# A Genomic Algorithm for the Molecular Classification of Common Renal Cortical Neoplasms: Development and Validation

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**Purpose:** Accurate discrimination of benign oncocytoma and malignant renal cell carcinoma is useful for planning appropriate treatment strategies for patients with renal masses. Classification of renal neoplasms solely based on histopathology can be challenging, especially the distinction between chromophobe renal cell carcinoma and oncocytoma. In this study we develop and validate an algorithm based on genomic alterations for the classification of common renal neoplasms.

**Materials and Methods:** Using TCGA renal cell carcinoma copy number profiles and the published literature, a classification algorithm was developed and scoring criteria were established for the presence of each genomic marker. As validation, 191 surgically resected formalin fixed paraffin embedded renal neoplasms were blindly submitted to targeted array comparative genomic hybridization and classified according to the algorithm. *CCND1* rearrangement was assessed by fluorescence in situ hybridization.

**Results:** The optimal classification algorithm comprised 15 genomic markers, and involved loss of *VHL*, 3p21 and 8p, and chromosomes 1, 2, 6, 10 and 17, and gain of 5qter, 16p, 17q and 20q, and chromosomes 3, 7 and 12. On histological rereview (leading to the exclusion of 3 specimens) and using histology as the gold standard, 58 of 62 (93%) clear cell, 51 of 56 (91%) papillary and 33 of 34 (97%) chromophobe renal cell carcinomas were classified correctly. Of the 36 oncocytoma specimens 33 were classified as oncocytoma (17 by array comparative genomic hybridization and 10 by array comparative genomic hybridization plus fluorescence in situ hybridization) or benign (6). Overall 93% diagnostic sensitivity and 97% specificity were achieved.

**Abbreviations and Acronyms**

aCGH = array comparative genomic hybridization

ccRCC = clear cell renal cell carcinoma

chrRCC = chromophobe renal cell carcinoma

CNA = copy number alteration

FFPE = formalin fixed, paraffin embedded

FISH = fluorescence in situ hybridization

OC = oncocytoma

pRCC = papillary renal cell carcinoma

QPCR = quantitative polymerase chain reaction

RCC = renal cell carcinoma

TCGA = The Cancer Genome Atlas

**Editor's Note:** This article is the first of 5 published in this issue for which category 1 CME credits can be earned. Instructions for obtaining credits are given with the questions on pages 1732 and 1733.

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**Conclusions:** In a clinical diagnostic setting the implementation of genome based molecular classification could serve as an ancillary assay to assist in the histological classification of common renal neoplasms.

**Key Words:** carcinoma, renal cell; comparative genomic hybridization; DNA copy number variations; classification; oncocytoma, renal

ACCURATE diagnostic discrimination of major renal cortical neoplasm subtypes is not only useful to guide appropriate treatment strategies but also to estimate prognosis. While histology and associated immunohistochemical assays of these neoplasms serve as the gold standard, in the era of precision medicine, classification based on molecular biomarkers is appealing, well suited for use with specimens with limited tissue availability and may reveal novel molecular targets for therapy.[1–3] Common renal cortical neoplasms include malignant renal cell carcinoma (clear cell, papillary and chromophobe) and benign subtypes such as oncocytoma. A clear discrimination between benign OC and the malignant eosinophilic variant of chrRCC is often difficult to achieve solely based on histology due to overlapping morphological features.[4,5]

Another histological challenge involves the up to 6% of cases that do not fit into an established diagnostic category, the so-called unclassified RCC.[6,7] The introduction of an ancillary assay to assist histology in achieving the correct renal tumor classification could impact the overall management and outcome of patients with RCC. Renal neoplasms are characterized by genomic CNAs and chromosomal rearrangements (for instance, *CCND1* rearrangement in OC), which could be used for diagnostic and prognostic purposes, as suggested by several studies.[8–16] Therefore, in this study we develop and validate a robust algorithm for the molecular classification of common renal cortical neoplasms based on genomic imbalance (copy number gain and loss) that could be implemented in a clinical diagnostic setting as an adjunct assay to assist in accurate diagnosis.

