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Clear Cell Type A and B Molecular Subtypes in Metastatic Clear Cell Renal Cell Carcinoma: Tumor Heterogeneity and Aggressiveness

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Abstract

Background—Intratumor molecular heterogeneity has been reported for primary clear cell renal cell carcinoma (ccRCC) tumors; however, heterogeneity in metastatic ccRCC tumors has not been explored.

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Objective—To evaluate intra- and intertumor molecular heterogeneity in resected metastatic ccRCC tumors.

Design, setting, and participants—We identified 111 patients who had tissue available from their primary tumor and at least one metastasis. ClearCode34 genes were analyzed for all tumors.

Outcome measurements and statistical analysis—Primary and metastatic tumors were classified as clear cell type A (ccA) or B (ccB) subtypes. Logistic and Cox regression were used to evaluate associations with pathologic features and survival.

Results and limitations—Intratumor heterogeneity of ccA/ccB subtypes was observed in 22% (95% confidence interval [CI] 3–60%) of metastatic tumors. Subtype differed across longitudinal metastatic tumors from the same patient in 23% (95% CI 10–42%) of patients and across patient-matched primary and metastatic tumors in 43% (95% CI 32–55%) of patients. Association of subtype with survival was validated in primary ccRCC tumors. The ccA/ccB subtype in metastatic tumors was significantly associated with metastatic tumor location, metastatic tumor grade, and presence of tumor necrosis. A limitation of this study is that we only analyzed patients who had both a nephrectomy and metastasectomy.

Conclusions—Approximately one quarter of metastatic tumors displayed intratumor heterogeneity; a similar rate of heterogeneity was observed across longitudinal metastatic tumors. Thus, for biomarker studies it is likely adequate to analyze a single sample per metastatic tumor provided that pathologic review is incorporated into the study design. Subtypes across patient-matched primary and metastatic tumors differed 43% of the time, suggesting that the primary tumor is not a good surrogate for the metastatic tumor.

Patient summary—Primary and secondary/metastatic cancers of the kidney differed in nearly one half of ccRCC patients. The pattern of this relationship may affect tumor growth and the most suitable treatment.

Keywords

Kidney; ClearCode34; Subtype; Formalin-fixed; paraffin-embedded tissue; Biomarker

1. Introduction

Intratumor molecular heterogeneity has been reported for primary clear cell renal cell carcinoma (ccRCC) tumors, and as a result it has been suggested that multiple biopsies should be analyzed from each tumor to adequately molecularly profile ccRCC tumors [1–3]. With respect to personalized medicine, prognosis and treatment should be based on the most aggressive subclone identified from the multiple biopsies [4]. Conversely, analysis of resected ccRCC tumors allows the pathologist to review and identify the section of each tumor with the most adverse pathologic features (eg, highest tumor grade and presence of necrosis). In fact, it has been shown that ccRCC prognostic biomarkers are often enriched for pathologic features of aggressiveness, and thus when identifying candidate prognostic biomarkers sections with the most adverse features should be analyzed [5]. To determine optimal experimental designs for prognostic studies, it is important to evaluate intratumor heterogeneity across sections of a tumor with equivalent pathologic features. Such studies

would determine if a single sample is adequate or if multiple samples need to be analyzed from each resected tumor. Furthermore, while studies investigating intratumor heterogeneity to date have focused on primary ccRCC tumors, little is known regarding heterogeneity of the more-lethal and therapeutically-relevant metastatic ccRCC tumor.

Our objective was to evaluate both intra- and intertumor molecular heterogeneity in a large cohort of resected metastatic ccRCC tumors. As a cost-effective approach, we analyzed 34 genes that comprise the ClearCode34 model [6] and evaluated heterogeneity associated with the clear cell type A (ccA) and B (ccB) expression subtypes, which are associated with ccRCC prognosis [7]. In fact, intratumor heterogeneity in primary ccRCC tumors has similarly been evaluated using the ccA/ccB subtypes, where it was observed that most primary ccRCC tumors display both the ccA and ccB subtype [3]. We aimed to determine if similar heterogeneity exists in metastatic ccRCC tumors. In particular, we wanted to evaluate if molecular subtypes were maintained from primary to metastatic disease or whether progression to metastatic disease results in development of the ccA subtype. We furthermore evaluated concordance of the ccA/ccB subtypes across patient-matched primary and metastatic tumors. All assays were performed on resected formalin-fixed paraffin-embedded tissues.

