysis using a murine cytokine array panel established that increased IL-12

and IL-6 levels were also detected in pooled metastatic serum. While encouraging and consistent with previous reports about increased IL-12 and IL-6 expression in RCC [68,69], this data highlights one of the major challenges in using proteomics profiling to delineate mouse serum from human explant models. Is the signal detected really specific to mouse or human species? Analysis of serum proteins by standard methods is even more confounding. The high sequence homology of serum proteins in mice and humans makes interpretation of species specific protein level changes difficult to access. As summarized in Table 2, common serum proteins are listed with their comparative levels in pooled serum samples from normal tissue, non-metastatic tumor and tumor tissues determined by spectral counting. The protein names are listed under each sub-group in which the highest level of expression was detected. As would be expected, major serum proteins were detected (albumin and immunoglobulins were excluded), and included many of the potential biomarkers described for ccRCC. Of note is the striking difference in the levels of different complement proteins (C3, factor B, factor H, factor I) detected in mouse serum from animals implanted with normal renal tissues relative to tumor tissues. The lack of complement protein changes associated with the tumor tissues could lead to different approaches for evaluating and comparing the immune response to ccRCC in humans. Expression of gelsolin and ceruloplasmin as determined by spectral counting were verified by western blotting (Figure 2a and 2b) confirming loss of gelsolin expression and increased ceruloplasmin expression in serum samples from mice bearing human metastatic ccRCC tissue. Analysis of tissue mRNA expression in a separate patient matched data set was also performed but the results did not correlate with serum protein expression levels (Figure 2c and 2d). Further analysis of serum samples will require the use of antibodies vetted for species specificity, and more extensive fractionation of the serum prior to MS detection to get into the lower abundance proteins present, potentially focusing on N-glycoproteins for parallel depletion/enrichment of tumor secreted proteins. As this explant model has the potential to provide great utility in testing and evaluating potential ccRCC therapeutics, this baseline type of data is critical in interpreting future results. Being able to identify host murine protein changes in response to human tumor implantation, relative to changes in the secretome of human tumor proteins, could lead to novel serum based biomarker candidates for use in human studies.



**Figure 1: Cancer biomarker antibody array analysis of sera from mouse renal tissue explants.** Individual sera from control mice (n=2), normal renal tissue (n=3), non-metastatic tumor (n=5) and metastatic tumor (n=4) were analyzed on a Cancer Biomarker antibody array slide and detected using a SilverQuant detection kit (both from Gentel Biosciences). Intensity values for each of the 42 proteins analyzed per sample were averaged for each sample group.

## MicroRNA biomarkers of ccRCC

One of the most current forms of molecular characterization of tumors is that of microRNA (miRNA) profiling. miRNAs are naturally occurring, non-coding RNA sequences of 18-25 nucleotides in length that target mRNA to cause its degradation or repression of translation and subsequent decrease in specific protein expression. Investigations have identified that the aberrant expression of miRNAs is involved in the pathogenesis of many different human cancers [70-73]. The recent identification of miRNAs present in human plasma [74-76] and urine [76-79] raises the exciting possibility that circulating miRNA profiles could be used for clarification of tumor histotype, stage and metastatic potential. For instance, studies in breast cancer show significant increases in blood derived miR-34a in patients with advanced stage disease compared to early tumor stages and that the increase of miR-10b, miR-34a and miR-155 in serum correlated with the presence of metastases [80].

Studies in renal cancer have identified that the expression of 6 specific miRNAs can distinguish between the 4 subtypes of RCC [81] while others provided miRNA signatures for RCC subtype classification [82]. Expression profiles of miRNA in patient ccRCC tumor samples have identified miRNAs that distinguish all stages of ccRCC from patient matched normal tissue [83-86]. Interestingly from these studies, the majority of these differentially expressed miRNAs were down-regulated in ccRCC when compared to normal control tissues, a phenomenon that has been previously identified in tumor tissue [73]. Furthermore, miRNA signatures for favorable versus unfavorable outcome have been defined for ccRCC with the over-expression of miR-32 being associated with poor outcome [87]. In another study, expression of miR-106b is reported as a potential biomarker for the detection of early metastasis in patients after nephrectomy due to the observed expression levels being lower in patients with metastatic disease compared to patients in disease remission [88].