## MATERIALS AND METHODS

### FFPE Specimens
A total of 191 surgically resected renal cortical neoplasm specimens obtained from 191 patients as part of their routine care at the Cleveland Clinic were used in this institutional review board approved study (supplementary table 1, http://jurology.com/). The specimens were selected based on the presence of at least 80% tumor burden (as evaluated by hematoxylin and eosin staining, 1 section per cm tumor diameter) and pathological diagnosis according to WHO classification (as per pathology report), as having approximately equal numbers of ccRCC, pRCC, chrRCC and OC.[7] The specimens ranged from 12 to 150 mm with a median of 42 (median ccRCC 48 mm, median pRCC 34 mm, median chrRCC 54 mm, median OC 35.5 mm).

### Targeted and Whole Genome aCGH
Specimen DNA was extracted and when greater than 800 bp in size was subjected to heat fragmentation at 95C until the bulk of the DNA fragments reached 400 to 800 bp. Using similarly fragmented sex-matched normal male/female gDNA (Promega, Madison, Wisconsin), aCGH was performed and analyzed as described in the supplementary material (http://jurology.com/).

### FISH and QPCR
FISH was performed on 4 micron sections to assess *CCND1* rearrangement and gain of 17q. TaqMan® based QPCR copy number assays were performed on extracted DNA to assess gain of 17q as described in the supplementary material (http://jurology.com/).

## RESULTS

### Development of a RCC Classification Algorithm
To construct a robust algorithm by which common renal neoplasms (malignant and benign) could be subtyped based on genomic imbalance, we first identified CNAs diagnostic of each of the 3 malignant subtypes that in a hierarchical manner could be optimized for classification using the copy number profiles for 489 ccRCC, 75 pRCC and 65 chrRCC available in TCGA (http://cancergenome.nih.gov).[17] Since ccRCC is the most predominant RCC subtype and is characterized by loss of *VHL*,[18] all 629 TCGA specimens were initially categorized into the 2 major groups of 450 (71%) with *VHL* loss (3p25 locus, 10.1-10.2 Mb) and 179 without. In the latter group 68 specimens were positive for 5qter gain (169-181 Mb), a CNA also predominantly observed in ccRCC. There were 34 ccRCCs among the 111 samples without *VHL* loss or 5qter gain. Of the 518 cases positive for *VHL* loss or 5qter gain 455 were ccRCC, 15 were pRCC (9 with *VHL* loss) and 48 were chrRCC (15 with *VHL* loss). Thus, while the sensitivity of ccRCC classification was 92% based on these 2 aberrations, the specificity was poor at 55%. Clearly the consideration of other biomarkers was required to optimize the algorithm.

It has been well documented that pRCC exhibits gain, in particular of chromosomes 3, 7, 12, 16, 17 (mostly 17q) and 20, and chrRCC exhibits loss, often involving chromosomes 2, 6, 10 and 17.[16,18–22] Thus, the gain/loss status of chromosome 17 was

considered important to discriminate between these 2 subtypes, and together with *VHL* loss and 5qter gain, served as the backbone for the final algorithm. Based on the literature as well as the relative abundance, commonality and size of the CNAs in each RCC subtype of TCGA data set, a RCC subtyping algorithm was constructed in an iterative manner to optimize the sensitivity and specificity of the classification of the 3 malignant subtypes.

Figure 1 shows the classification schema developed in which an additional 9 aberrations were included, such as loss of 8p in ccRCC; gain of chromosomes 7, 12, 16p and 20q for pRCC; loss of chromosomes 2, 6 and 10 for chrRCC subtype, and gain of chromosome 3 to distinguish ccRCC from the other 2 RCC subtypes. Also included in the algorithm was a low branch allowing for the classification of OC based on the reported loss of chromosome 1 in this benign renal neoplasm, and loss of a small locus at 3p21 (without concurrent loss of *VHL*) recently reported by FISH.[23] Thus, overall the algorithm used 15 diagnostic CNAs to classify specimens. Specimens that displayed aberrations that were not consistent with a specific subtype according to the algorithm were called "not classifiable." This allowed the distinction from those specimens classified as benign that did not display any genomic imbalance greater than 2 Mb other

than known common variants and that were not definitively classified as OC (fig. 1).

Using the developed algorithm the overall concordance achieved for all 629 malignant RCCs in TCGA was 87%. Table 1 lists the classifications and sensitivity achieved in each subtype. Cross-reference of the current 489 ccRCC data set to those cases reported by TCGA network[17] revealed that 52 were not confirmed as ccRCC on histological consensus rereview. Of these cases 23 had been misclassified by the algorithm, predominantly as chrRCC. Reexamination of the algorithm indicated that removal of these 52 cases did not impact the structure of the optimal classification algorithm. Due to the limited availability of data sets examining CNAs in OC and the inability to assess genomic imbalance in individual samples, the overall concordance for OC classification by the algorithm could not be determined. Thus, the lower branch (circled in fig. 1) allowing for OC classification may require further optimization.