2. Patients and methods

2.1. Patient selection and pathology review

We identified 111 patients who were treated surgically for ccRCC between 1990 and 2005, had synchronous (M1) or metachronous (M0 at presentation) ccRCC metastases, underwent metastasectomy for at least one of their metastatic tumors, and had formalin-fixed, paraffin-embedded (FFPE) tissue available from their primary tumor and at least one metastatic tumor. Multifocal renal tumors and contralateral renal tumors were not considered metastatic. A single pathologist reviewed all tumors to confirm histologic subtype (1997 American Joint Commission on Cancer [AJCC]/International Union Against Cancer [UICC] classification), 2010 tumor stage, 2012 International Society of Urological Pathology (ISUP) tumor grade, tumor size, and the presence of coagulative tumor necrosis and sarcomatoid differentiation. Pathologic review was performed over the study period. The FFPE block(s) that was most representative of the tumor (highest grade and presence of necrosis) was identified. If multiple blocks were identified as representative of the tumor, then all blocks were analyzed to evaluate intratumor heterogeneity across regions of the metastatic tumor that have equivalent pathologic features. This study was approved by the Mayo Clinic Institutional Review Board.

2.2. Nanostring

Total RNA was isolated from three slides per sample at 10 μ m sections of tumor-rich areas of FFPE tissue blocks using the AllPrep DNA/RNA FFPE kit reagents (Qiagen). Gene expression on the NanoString platform was assessed using a custom gene panel which included the 34 genes that comprise the ClearCode34 model [6]. Gene expression was quantified on the NanoString nCounter and raw counts were generated with nSolver.

2.3. The Cancer Genome Atlas (TCGA) ccRCC data

Level 3 RNA-sequencing by expectation maximization (RSEM) [8] normalized RNASeqV2 files were downloaded on January 8, 2013, from TCGA Data Portal for 481 ccRCC samples.

2.4. Statistical analysis

To assign the primary and metastatic tumor samples into the ccA or ccB subtype, TCGA ccRCC RNA sequencing data were used to develop a classification model using the prediction analysis for microarrays (PAM) method [9]. The normalized data were base-two logarithm transformed and median centered. The true ccA/ccB subtypes and corresponding probabilities were obtained from Brooks et al [6] for 379 samples that had RNA sequencing data. To increase the accuracy of the model, before developing the classification model we removed 12 ccA samples that had a published ccB probability >0.10 and five ccB samples that had a ccB probability <0.90 . The classification model was applied to the normalized and median centered nanostring data to assign all primary and metastatic tumor samples as ccA or ccB. The nanostring data were corrected using positive and negative spike-in controls and normalized using six housekeeping genes (*GUSB*, *HMBS*, *POLR2A*, *PPIA*, *TFRC*, and *UBC*) as previously described [10]. Unsupervised hierarchical clustering (average clustering and correlation distance) was applied via *hclust* in R to the nanostring data on the combined primary and metastatic tumor samples. Concordance between the PAM-classified ccA/ccB subtypes and the unsupervised clustering results was evaluated. Concordance was also evaluated across replicate blocks from the same metastatic tumor, across longitudinal metastatic tumors from the same patient, and across patient-matched primary and metastatic tumors; 95% confidence intervals (CIs) were calculated using the exact method. Cancer-specific survival and overall survival were assessed by the Kaplan-Meier method from date of nephrectomy to death (cancer-specific and overall, respectively). Cox proportional hazards regression was used to evaluate the association of ccA/ccB subtype in primary tumors with cancer-specific survival and overall survival, adjusting for age at nephrectomy and gender. Cox regression was also used to determine if ccA/ccB subtype in metastatic tumors was associated with cancer-specific and overall survival (from date of metastasectomy to death), adjusting for age at nephrectomy and gender; ccA/ccB subtype was modeled as a time-dependent covariate. If simultaneous metastatic tumor samples (metastatic tumors resected at the same time-point) had discrepant ccA/ccB subtype, then the sample was assigned to ccB [4,5]. Logistic regression was used to evaluate associations between ccA/ccB subtype and metastatic tumor location, metastatic tumor grade, and presence of necrosis. In the logistic models two terms were included: random intercept for patient and a main effect for metastatic tumor nested within patient. A p value <0.05 was considered statistically significant.

