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# Toward Biological Subtyping of Papillary Renal Cell Carcinoma With Clinical Implications Through Histologic, Immunohistochemical, and Molecular Analysis

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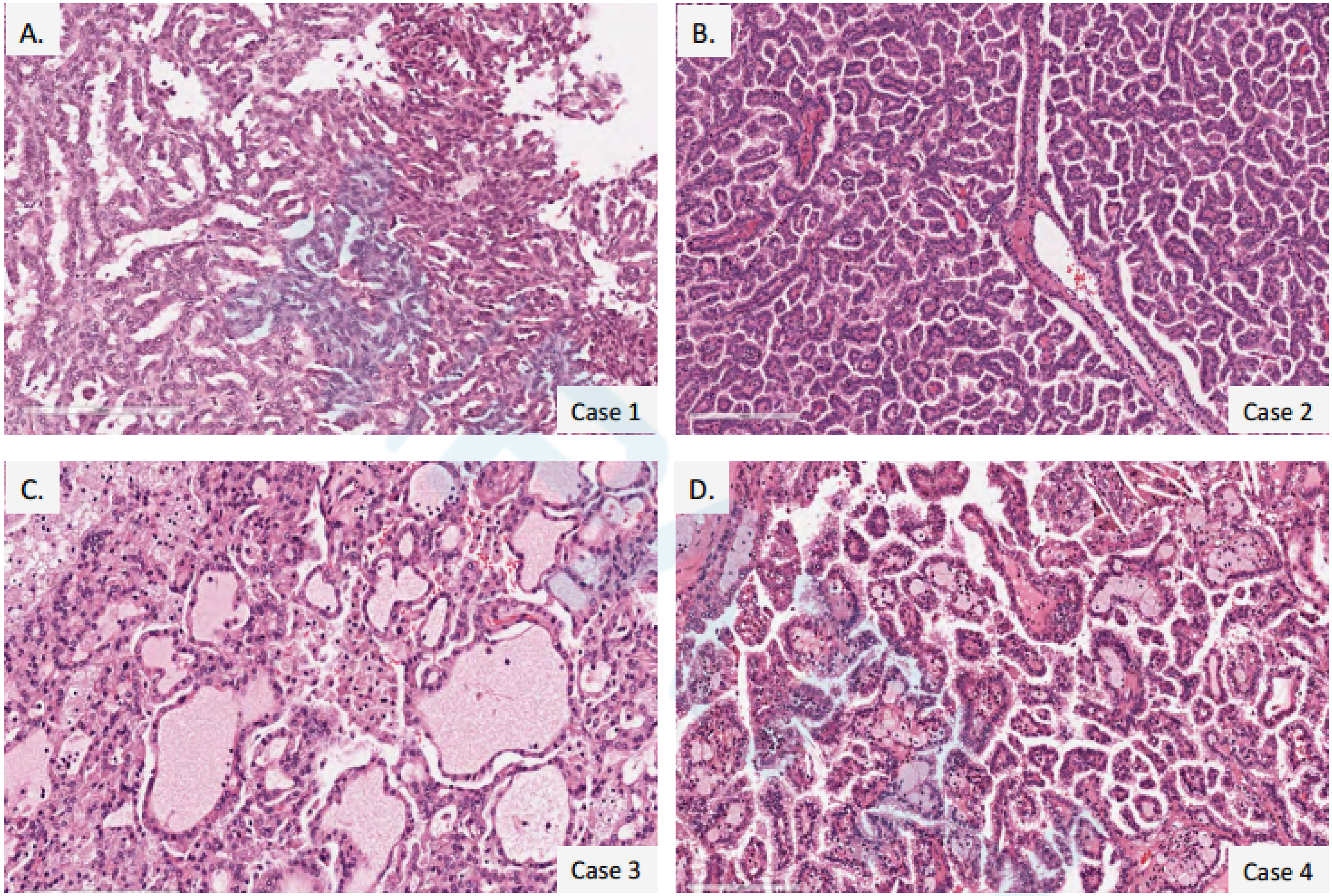
# BACKGROUND

