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Patients with ClearCode34-Identified Molecular Subtypes of Clear Cell Renal Cell Carcinoma Represent Unique Populations with Distinct Comorbidities

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Abstract

Purpose—The 34-gene classifier, ClearCode34, identifies prognostically distinct molecular subtypes of clear cell renal cell carcinoma (ccRCC) termed ccA and ccB. The primary objective of this study was to describe clinical characteristics and comorbidities of relevance in patients stratified by ClearCode34.

Patients and Methods—In this retrospective analysis, 282 patients from Moffitt Cancer Center with ccRCC with gene expression analyses of the primary tumor were identified and ClearCode34 was applied to identify tumors as ccA or ccB. The medical record and institutional databases were queried to define patient characteristics, comorbidities, and outcomes.

Results—We validated in this external cohort the superior overall survival (OS), cancer specific survival (CSS), and recurrence-free survival of ccA patients relative to ccB patients ($p < 0.001$). Addressing other clinical characteristics, the ccA patients were more likely to be obese (48%

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versus 34%, $p=0.021$) and diabetic (26% versus 13%, $p=0.035$). The ccA patients also trended towards having been more frequent users of angiotensin system inhibitors (ASIs) (71% versus 52%, $p=0.055$). In multivariate analyses, ccB status is independently associated with inferior CSS (HR 3.26, 95% CI 1.84–5.79) and OS (HR 2.50, 95% CI 1.53–4.08).

Conclusions—ClearCode34, after considering distinct patterns of comorbidities in each molecular subtype, remains a strong prognostic tool in ccRCC patients. Obesity and DM emerged as factors that may influence ccRCC phenotypes and further studies investigating the impact of these metabolic conditions functionally onto tumor biology are warranted. Additionally, use of ASI could be studied in the context of ccRCC molecular classification in future studies to better understand its impact on ccRCC outcomes.

Keywords

ClearCode34; clear cell renal cell carcinoma; obesity; diabetes mellitus

1. Introduction

Cancers of the kidney and renal pelvis are among the more common cancers diagnosed in the United States with an estimated 61,560 new cases and 14,080 deaths for 2015 [1]. In adults, the overwhelming majority of these cases were renal cell carcinoma (RCC). Significant effort has been expended in developing ways to stratify RCC patients into clinically meaningful categories in order to inform clinical decisions. Classically, anatomical features (e.g. TNM classification or Fuhrman nuclear grade) were used to predict more aggressive disease [2]. Clinical prognostic systems are commonly used and include both clinical factors and laboratory data [3, 4]. Models including molecular testing have also received much attention in the prognostication of RCC, including multi-gene expression signatures [5–7]. The most comprehensive molecular analysis to date of clear cell RCC (ccRCC), the most common RCC histologic subtype, was The Cancer Genome Atlas (TCGA) study. The TCGA group analyzed over 400 tumors on multiple platforms and described numerous molecular changes that correlated significantly with patient outcomes [8]. However, despite these intense efforts, no models using molecular analyses are routinely utilized in the clinic to direct patient care.

Brannon et al. and others used gene expression microarrays to describe two dominant molecular subtypes of ccRCC tumors termed clear cell A (ccA) and clear cell B (ccB) [6, 8, 9]. The ccA patients demonstrated superior overall survival (OS), cancer-specific survival (CSS), and recurrence free survival (RFS) [6, 7]. However, these studies, in utilizing frozen tissue and large numbers of gene probes, have significant barriers to translational and clinical applications. Brooks et al. published a method that attempts to circumvent these barriers [7]. In their work, a model using 34 genes (coined ClearCode34) was developed that reliably classified individual tumors as ccA and ccB from formalin-fixed, paraffin embedded tumor specimens (Table 1). Prognostic models containing this signature outperformed traditional clinical nomograms [7]. Thus, ClearCode34 is poised for further study in readily available specimens.

While new technology will facilitate translational investigations, much remains to be learned regarding the patients who constitute the ccA and ccB cohorts. Some clinical annotation has previously been described, such as lower stage and Fuhrman nuclear grade among ccA patients [6]. However, further clinical description, for example the anatomic metastatic pattern, of these patient cohorts is lacking. Furthermore, while evidence emerges that comorbidities and their treatment may influence kidney cancer outcomes [10, 11], the distribution of these among ccA and ccB patients remain to be addressed. These are analyses which may prove important in understanding the biology and clinical relevance of these ccRCC molecular subtypes.