### Validation of the Algorithm in an Independent Data Set

For validation purposes and subsequent application in a diagnostic setting, a targeted array was designed to provide coverage of genomic regions reported to be involved in urogenital cancers (supplementary



**Figure 1**. Classification algorithm for copy number based subtyping of renal neoplasms. Schematic representation of step-wise classification of ccRCC, pRCC, chrRCC and OC based on 15 diagnostic CNAs. Circled branch required further optimization for OC classification. Underlined criterion in circled branch indicates modification made to algorithm to optimize OC classification. *chrs*, chromosomes.

**Table 1.** *Optimized classification of 629 malignant RCCs (TCGA)*

| Algorithm Classification Subtype | ccRCC (489) | ccRCC (437)* | pRCC (75) | chrRCC (65) |
|---|---|---|---|---|
| ccRCC | 442 (90%) | 413 (94%) | 9 | 3 |
| pRCC | 22 | 15 | 52 (69%) | 1 |
| chrRCC | 15 | 3 | 3 | 55 (85%) |
| OC | 2 | 1 | 3 | 2 |
| Not classifiable | 3 | 3 | 6 | — |
| Benign | 5 | 2 | 2 | 4 |

* Excluding 52 ccRCCs according to TCGA.

table 2, http://jurology.com/). Those aberrations in the algorithm that involved assessment of smaller chromosomal loci (deletions involving *VHL*, 3p21 and 8p loci, and gain of 5qter) were represented at a higher resolution, and for those involving whole or arms of chromosomes, often several targeted regions were represented. Based on the minimum length of the aberrations observed in TCGA, criteria were established to score specimens positive or negative for the presence of each of the 15 genomic markers (supplementary table 3, http://jurology.com/). For loss of chromosome 1 and 3p21 in OC, the criteria were established based on the limited published literature.[23,24]

To validate the classification algorithm a panel of 191 FFPE specimens comprising the 4 main renal tumor subtypes was blindly submitted to targeted aCGH. The 15 diagnostic CNAs were scored as previously described (supplementary table 3, http://jurology.com/). Given the overall poorer quality of aCGH for DNA from FFPE specimens, those CNAs with an average $\log_2$ ratio close to the cutoff (between 0.15 and 0.25 for gain, and $-0.15$ and $-0.25$ for loss) were recorded as low. Verification of these low level aberrations was provided by a combination of whole genome aCGH, FISH and QPCR assays, which overall suggested that low level 17q gain when detected as a sole aberration should be considered as an artifact (supplementary material, www.jurology.com/). Each specimen was then classified according to the algorithm (fig. 1 and supplementary table 1, http://jurology.com/), after which the respective histopathological classifications were revealed and all cases microscopically rereviewed by an independent pathologist (CM-G).

As expected, OC classification did require optimization since 6 of the 36 OCs were misclassified as pRCC due to the presence of a gain of 1 of chromosomes 7, 17q or 20q (circled in fig. 1). Five of these also exhibited loss or partial loss of chr1, which is commonly observed in OC and to some extent in ccRCC, but rarely in pRCC.[8] Thus, one modification of the algorithm was made (underlined in circled branch in fig. 1), and all specimens were reclassified (supplementary table 1, http://jurology.com/) and summarized in table 2.

***ccRCC classification.*** Of the 63 ccRCC specimens 58 (92%) were similarly classified (table 1). Two were classified as pRCC (CGI-051 and CGI-190) which, on pathological rereview, indicated ccRCC with necrosis and rhabdoid features. Another specimen was classified as benign (CGI-120). It is likely that other genetic (eg mutation and/or rearrangement) and epigenetic events not detected by aCGH might underlie the disease in this specimen. One other sample (CGI-115) was misclassified as OC due to the loss at the 3p21 locus (45-51 Mb) without any change at the 3p25 locus (*VHL*, 10.1-10.2 Mb). While a prior FISH study of ex vivo needle biopsies of resected specimens indicated that loss of 3p21 without loss of 3p25 was observed in 2 of 10 OC specimens evaluated, the current study indicates that this combination of genomic imbalance is also observed in ccRCC and may target the *PBRM1* gene.[25] On histopathological rereview 1 specimen not classifiable (CGI-137) was identified as clear cell tubulopapillary RCC, an emerging RCC subtype with an as yet unclear genomic profile.[26]