There is also the potential that targeting the expression of specific miRNAs could lead to potential therapeutic of disease [89]. Therapies to inhibit or reinstate specific miRNAs in cancer could lead to decreases in tumor cell growth as identified *in vitro* and *in vivo* [90,91]. In ccRCC, Chow *et al.* have identified that the miR-71-92 cluster that contains 7 miRNAs, is over-expressed and that individual inhibition of two of these miRNAs (miR-20a and miR-17-5p) in the ACHN cell line inhibited cell proliferation [92]. This group furthered the understanding of miRNA function in ccRCC by predicting that HIFs, mTOR, VEGF and VHL are all potential targets for dysregulated miRNAs [6,92]. Other miRNAs identified to be potential targets for therapy in ccRCC include miR-141 and miR-200c whose expression is down-regulated when compared to normal renal tissue [84,85]. Re-expression of these miRNAs in ccRCC cell lines lead to re-expression of E-cadherin [84], suggesting a potential tumor suppressor function. A separate study looking at targets of miR-200c observed an anti-correlation with mRNA expression of VEGFA suggesting loss of this miRNA in ccRCC plays a role in increases in VEGFA expression levels [93]. As of the time of publication there is one ongoing clinical trial (Clinical Trial Number: NCT00743054) for the identification of miRNAs in renal cell carcinoma patients, the roles that these miRNAs play on the formation of RCC and discovery of potential biomarkers.

## Single Nucleotide Polymorphism (SNP) genotyping and ccRCC

Recent studies have set forth to identify cytogenetic variants that are capable of predicting patient prognosis in ccRCC patients. Through

the use of genome wide single nucleotide polymorph gene arrays, copy number abnormalities and microdeletions have been identified within the genome of these patients. Polymorphs in the promoter of the IL-4 gene analyzed in a cohort of 80 patients with metastatic RCC have emerged as prognostic factors for survival [94]. Associations with survival have also been observed with SNP polymorphisms in the vascular endothelial growth factor (VEGF) gene [95]. Loss of the p arm of chromosome 9 and chromosome 14q have been frequently observed in ccRCC and when both occur together, have been correlated with a poor prognosis [96]. Singularly, deletion of the 9p chromosome leads to an aggressive phenotype of ccRCC [97] whilst loss of chromosome 14q is associated with high stage, increased risk of reoccurrence and decreased overall survival [96]. Gain of 8q is also associated with decreased survival [96] whilst one publication showed that gain of 5q predicts a favorable prognosis in a distinct clinical phenotype of ccRCC [98]. A study carried out in 2010 identified that each subtype of RCC has a distinct cytogenetic profile; -3p, +5q and -8p are only observed in ccRCC, with -3p and +5q appearing in all ccRCC tumors independent of grade or stage [99]. This study also identified cytogenetic alterations that take place in early and late stages of ccRCC, alterations that are associated with tumor grade, and alterations associated with patient survival and clinical or pathological factors [99]. As can be seen from these studies, cytogenetic profiling has the potential to determine subtype, stage, grade and patient prognosis in ccRCC.