2. Patients and methods

2.1. Patients and clinical samples

Gene expression data was obtained from primary ccRCC tumors of patients treated with nephrectomy at Moffitt Cancer Center (MCC) from 1/1/1998 to 10/25/2011 and organized through the Total Cancer Care (TCC) initiative [12]. Priority was given to samples with sufficient quantity to satisfy both clinical and research requirements. Tissue was collected and snap frozen within 15 minutes of surgical extirpation with cold ischemia times typically <5 minutes. Subsequently, frozen sections were prepared and stained. In order to be eligible for molecular analysis, tissue was evaluated by TCC pathologists for quality control and central pathology review. Macro-dissection was performed in liquid nitrogen to maintain the frozen tissue and enrich tumor. RNA extraction was performed and specimens arrayed on Affymetrix HuRSTA-2a520709 GeneChips (Affymetrix, Santa Clara, CA) chips with over 60,000 probe sets representing over 22,000 unique genes. Further details on the MCC TCC initiative and protocol were previously published [12]. Inclusion criteria included adult patients (>18 years old) with ccRCC. A total of 282 ccRCC primary tumors with gene expression data in TCC were identified; follow-up data including survival and vital status were obtained as part of the TCC initiative. Median follow-up was 74 months for all patients (74 for ccA and 76 for ccB). Patients' medical records were reviewed to further define study variables. Clinical characteristics such as age, BMI [weight (kg)/height² (m²)], and comorbidity status and treatment were gathered at time of diagnosis. A patient was classified as obese if BMI was 30 kg/m² or greater. Patients were considered to have hypertension, hyperlipidemia, or diabetes mellitus (DM) if they reported use of a medication typically used to treat the condition. All samples and data were obtained with appropriate institutional review board approvals.

2.2. Application of gene expression classifier of ccA or ccB

The ClearCode34 consists of a 34 gene panel (Table 1) that was previously published [7] and assigns tumors as either ccA or ccB according to gene expression level. Tumor classification was performed in R program (www.r-project.org) using Prediction Analysis of Microarrays for R (PAMR), a centroid-based classification algorithm [7].

2.3. Statistical analysis

All continuous variables were described with the median and range values. OS was defined as the time from diagnosis to death of any cause. CSS was defined as the time from

diagnosis to death from ccRCC; patients who remained alive, died of other causes, or had unknown cause of death were censored at the time of last follow-up or death. RFS was defined as the date from nephrectomy to the earliest date that recurrence or metastasis was described by imaging or pathology report. The probability of death or recurrence was determined by using the Kaplan-Meier method, and comparisons were performed using a log-rank test. Significance of categorical analyses was determined with two-tail Fisher's exact test (two variables) or chi-squared (three variables). Significance of medians was evaluated with Mann-Whitney U test. Cox regression multivariate analyses were performed with SPSS version 21.

3. Results

3.1. Patient characteristics of molecular subtypes

Of the 282 ccRCC tumors studied, 226 (80%) were classified as ccA and the remainder as ccB (Table 2). Univariate analyses (UVAs) demonstrated that ccA patients, relative to ccB patients, presented with lower Fuhrman nuclear grade ($p<0.001$) and smaller tumors (median 4.6 cm versus 7.2 cm, $p=0.003$) and were less likely to have nodal involvement (4% versus 11%, $p=0.038$) or metastatic disease (23% versus 60%, $p=0.003$). There were no statistically significant distinctions in anatomic pattern of metastatic spread between the ccA and ccB patients: lung-only (19% versus 31%, $p=0.201$), lung (79% versus 63%, $p=0.130$), bone (45% versus 45%, $p=1.000$), brain (21% versus 13%, $p=0.393$), and liver (9% versus 13%, $p=0.723$).

3.2. Overall survival, cancer specific survival, and recurrence-free survival of molecular subtypes

Consistent with prior publications, the ccB patients demonstrated inferior OS relative to ccA patients (median OS 151 months versus 31 months, log-rank $p<0.001$) with a hazard ratio (HR) of 5.52 (95% CI, 3.17–9.61) (Fig. 1a) as well as inferior CSS (median CSS 253 versus 33 months, $p<0.001$) with a HR of 12.45 (95% CI, 6.45–24.03) (Fig. 1b). The ccB patients were also inferior for recurrence-free survival (RFS) in non-metastatic patients after nephrectomy (median not reached, $p<0.001$) with a HR of 12.20 (95% CI, 4.48–33.17) (Fig. 1c).