**Table 2.** *Subtype classification of 191 renal cortical neoplasms according to the classification algorithm based on aberrations detected by targeted aCGH*

| Decision Algorithm Classification | No. ccRCC (63) | No. pRCC (57) | No. chrRCC (35) | No. OC (36) | No. OC (+*CCND1*) (36)* |
|---|---|---|---|---|---|
| ccRCC | 58 | 3 | — | 1 | 1 |
| pRCC | 2 | 51 | 1 | 1 | 1 |
| chrRCC | — | — | 33 | — | — |
| OC | 1 | — | — | 17 | 27 |
| Not classifiable | 1 | 2 | — | 3 | 1 |
| Benign | 1 | 1 | 1 | 14 | 6 |

* FISH was performed to assess *CCND1* rearrangement on the 17 not classifiable and benign OC specimens, and permitted reclassification of 10 as OC.

***pRCC classification.*** The algorithm afforded classification of 51 of the 57 pRCC specimens as pRCC (89%) (table 2). Two specimens (CGI-039 and CGI-081) were designated not classifiable due to the detection of CNAs rarely reported in this subtype. Indeed CGI-039 showed an alteration only in chr2 involving a gain of 2q24.3-q33.3, and CGI-081 exhibited a loss of 7q22.3-31.2, each of which was also detected in 1 of 65 TCGA pRCC specimens, the significance of which is unknown. Three samples classified as ccRCC (CGI-092, CGI-129 and CGI-150) had genomic markers commonly observed in ccRCC and pRCC subtypes. Lastly, 1 case (CGI-110) was classified as benign, which on histological rereview was called an oncocytic neoplasm with papillary features. CGI-110 was also negative by FISH for gain of chr7 and 17q (data not shown), supporting the aCGH results.

***chrRCC classification.*** Of the 35 chrRCC specimens 33 were classified as such (table 2). The absence of widespread monosomies and the presence of a low level chr12 gain led to the classification of 1 chrRCC specimen (CGI-037) as pRCC. One other chrRCC (CGI-064) did not exhibit any genomic imbalance and was classified as benign. Histological rereview of this specimen indeed confirmed the submission of benign tissue instead of lesional tissue for aCGH analysis.

***OC classification.*** Of 36 OC specimens 12 were classified as OC using the decision algorithm. One OC (CGI-005) exhibited a clear pattern of alterations consistent with ccRCC, including loss of *VHL*, for which no clinical followup was available. Fourteen cases were classified as benign, lacking any genomic imbalance. Another 3 displayed aberrations other than the 15 used in the current classification scheme and, thus, were not classifiable (table 2 and supplementary table 1, http://jurology.com/). Since a subset of OC has been reported in the literature to possess rearrangement at the 11q13 (*CCND1*) locus, a genomic alteration cannot be assessed reliably by aCGH.[11,27] FISH was performed to detect break apart of *CCND1*. Of the 17 specimens 10 (8 benign and 2 not classifiable) were found to display this alteration and, thus, were reclassified as OC (supplementary table 3, http://jurology.com/). Figure 2 shows a representative specimen (CGI-021) that by aCGH was not classifiable but showed *CCND1* rearrangement on FISH.

### Sensitivity and Specificity of Molecular Classification

To calculate the sensitivity and specificity of the algorithm 3 specimens were excluded from study, namely CGI-137, clear cell tubulopapillary RCC; CGI-110, the oncocytic neoplasm with papillary