3. Results

3.1. Clinical characteristics of patient-matched primary-metastatic cohort

Of 111 patients who had a primary ccRCC tumor and at least one ccRCC metastatic tumor available for molecular analysis, the nanostring assay was successful on the primary tumor for 91 patients (Table 1). Of these 91 patients, 79 experienced an RCC-specific death and the median follow-up after nephrectomy for patients who survived was 144.4 mo. A total of 123

metastatic tumors were analyzed representing 90 patients (Supplementary Table 1). Pulmonary metastases were the most common, accounting for 40% of all metastases analyzed. Median time from nephrectomy to first metachronous metastasis was 1.6 yr (minimum 37 d, maximum 10.8 yr). The median follow-up after first metastasectomy for patients who survived was 126.2 mo. None of the patients received systemic therapy before their first metastasis.

3.2 Assigning ccA/ccB molecular subtypes to the primary and metastatic tumor samples

Using TCGA ccRCC samples to develop a classification model, primary and metastatic tumor samples were assigned to the ccA/ccB subtypes (Fig. 1 and Supplementary Figs. 1 and 2). Previous investigations have shown that patients assigned to the ccA subtype have improved survival [6,7]. We independently validated these results in primary tumors; patients classified to ccB subtype had significantly worse cancer specific survival (age- and gender-adjusted hazard ratio [HR] 1.70, 95% confidence interval [CI] 1.07–2.71, $p = 0.025$; Fig. 2) and overall survival (age- and gender-adjusted HR 1.73, 95% CI 1.10–2.73, $p = 0.018$) in comparison to ccA.

As a complementary approach, unsupervised clustering was performed on patient-matched primary and metastatic tumor samples and two primary clusters were obtained (Fig. 1). The concordance of these two clusters with the ccA/ccB subtypes was high; 86% and 85% for primary and metastatic tumors, respectively (Supplementary Table 2).

3.3. Intra- and intermetastatic tumor molecular heterogeneity

Of 90 patients who had nanostring data for at least one of their metastatic tumors, nine had replicate blocks available from the same metastatic tumor in which to evaluate intra-metastatic tumor heterogeneity (minimum 2, maximum 5 replicate blocks per metastatic tumor). All replicate blocks from the same tumor had equivalent tumor grade and necrosis status. Thus, our objective was to determine if intra-metastatic tumor heterogeneity existed across pathologically similar sections of a resected tumor. Only two of the nine (22%; 95% CI 3–60%) patients (P33, P44) had both ccA and ccB subtypes present within the same metastatic tumor (Fig. 3 and Supplementary Fig. 3).

We also evaluated intermetastatic tumor heterogeneity. Specifically, 30 patients had more than one ccRCC metastatic tumor analyzed (minimum 2, maximum 3 longitudinal metastatic tumors per patient). Seven of the 30 (23%, 95% CI 10–42%) had metastatic tumors with discordant ccA/ccB subtypes (Figs. 3 and 4 and Supplementary Fig. 3). Three of these seven patients (P28, P44, P101) had multiple metastatic tumors resected at the same time with discrepant subtypes (Fig. 3). Two patients (P17, P62) progressed from ccA to ccB over the course of their metastatic disease (Fig. 3). One patient (P12) progressed from a metastatic tumor classified as ccB to subsequently having another metastatic tumor classified as ccA (Fig. 3). One patient (P33) had a metastatic tumor that contained both ccA and ccB subtypes and subsequently another metastatic tumor classified as ccB (Fig. 3).