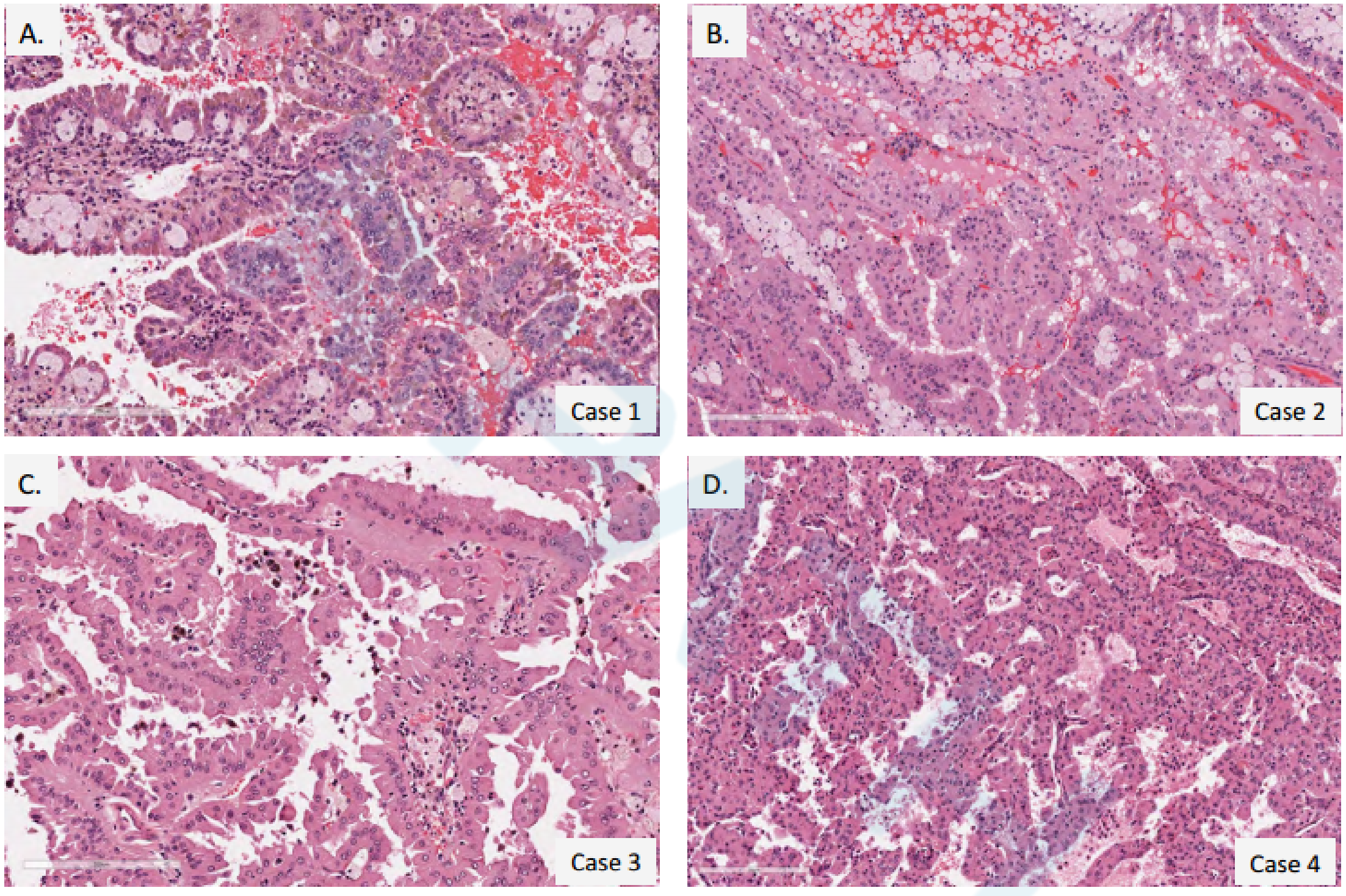
- PRCC (Papillary Renal Cell Carcinoma): The second most common type of renal cell carcinoma (RCC) following clear cell RCC.
- PRCC1 and PRCC2

➢ Clinically，PRCC2 is more aggressive than PRCC1

- ✓ higher TNM stage
- ✓ larger tumor size
- ✓ worse prognosis




**Supplemental** Fig. 1: **PRCC1** tumors; A-D) Morphologically the tumors correspond to the described PRCC1 features： small cells, scant cytoplasm, inconspicuous nucleolus, linear nuclear arrangement, lack of cellular crowding and lack of pseudostratification.