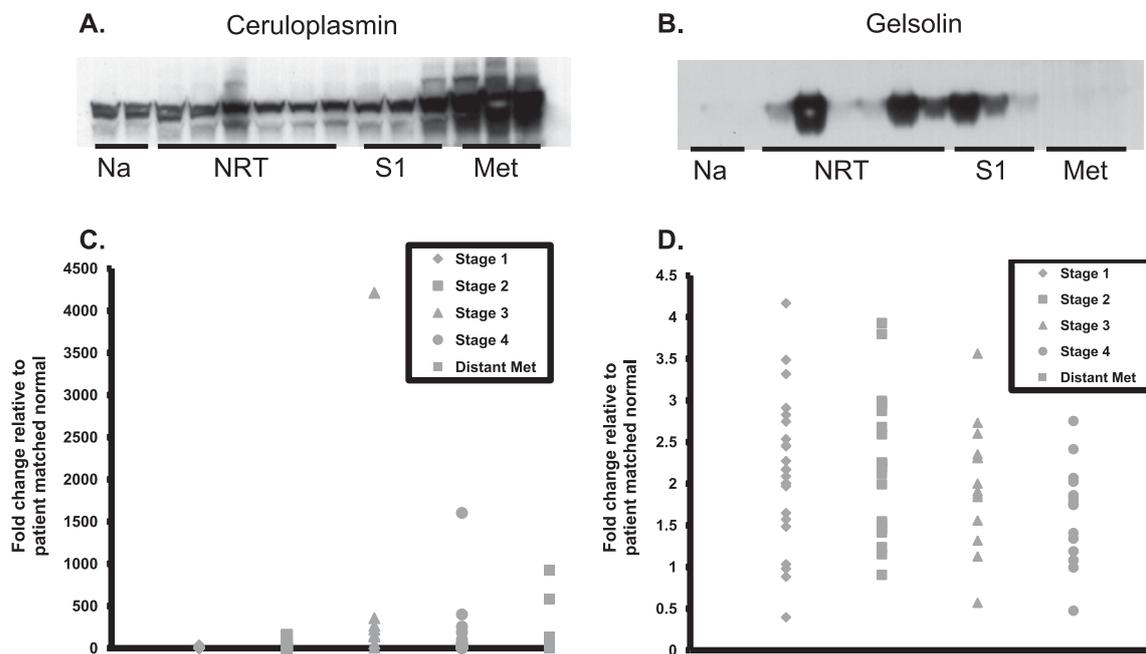
## Conclusions

As the incidence of ccRCC continues to increase each year, the continued identification of biomarkers for early detection and potential targets for therapeutic intervention are necessary. ccRCC is a fundamentally metabolic disease, and is a clear example of the Warburg effect in tumor metabolism and dramatic alterations in lipid metabolism. There is cumulative genetic, metabolomic, proteomic and lipidomic data that highlight this and point toward multiple targets of individual enzymes and pathways as molecular markers. Integrating and interpreting the breadth of this data remains one of the primary challenges in sorting out the most appropriate candidates for validation as biomarkers and development of diagnostic or prognostic assays. Because ccRCC is so fundamentally a metabolic disorder, sorting out aberrant metabolites that differ from those expected to be present from normal metabolism and diet is an important challenge.

Previous research has identified an abundance of biomarkers for staging of disease, potential for metastasis and determination of patient survival, which along side traditional methods for staging and subtype determination could lead to a deeper understanding of ccRCC and a better prognosis for the patient. However, considerations must be made to tissue acquisition and storage in the continued efforts for biomarker analysis. As reviewed by Napoli and Signoretti, collection and storage, fixation, staining and analysis of patient tissue samples needs to be carefully undertaken so that biological markers of disease are not influenced, degraded or miss validated [100]. Tissue microarray (TMA) results should also be confirmed in stained whole tissue sections verifying that the tissue cores truly represent the overall tumor sample [101]. And finally, it should be noted that as ~98% of human genes are expressed in many different tissues, care must be taken to identify those biomarkers that are truly tissue specific [102]. Biological markers identified as potential biomarkers must be sensitive and specific; only found to be present in patients with cancer and never found in those without disease. It may be that a combination of several biomarkers is needed to achieve adequate sensitivity and specificity. These biomarkers identified in multiple systems and validated in large