3.4. Concordance between patient-matched primary and metastatic ccRCC tumors

We compared molecular subtypes of the metastatic tumor(s) with the corresponding patient-matched primary tumor. Of the 81 patients who had nanostring data on their primary and metastatic tumors, 35 (43%, 95% CI 32–55%) had discordant ccA/ccB subtypes (Figs. 3 and 4 and Supplementary Fig. 3). Of these 35 patients, 28 (80%) had a primary tumor that was classified as ccA and at least one metastatic tumor classified as ccB. Conversely, seven (20%) patients had a primary tumor classified as ccB and at least one metastatic tumor classified as ccA.

3.5. Association of metastatic tumor subtype with clinical and pathologic features

We observed a statistically significant association of ccA/ccB subtype with metastatic pathologic features (Table 2). Specifically, the odds of being ccB for a grade 3 metastatic tumor is approximately sevenfold larger than the odds for a grade 2 metastatic tumor (odds ratio [OR] 7.08, 95% CI 1.14–43.8, $p = 0.035$). Similarly, the odds of being ccB for a grade 4 metastatic tumor are much greater than the odds of a grade 2 metastatic tumor (OR 49.2, 95% CI 3.51–691.4, $p = 0.004$). Metastatic tumors with the presence of necrosis are also more likely to be ccB (OR 9.88, 95% CI 2.15–45.5, $p = 0.003$). Conversely, metastases to the contralateral adrenal gland, pancreas, and bone are less likely to be ccB in comparison to metastases to pulmonary tissue (Table 2). Adjusting for age at nephrectomy and gender, we did not observe a significant association between ccA/ccB subtype in metastatic tumors and ccRCC-specific survival ($p = 0.2$) or overall survival ($p = 0.6$).

4. Discussion

Molecular subtyping of primary ccRCC tumors based on gene expression profiles has shown that ccRCC tumors can be classified into molecular subtypes that are significantly associated with outcome [6,7,11,12]. While this work was revolutionary and helps to understand the molecular underpinnings of ccRCC, there are important practical limitations. In particular, classification algorithms—together with corresponding predictive accuracy measures—are not available to classify patients into these subtypes. In addition, subtypes have not been adequately independently validated. Thus, to classify the 91 primary ccRCC tumors into the ccA/ccB subtypes we applied the PAM approach [9] to the ClearCode34 genes to develop our own classification model [6,7]. In doing so we independently validated that patients assigned to the ccA subtype have improved survival. Furthermore, we performed unsupervised clustering and obtained two clusters that had high concordance with the ccA/ccB classifications, further supporting the presence of these two molecular subtypes in primary ccRCC tumors.

Recently, Gulati et al [3] evaluated intratumor heterogeneity of primary ccRCC tumors by determining if both ccA and ccB subtypes existed within the same tumor. They evaluated multiple biopsy samples from each tumor and observed that eight of ten (80%) patients had a primary tumor that exhibited both ccA and ccB subtypes. Similar observations have been made using this same cohort for other molecular markers [1,2]. Thus, it has been concluded that multiple samples should be analyzed from every tumor so as to identify poor prognostic clones [1–3]. Importantly, these investigations analyzed intratumor heterogeneity by

comparing multiple biopsy samples from the same tumor without accounting for the varying pathologic features of the different biopsies. Our objective was to evaluate intratumor heterogeneity across multiple sections of a resected metastatic tumor that had similar pathologic features. In particular, it has been suggested that regions of a tumor that have the most aggressive pathologic features (eg, highest grade) should be interrogated in biomarker studies because it is well known that these features drive aggressiveness [5]. Thus, from an experimental design standpoint, it is important to know if intratumor heterogeneity exists within pathologically similar sections of the same tumor. Using this approach we observed minimal heterogeneity in resected metastatic ccRCC tumors; two of nine (22%) patients demonstrated intratumor heterogeneity. Thus, for biomarker studies, if strict pathologic review is incorporated then one sample per tumor should be sufficient. While these analyses were performed on metastatic ccRCC tumors, we expect that similar results would be obtained from primary ccRCC tumors; however, similar analyses should be conducted on larger cohorts of primary ccRCC tumors to verify this assumption. These results will not be applicable if the objective is to molecularly characterize a tumor. In this scenario, as others have reported [1–3], multiple samples are likely necessary to characterize both low and high-grade portions of a given tumor. In addition to evaluating intrametastatic tumor heterogeneity, we also evaluated intertumor heterogeneity by comparing ccA/ccB subtypes across longitudinal metastatic tumors from the same patient. Interestingly, we observed similar rates of intra- and intertumor heterogeneity. We additionally compared ccA/ccB subtypes across patient-matched primary and metastatic ccRCC tumors and observed discordance in 43% of patients. Not surprisingly, of the patients with discordant subtypes, 80% progressed from ccA to ccB, suggestive of transformation to a more aggressive phenotype.