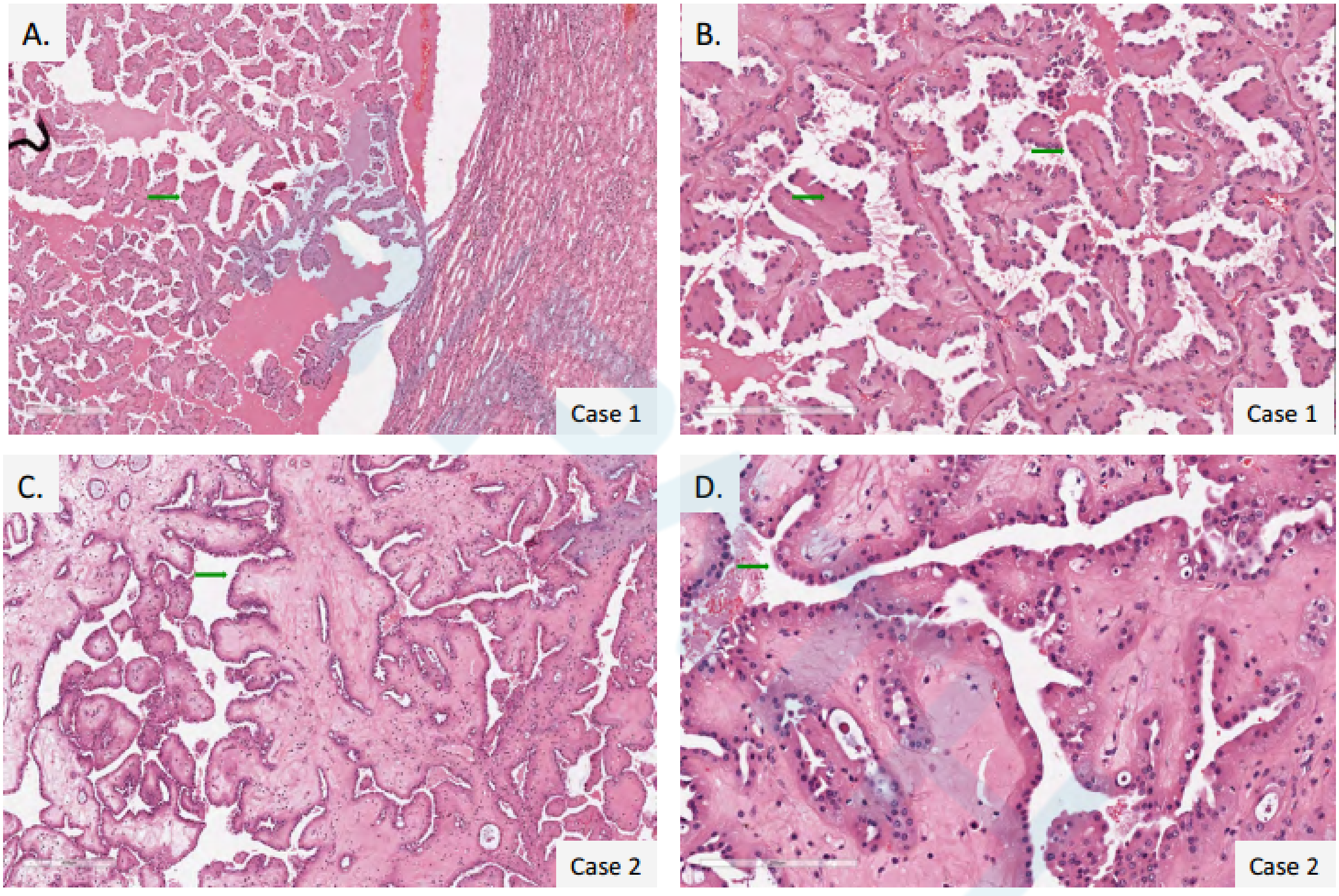

**Supplemental** Fig. 2: **PRCC2** tumors; A-D) Morphologically the tumors correspond to the described PRCC2 features： large cells, abundant cytoplasm, very prominent nucleoli and pseudostratification.

# BACKGROUND

- PRCC1 and PRCC2
  - Molecularly:
    - ✓ PRCC1 harbors gains in chromosomes 7, 17, 16, and 20 while loss in chromosome Y. MET pathway activation is frequently implicated in PRCC1.
    - ✓ PRCC2 has a more heterogenous spectrum of chromosomal gains and losses. 8q gains have been reported in particular as being associated with poor prognosis in that type.Additional gains and losses reported in PRCC2 involve chromosomes 1, 3, 4, 5, 6, 9, 14, and 15. Repeatedly, though the NRFARE2 pathway was shown to be enriched in PRCC2.

# BACKGROUND

- PRCC (OLG) ：An oncocytic low grade variant.
  - Immunophenotype：comparable with PRCC2
  - Clinically：closer to PRCC1, indolent and showed no disease progression.
  - Molecularly ：closer to PRCC1, similar gains of chromosomes 7 and 17




**Supplemental** Fig. 4: **PRCC OLG** tumors; A-D)： Large oncocytic cells, low-grade nuclei, and diffuse nuclear distribution in a linear manner away from the basal aspect of the cells (green arrows)

# BACKGROUND

- PRCC NOS: These tumors have been referred to as mixed, unclassified, overlapping or not otherwise specified (NOS).Frequencies to be about half of the tumor cohort (47%).

- PRCC NOS cases are problematic in clinical practice, as there are currently no established markers to accurately subclassify them which can leave clinicians unsure of how to best manage individual patients.