3.3. Comorbidities in relationship to ClearCode34 subtypes

There is a growing appreciation for the impact of comorbidities, such as hypertension and hyperlipidemia, and their treatment on RCC outcomes. Therefore, we sought to evaluate whether comorbidities and their treatments at time of diagnosis contribute to the divergent outcomes observed for the ClearCode34 subtypes. Intriguingly, the ccA patients have a higher median BMI at diagnosis relative to the ccB patients (29.7 versus 27.7, $p=0.019$) and a higher frequency of obesity (48% versus 34%, $p=0.021$; Table 3). In addition, there was a higher rate of DM among ccA patients relative to ccB patients (26% versus 13%, $p=0.035$). The risk of RCC in patients with hypertension has been observed to be related to the degree of hypertension [13–16]. However, similar rate of hypertension was observed in each subtype (63% versus 56%, $p=0.355$) though there was a trend towards increased utilization of ASI among the ccA patients with hypertension (71% versus 52%, $p=0.055$). Similar rates

of hyperlipidemia and statin utilization were observed. Despite being associated with advanced RCC [17], smoking rates were similar in each subtype (Table 3).

3.4. Univariate and multivariate Cox proportional hazards regression for cancer-specific survival and overall survival

While prior work showed ccA patients have a lower stage and grade of disease, the current study also identified a higher BMI and rate of obesity, higher rate of DM, and of a trend towards increased utilization of ASIs in the ccA patients. As BMI and ASI use are reported to be associated with improved outcomes in kidney cancer, we sought to determine whether comorbidities and their treatment were confounding the survival analyses for ClearCode34 subtypes and could thus explain the improved outcomes seen in ccA patients. UVA and multivariate analysis (MVA) using the Cox regression model for OS and CSS included these novel variables (BMI, DM status, ASI utilization) as well as established variables (age, grade, tumor size, nodal status, metastatic disease; Tables 4 and 5). In the CSS analysis, ccB status was associated with inferior survival relative to ccA patients in both UVA and MVA (UVA: $p < 0.001$; HR 5.01, 95% CI 3.14–8.00 and MVA: $p < 0.001$; HR 3.26, 95% CI 1.84–5.79). Similarly, in the OS analysis, ccB status was also associated with inferior survival in both UVA and MVA (UVA: $p < 0.001$; HR 3.23, 95% CI 2.16–4.86 and MVA: $p < 0.001$; HR 2.50, 95% CI 1.53–4.08). Among the novel variables, the UVA showed DM was not associated with survival, neither for CSS ($p = 0.125$, HR 0.84, 95% CI 0.47–1.51) nor for OS ($p = 0.484$; HR 1.17, 95% CI 0.75–1.83). However, in the MVA, DM was associated with inferior CSS ($p = 0.011$; HR 2.56, 95% CI 1.24–5.30) and inferior OS ($p = 0.001$; HR 2.43, 95% CI 1.41–4.18). The use of ASI was not associated with survival in any of these analyses.

4. Discussion

This large, single-institution, retrospective study validated the prognostic significance of the ClearCode34 subtypes ccA and ccB in an independent cohort of ccRCC patients. In addition, we provide a novel description of the clinical characteristics of the patients that constitute these ccRCC subtypes. The ccA patients are more likely to be obese and diabetic. The ccB patients have an inferior prognosis, larger tumors, higher Fuhrman nuclear grade, increased nodal involvement, and are more likely to have tumor that is or becomes metastatic. Importantly, multivariate analyses for both OS and CSS demonstrate that even after considering factors such as tumor size, grade, and comorbidities, ccA/ccB distinction makes a significant and robust contribution to survival models. This work reinforces the concept that ccA and ccB defines distinct clinical cohorts with unique tumor biology.

In the current study, the prognostically superior ccA group has a higher rate of obesity while the ccB patients have a rate of obesity similar to other Americans of a similar age (Table 3) [18]. This observation highlights a paradox involving BMI and ccRCC. Obesity is a risk factor for RCC in both men and women [19] yet obese RCC patients have lower stage disease, improved survival, and improved outcomes when treated with targeted therapies relative to non-obese patients [20, 21]. Thus, our observation that ccA patients, with a higher BMI and rate of obesity, have superior outcomes is consistent with those observations. However, this retrospective data is not sufficient to conclude that obesity directly causes

development of ccA tumors. Future studies should further explore how obesity impacts kidney tumor biology and how best to exploit this unique biology for the benefit of patients.