While the ccA/ccB subtypes have been shown to be associated with outcome in primary ccRCC tumors [6,7], the clinical relevance of these molecular subtypes has not been evaluated within metastatic tumors. We observed that the ccA/ccB subtypes are significantly associated with metastatic tumor pathologic features; the ccB subtype is enriched for higher-grade metastatic tumors and metastatic tumors with tumor necrosis. However, we were unable to observe a significant association with outcome in metastatic ccRCC tumors.

We acknowledge that there may be systematic differences between subjects who underwent metastasectomy and subjects with distant metastases that were not treated surgically. However, tissue will only be available from patients undergoing metastasectomy, and this limitation is therefore unavoidable. In general, all patients with metastatic ccRCC were considered for metastasectomy if they were determined to have surgically resectable metastases with acceptable anticipated morbidity. Our practice has been to recommend metastasectomy if all known disease can be removed, whether a solitary site or in multiple organs [13]. In addition, we only analyzed metastatic tumors from patients whom we also had a patient-matched primary ccRCC tumor available for analysis. Because we ignored metastatic tumors from patients who did not have a nephrectomy at our institution, we may have a biased sample of metastatic tumors. Thus, future studies examining the molecular characteristics of metastatic tumors and identifying prognostic markers should evaluate all available metastatic tumors.

5. Conclusions

We validated the existence of the ccA/ccB subtypes within primary ccRCC tumors and their association with prognosis. In addition, we demonstrated for the first time that the ccA/ccB subtypes are significantly associated with metastatic tumor pathologic features of aggressiveness. With respect to tumor heterogeneity, we observed that nearly one quarter of patients demonstrated intrametastatic tumor heterogeneity across sections of a metastatic ccRCC tumor with similar pathologic features. Thus, for biomarker studies it should be adequate to analyze a single sample per metastatic tumor provided that strict pathologic review is incorporated into the study design. However, multiple samples will be necessary if the objective is to molecularly characterize a metastatic tumor. Patient-matched primary and metastatic tumors displayed different molecular subtypes 43% of the time, suggesting that the primary tumor is not a good surrogate for the more lethal and therapeutically relevant metastatic tumor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Heatmap displaying gene expression of the ClearCode34 genes [6,7] for 91 primary tumors and 123 metastatic tumors. The bars on the top of the heatmap represent clear cell type A (ccA) and B (ccB) subtypes as assigned using the prediction analysis for microarrays (PAM) method [9] (blue denotes ccA, red denotes ccB), the results of performing unsupervised clustering on the primary and metastatic tumor samples (purple denotes Cluster 1, orange denotes Cluster 2), and whether the sample denotes a primary or metastatic tumor (aqua denotes primary tumor, pink denotes metastatic tumor).

Fig. 2.

Renal cell carcinoma (RCC)–specific survival from the time of primary nephrectomy for patients assigned to ccA subtype compared to patients assigned to ccB subtype. Subtype assignment was based on gene expression profiles obtained from primary clear cell RCC (ccRCC) tumors.

Fig. 3.

Molecular heterogeneity of ccA/ccB subtypes [6,7]. Triangles denote the primary ccRCC tumor and squares denote metastatic tumors. A dot represents a sample analyzed from each tumor; blue denotes ccA subtype and red denotes ccB subtype. The number of dots denotes the number of samples that were analyzed from the corresponding tumor; replicate samples all had the same pathologic features (tumor grade and presence of necrosis). Tumor grade and tumor necrosis status is provided for the primary tumors. Tumor grade, necrosis status, and metastatic site are provided for each metastatic tumor. Vertical arrow with time (yr) denotes the amount of elapsed time between each tumor (resection date). Multiple tumors on the same horizontal axis for a patient imply that the patient had multiple tumors resected at the same time. Patient 28 (P28) is an example of a patient for whom the nanostring assay was not successful on the primary ccRCC tumor. Data for all 100 patients are provided in Supplementary Fig. 3.