**CCND1** BA (11q13) (1R1G1F)
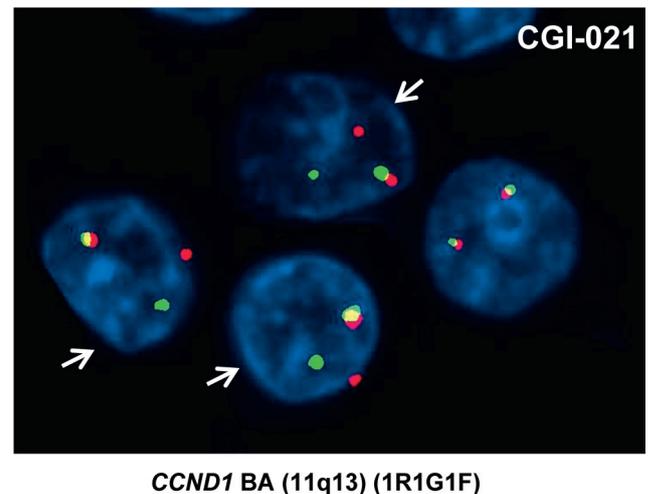
**Figure 2.** *CCND1* break apart FISH. Representative FISH image of nuclei from OC specimen (CGI-021) showing 11q13 (*CCND1*) rearrangement with separated 5′-*CCND1* (red) and 3′-*CCND1* (green) signals, and 1 intact normal fusion (yellow [F]) signal.

features; and CGI-064, which comprised benign tissue as previously described. Thus, the final analyzable cohort of 188 specimens comprised ccRCC (62), pRCC (56), chrRCC (34) and OC (36). Table 3 lists the sensitivity and specificity calculated for each of the 4 subtypes by aCGH alone using the classification algorithm, and also including the *CCND1* rearrangement status assessed by FISH for oncocytoma classification. Specimens classified as benign were considered correct for OC samples. Overall the algorithm based on genomic imbalance correctly classified 173 of 188 specimens using histology as the gold standard, yielding a diagnostic sensitivity of 92%. Upon inclusion of the *CCND1* rearrangement results, the overall diagnostic sensitivity increased to 93% (175 of 188).

## DISCUSSION

Given the clinical need for the accurate classification of renal neoplasms, multiple biomarkers including genomic alterations have been evaluated as ancillary assays in the diagnostic realm. However, for the most part they have as yet to be

**Table 3.** *Overall diagnostic sensitivity and specificity of renal tumor classification*

|  | No./Total No. Sensitivity (%) | No./Total No. Specificity (%) |
| --- | --- | --- |
| ccRCC | 58/62 (93) | 122/126 (97) |
| pRCC | 51/56 (91) | 128/132 (97) |
| chrRCC | 33/34 (97) | 154/154 (100) |
| OC* | 31/36 (86) | 151/152 (99) |
| OC (+*CCND1*)* | 33/36 (92) | 151/152 (99) |

* Included samples classified as OC and benign.

successfully implemented in a clinical setting. In the current study a classification algorithm comprising 15 genomic imbalance markers with well-defined scoring criteria was developed and validated in large specimen data sets by aCGH. Unlike other classification strategies based on immunohistochemistry, expression and miRNA profiling and cytogenetic evaluation, the current assay provided a high diagnostic yield and reproducibility.[14,15,23,28–30] The final genomic algorithm constructed in this study afforded a 93% sensitivity for the diagnosis of common malignant RCC subtypes. Importantly, high sensitivity was achieved in the discrimination of malignant chrRCC, in particular, from benign OC, although definitive diagnosis as OC vs benign was enhanced with the inclusion of assessment of *CCND1* rearrangement by FISH.

While algorithm based aCGH classification exhibits reduced analytical sensitivity, an inability to assess rearrangements and poor classification of rare neoplasms, such limitations are also exhibited by other molecular classification methodologies. Intratumor heterogeneity could also impact conclusive classification based on molecular biomarkers in general, compared with conventional histology. Transition of the algorithm in a diagnostic setting to a targeted massively parallel sequencing approach would not only increase the overall analytical sensitivity offered by aCGH, but would also permit the co-assessment of diagnostic translocations and low burden subclonal populations.

This study represents the initial step toward the implementation of the genomic algorithm for molecular classification in a clinical diagnostic setting to assist histology in the diagnosis of renal neoplasms. Prospective validation studies are currently under way to assess the overall clinical usefulness, particularly in needle core biopsies of small renal masses where multiple sampling may minimize the impact of intratumor heterogeneity, as well as in unusual renal tumors that are difficult to classify for which accurate diagnosis could impact treatment strategies and patient management.

## CONCLUSIONS

The genomic algorithm developed in the current study achieved a high sensitivity and specificity of classification of benign and malignant neoplasms using histology as the gold standard. Inclusion of the algorithm based classification as an ancillary assay to histology may be beneficial in the overall diagnosis and clinical management of renal masses.

## ACKNOWLEDGMENTS

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