Increased incidence of solid tumors has long been recognized in patients with DM [22]. However, DM as a risk factor for RCC, and its impact on RCC outcomes, is controversial [23–25]. In the current study, ccA patients had increased incidence of DM at time of diagnosis relative to ccB patients. Interestingly, in MVA, DM was associated in inferior CSS and OS despite being associated with the superior prognostic group, ccA. Despite this conundrum, the prognostic validity of ClearCode34 persists as ccA status continues to correlate with improved CSS and OS in MVA that included DM. Given the increased incidence of obesity and DM in ccA patients, the question of whether host features can impact molecular subtypes and tumor biology is raised. Future, ideally prospective studies, are needed to address this question further.

As ccA patients are more likely to have DM, it is not surprising that a trend was observed towards increased ASI use in ccA patients (71% of ccA patients versus 52% of ccB patients, $p=0.055$). Due to their proven benefit, guidelines for therapy of DM list multiple indications for ASIs in DM patients, including hypertension [26]. The current study did not support a protective effect for ASI in ccRCC (Tables 4 and 5). However, other studies have found that ASIs may offer survival benefits in kidney cancer in the targeted treatment era [11, 27]. Possibilities for this discrepancy include insufficient power in the current study to detect a benefit from ASIs. Another possibility is that ClearCode34 status was confounding the results of the other studies since ccA patients, a group with superior survival, may have increased rates of DM and ASI use. Thus, the benefit from ASI seen in other studies may have been partly due to an enrichment of ccA patients among ASI users. Further, ideally prospective, studies are needed to resolve this uncertainty regarding the benefits of ASIs in kidney cancer patients.

Limitations of the current study include the sample size as well as the retrospective nature of the study. In addition, the study period was long in order to maximize sample size. A prospective cohort study would better capture details of comorbidities in patients during the time of tumorigenesis (i.e. years prior to diagnosis of kidney cancer) but would require a very large sample size. Given practice patterns, the MCC electronic medical record lacks some data that would further illuminate the questions raised in the current study regarding comorbidities (e.g. hemoglobin A1C values). In addition, we identified more ccA tumors than ccB. While other studies too have observed a greater incidence of ccA relative to ccB [6, 8, 9], we cannot exclude unintended bias impacting selection of which patients go to surgery and which tumors get selected for study. Finally, heterogeneity within tumors has been documented for gene expression profiles (GEPs) [28]. Whether a scoring system integrating ClearCode34 classification from multiple sites of the primary tumor, or other strategies to capture the heterogeneity of an individual tumor, would impact the prognostic ability of this GEP is an important question going forward.

This work validates, in an external dataset, the ability of ClearCode34 to provide prognostic information in ccRCC [7]. The increased prevalence of obesity and DM in the ccA patients raises the question of whether comorbidities influence tumor biology and predispose to the

development of a particular molecular subtype, or could, conversely, provide a protective effect that hinders disease progression. In addition, it is interesting that in the current MVAs, DM status is associated with inferior survival. While this observation requires validation, if true, the mechanism of this effect becomes an important research question with possibilities including tumor-promoting changes in the tumor microenvironment (i.e. diabetic nephropathy) versus a potential DM-associated metabolic effect. Furthermore, while other studies have demonstrated improved survival in RCC with ASI use, the current study fails to observe such effect. Since ASI users may enrich for prognostically superior ccA patients, this is a potential source of bias and thus ClearCode34 status may enhance future studies evaluating ASI benefit in ccRCC.

5. Conclusion

ClearCode34 identifies biologically-divergent molecular subtypes of kidney cancer that originates in patients with distinct comorbidities.

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Highlights

- ClearCode34 subtypes clear cell RCC tumors in molecular subtypes, ccA and ccB
- ccA patients have higher rate of obesity and diabetes mellitus
- After considering host comorbidities, ccA patients remain prognostically superior