Fig. 4.

Molecular subtype of patient-matched primary and metastatic tumors for 100 ccRCC patients. A blue box denotes that the tumor was classified as ccA, a red box denotes that the tumor was classified as ccB, a purple box denotes that multiple blocks were analyzed from the corresponding metastatic tumor with discordant molecular subtypes, and a grey box denotes that the nanostring assay was not successful for that tumor. A black dot in the primary tumor denotes that the patient had synchronous (M1) ccRCC metastases; otherwise, the patient had metachronous (M0) metastases. Met 1, Met 2, and Met 3 denote that up to three longitudinal metastatic tumors were analyzed for each patient (Supplementary Fig. 3).

Table 1

Clinical characteristics of the primary-metastatic cohort and pathologic information associated with the primary clear cell renal cell carcinoma (ccRCC) tumor

	M0 at presentation (n = 46)	M1 (n = 45)	Total (n = 91)
Gender			
Female	13 (28%)	11 (24%)	24 (26%)
Male	33 (72%)	34 (76%)	67 (74%)
Age at nephrectomy (yr)			
Mean	62.9	57.7	60.3
Median	65.5	58.6	61.4
Interquartile range	(56.9, 70.0)	(50.7, 64.4)	(54.6, 68.2)
Max tumor size (cm ²)			
Mean	9.6	11.0	10.3
Median	9.0	10.0	9.5
Interquartile range	(6.5, 12.8)	(8.0, 13.3)	(7.0, 12.8)
2010 pT			
Missing	0	1	1
1A	3 (7%)	1 (2%)	4 (4%)
1B	6 (13%)	6 (14%)	12 (13%)
2A	12 (26%)	6 (14%)	18 (20%)
2B	2 (4%)	5 (11%)	7 (8%)
3A	14 (30%)	15 (34%)	29 (32%)
3B	6 (13%)	4 (9%)	10 (11%)
3C	2 (4%)	1 (2%)	3 (3%)
4	1 (2%)	6 (14%)	7 (8%)
2010 pN			
0	15 (33%)	17 (38%)	32 (35%)
1	4 (9%)	9 (20%)	13 (14%)
X	27 (59%)	19 (42%)	46 (51%)
TNM stage			
1	9 (20%)	0 (0%)	9 (10%)
2	14 (30%)	0 (0%)	14 (15%)
3	22 (48%)	0 (0%)	22 (24%)
4	1 (2%)	45 (100%)	46 (51%)
Grade			
2	9 (20%)	4 (9%)	13 (14%)
3	29 (63%)	25 (56%)	54 (59%)
4	8 (17%)	16 (36%)	24 (26%)
Number of metastases			
1	25 (54%)	36 (80%)	61 (67%)
2	13 (28%)	9 (20%)	22 (24%)
3	6 (13%)	0 (0%)	6 (7%)

	M0 at presentation (<i>n</i> = 46)	M1 (<i>n</i> = 45)	Total (<i>n</i> = 91)
4	2 (4%)	0 (0%)	2 (2%)

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Table 2

Association of ccA/ccB subtype with metastatic pathologic features

	Odds ratio	95% confidence interval	p value
Grade			
2	Reference		
3	7.08	(1.14–43.8)	0.035
4	49.2	(3.51–691.4)	0.004
Necrosis			
No	Reference		
Yes	9.88	(2.15–45.5)	0.003
Location			
Pulmonary	Reference		
Contralateral adrenal	0.01	(0.0002–0.65)	0.030
Ipsilateral adrenal	0.13	(0.01–1.61)	0.11
Pancreas	0.03	(0.002–0.80)	0.036
Non-regional nodes	0.34	(0.02–4.81)	0.4
Liver	0.71	(0.03–14.7)	0.8
Bone	0.08	(0.009–0.79)	0.030
Brain	0.05	(0.002–1.17)	0.063
Other	0.40	(0.06–2.63)	0.3