Highest in Normal	Normal Tissue		Primary Tissue		Metastatic Tissue	
	Peptide #	Spectral Count	peptide #	Spectral Count	peptide #	Spectral Count
Alpha-2-macroglobulin	36	410	23	101	28	188
Serotransferrin	36	260	18	80	27	185
Serum albumin	28	192	10	33	18	70
Hemopexin	18	191	11	38	10	75
Alpha-1-antitrypsin 1-1	10	186	8	65	7	74
Alpha-1-antitrypsin 1-2	9	186	7	73	5	85
Alpha-1-antitrypsin 1-4	8	159	7	70	6	51
Complement C3	35	132	17	36	30	69
Murineoglobulin-1	28	128	20	55	22	52
Apolipoprotein A-I	17	123	5	31	7	39
Plasminogen	22	72	7	7	8	11
Haptoglobin	11	65	3	3	6	15
Apolipoprotein A-II	4	63	3	19	4	15
Fibronectin	28	62	13	17	18	23
Liver carboxylesterase N	12	59	5	10	8	11
Alpha-2-HS-glycoprotein	6	59	5	15	6	38
Apolipoprotein C-III	3	58	2	19	3	16
Complement factor H	24	57	8	9	13	16
Kininogen-1	13	36	6	8	12	17
Vitamin D-binding protein	13	32	7	8	12	24
Apolipoprotein E	7	31	4	5	5	8
Inter-alpha-trypsin inhibitor heavy chain H3	10	28	5	5	10	16
Fetuin-B	6	28	3	5	4	8
Antithrombin-III	14	24	5	5	9	10
Afamin	9	19	3	4	7	8
Serine protease inhibitor A3N	9	16	3	7	4	14
Complement factor B	11	15	5	5	7	8
Vitronectin	8	15	2	3	4	9
Gelsolin	8	13	4	4	4	4
Prothrombin	8	12	4	5	4	4
Inter-alpha-trypsin inhibitor heavy chain H1	8	12	7	9	4	7
Clusterin	8	11	ND	ND	3	4
Plasma kallikrein	8	10	4	4	4	4
Carboxypeptidase N catalytic chain	7	10	2	2	2	2
Corticosteroid-binding globulin	6	8	ND	ND	4	4
Carboxypeptidase N subunit 2	4	8	3	4	3	4
Complement factor I	7	8	3	4	5	6
Serum paraoxonase/arylesterase 1	4	7	ND	ND	2	2
Serum amyloid A-1 protein	3	5	ND	ND	3	3
Heparin cofactor 2	4	5	3	3	2	3
C4b-binding protein	3	4	ND	ND	3	4
Coagulation factor X	3	4	2	2	ND	ND
<b>Highest in Non-Met Tumor</b>						
Serine protease inhibitor A3M	8	61	7	243	6	51
Serine protease inhibitor A3K	15	75	15	125	13	74
<b>Highest in Metastatic</b>						
Ceruloplasmin	27	96	10	18	27	98
Apolipoprotein A-IV	8	18	5	40	9	69
Alpha-1-acid glycoprotein 1	3	35	3	5	5	36
Hemoglobin subunit alpha	2	7	4	16	4	33
Complement C4-B	8	11	5	6	6	19
Inter-alpha-trypsin inhibitor heavy chain H2	5	6	3	5	7	12
Hemoglobin subunit beta-1	4	6	3	7	4	10

**Table 2: Spectral count and peptide number analysis of serum circulating human proteins obtained from pooled humanized ccRCC mouse tumor model systems.** Pooled mouse sera (n=5/group) from each of the three tissue explant groups were first depleted of albumin and immunoglobulins by a ProteoPrep AI spin column (Sigma-Aldrich). Following overnight trypsin digestion, peptides were analyzed using a 4-cycle automated two dimensional MudPIT separation on an LTQ Orbitrap-XL mass spectrometer as previously described [103]. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides per analyzed sample. Raw data was converted to m/z XML and searched by SEQUEST against a human or mouse IPI protein sequence database. Results are presented as the most abundantly expressed detected serum proteins in mice implanted with normal kidney tissue, primary ccRCC tumor tissue, or metastatic tumor tissue. Peptide numbers represent the number of individual peptides detected for each protein species, and the spectral count number represents the cumulative number of times a peptide from a given protein was detected across the chromatography run.



**Figure 2: Verification of gelsolin and ceruloplasmin expression levels in the serum of humanized mouse models.** As identified in Table 1, protein levels of human gelsolin were highly expressed in serum from mice bearing distant site, normal kidney tissue while human ceruloplasmin expression was highest in the serum of metastatic tumor bearing mice. Serum samples were prepared for western blot analysis and 30µg of fibrin and cell cleared serum loaded per lane. Human specific antibodies confirmed that ceruloplasmin was highly expressed in the serum of metastatic bearing mice (Figure 2a) and gelsolin expression was lost in the same group (Figure 2b). These observations held true when compared to stage 2 and 4 tumors serum samples (data not shown). Na=Naive; NRT=Normal renal tissue; S1=Stage 1 ccRCC tumor tissue; Met=Metastatic site tissue. Analysis of relative mRNA expression levels for ceruloplasmin (Figure 2c) and gelsolin (Figure 2d) in patient matched tumor tissues.

patient sample groups have the potential to further our understanding of the underlying biological mechanisms taking place in ccRCC, provide mechanisms for early detection of disease and further assist in identifying molecular pathways for targeted treatment options.

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