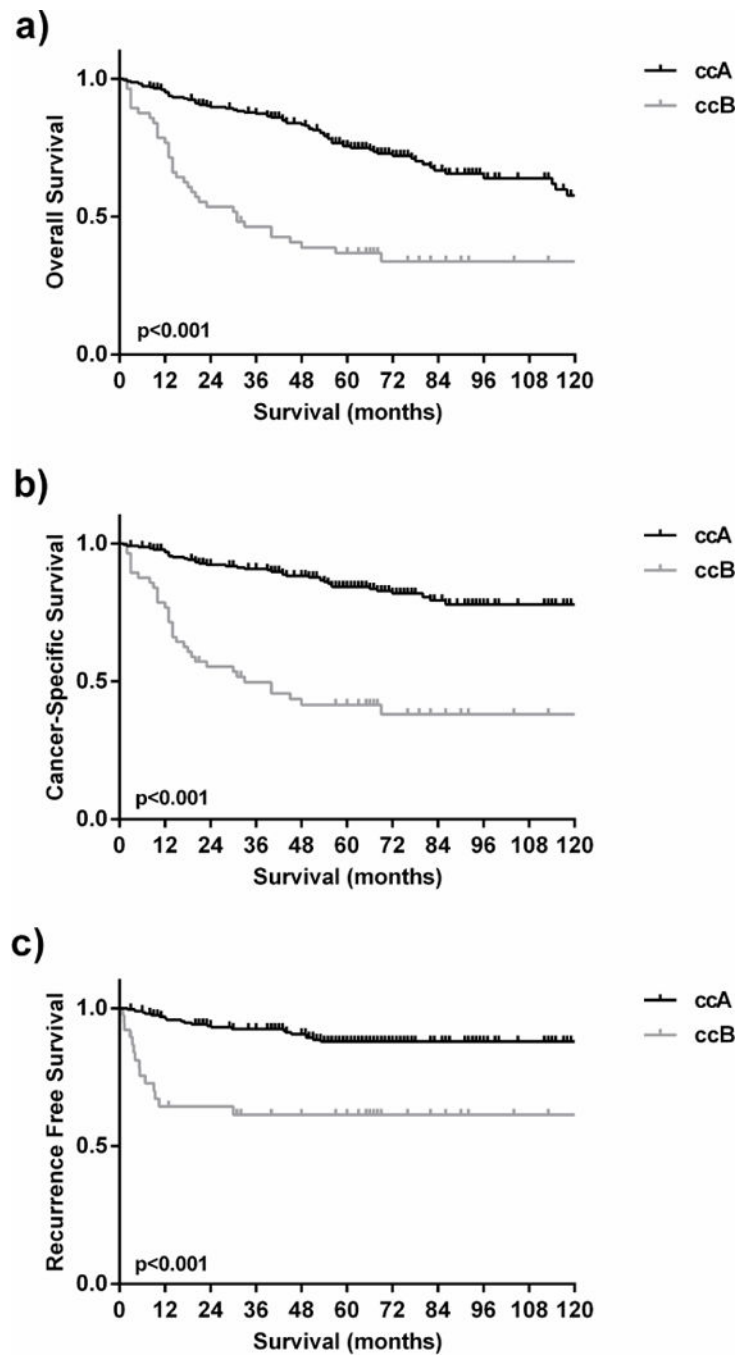


Figure 1. ClearCode34 defines ccRCC subtypes with significantly different patient outcomes
The ccA patients had superior **a)** overall survival, **b)** cancer-specific survival and **c)** recurrence free survival relative to ccB patients.

Table 1

ClearCode34 Gene Expression Profile

ccA		ccB
ARNT	NRP1	CCNO
BNIP3L	PDGFD	FLJ23867
C11orf1	PHYH	FOXM1
CDH5	PRKAA2	GALNT10
EHBP1	RGS5	GALNT4
EPAS1	SLC4A4	KCNN4
ESD	SPRYD7	MOXD1
FZD1	ST13	ROR2
GIPC2	STK32B	SERPINA3
LEPROTL1	TCEA3	SLC4A3
MAOB	TLR3	
MAPT	VCAM1	

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Table 2
Clear Cell Renal Cell Carcinoma Molecular Subtypes and Patient Characteristics

	ccA			ccB			<i>p</i>
	n	%	n	n	%	n	
Total	226	80	56	20			
Age (years), Median (\pm IQR)	62 (53–71)		68 (56–73)				0.090
Gender							0.539
Men	144	64	33	59			
Women	82	36	23	41			
Race							0.071
White	214	95	49	88			
Non-White	12	5	7	13			
Fuhrman Nuclear Grade							<0.001
I	5	2	2	4			
II	95	42	11	20			
III	106	47	22	39			
IV	19	8	21	38			
Tumor Size (cm), Median (\pm IQR)	4.6 (3.2–7.0)		7.2 (3.4–10.4)				0.003
Node Positive	8	4	6	11			0.038
Metastatic Disease	53	23	32	60			0.003
Metastatic Pattern							
Lung Only	10	19	10	31			0.201
Lung	42	79	20	63			0.130
Bone	24	45	14	44			1.000
Brain	11	21	4	13			0.393
Liver	5	9	4	13			0.723
Length of Follow-Up (months)	74		76				

ccA = clear cell renal cell carcinoma subtype A; ccB = clear cell renal cell carcinoma subtype B cm = centimeters; IQR = interquartile range

Table 3
Comorbidities in Relationship to Clear Cell Renal Cell Carcinoma Molecular Subtypes

	ccA		ccB		p value
	n	%	n	%	
BMI, Median (± IQR)	29.7 (25.8–35.1)		27.7 (23.8–32.3)		0.019
Rate of Obesity					0.021
BMI < 30	112	52	31	66	
BMI ≥ 30	103	48	16	34	
Diabetes Mellitus	58	26	7	13	0.035
Metformin	26	45	1	14	0.224
No Metformin	32	55	6	86	
Hypertlipidemia	83	37	17	31	0.436
Statin	77	93	16	94	0.464
No Statin	6	7	1	6	
Hypertension	142	63	31	56	0.355
ASI	101	71	16	52	0.055
Thiazide	40	28	7	23	0.658
Other Treatment	78	55	24	77	<0.001
No. Drugs = 1	65	46	14	45	1.000
No. Drugs ≥ 2	77	54	17	55	
Smoking					0.680
Ever Smoker	130	58	32	57	0.881
Current Smoker	34	15	6	11	0.523
No. of Pack-Years, Median (± IQR)	30 (26–76)		30 (15–60)		0.523

ccA = clear cell renal cell carcinoma subtype A; ccB = clear cell renal cell carcinoma subtype B IQR = interquartile range; BMI = body mass index; ASI = angiotensin system inhibitor

Table 4
Univariate and Multivariate Cox Proportional Hazards Regression for Cancer Specific Survival

	Univariate			Multivariate		
	HR	CI	P	HR	CI	P
Age	1.03	1.01–1.05	0.010	1.06	1.03–1.08	<0.001
Grade						
I–II	Referent			Referent		
III–IV	5.98	2.97–12.02	<0.001	3.92	1.50–10.23	0.005
Tumor Size	1.21	1.16–1.27	<0.001	1.11	1.04–1.19	0.002
Node Positive	5.80	2.93–11.47	<0.001	1.74	0.81–3.76	0.158
Metastatic Disease	7.10	4.45–11.31	<0.001	17.65	7.71–40.40	<0.001
ClearCode34						
ccA	Referent			Referent		
ccB	5.01	3.14–8.00	<0.001	3.26	1.84–5.79	<0.001
BMI	0.97	0.93–1.01	0.125	0.99	0.94–1.04	0.686
Diabetes Mellitus	0.84	0.47–1.51	0.556	2.56	1.24–5.30	0.011
ASI	0.64	0.39–1.04	0.074	1.13	0.61–2.09	0.700

ccA = clear cell renal cell carcinoma subtype A; ccB = clear cell renal cell carcinoma subtype B BMI = body mass index at diagnosis; ASI = angiotensin system inhibitor; CI = 95% confidence interval

Table 5
Univariate and Multivariate Cox Proportional Hazards Regression for Overall Survival

	Univariate			Multivariate		
	HR	CI	P	HR	CI	P
Age	1.05	1.03–1.06	<0.001	1.07	1.04–1.09	<0.001
Grade						
I-II	Referent			Referent		
III-IV	2.75	1.77–4.29	<0.001	1.99	1.15–3.44	0.014
Tumor Size	1.17	1.12–1.22	<0.001	1.09	1.03–1.16	0.004
Node Positive	5.46	2.95–10.11	<0.001	2.34	1.15–4.74	0.019
Metastatic Disease	3.92	2.62–5.87	<0.001	5.11	3.04–8.58	<0.001
ClearCode34						
ccA	Referent					
ccB	3.23	2.16–4.85	<0.001	2.50	1.53–4.08	<0.001
BMI	0.96	0.92–0.99	0.013	0.98	0.94–1.02	0.259
Diabetes Mellitus	1.17	0.75–1.83	0.484	2.43	1.41–4.18	0.001
ASI	0.86	0.58–1.26	0.433	0.95	0.58–1.54	0.832

ccA = clear cell renal cell carcinoma subtype A; ccB = clear cell renal cell carcinoma subtype B BMI = body mass index at diagnosis; ASI = angiotensin system inhibitor; CI = 95% confidence interval