# BACKGROUND

- We found that PRCC1 and PRCC2 had distinct molecular signatures and also identified a select number of biomarkers that were differentially expressed in each subtype and had the potential to resolve the PRCC NOS dilemma*.

- Purpose
  - validate the expression of these biomarkers via immunohistochemistry (IHC) on an independent PRCC cohort
  - correlating the IHC findings with clinical and survival parameters.

*： Saleeb RM, Plant P, Tawedrous E, et al. Integrated phenotypic/genotypic analysis of papillary renal cell carcinoma subtypes: identification of prognostic markers, cancer-related pathways, and implications for therapy. Eur Urol Focus. 2016.

# MATERIALS AND METHODS

- 108 cases was selected
  - St. Michael's Hospital (SMH) 25 cases
  - McGill University Health Centre (MUHC) 83 cases
- Tumors were classified according to the original PRCC subtyping criteria set by Delahunt and Eble
- The cases that did not meet all the criteria or lacked consensus were stated as NOS

# MATERIALS AND METHODS

- Immunohistochemistry
  - MRP2 (ABCC2), CA9, GATA3, SALL4, BCL2
  - ABCC2: ATP-binding cassette transporters C2 （ATP结合盒转运体 ），also called MRP2:multidrug resistance-associated protein2（多药耐药相关蛋白2）
  - Of the 5 IHC markers evaluated, BCL2 and SALL4 did not show differential staining between PRCC subtypes.

# MATERIALS AND METHODS

- DNA and RNA Extraction
- CNVs（Chromosomal Copy Number Variations）
  Expression：12 PRCC samples of the identified different histologic subtypes（4 PRCC1, 4 PRCC2, and 4 PRCC3）
  - The nCounter Human Karyotype panel (by Nanostring Technologies)
- miRNA Expression Analysis：3 PRCC OLG samples
  - Nanostring Human miRNA V.3 hybridization platform (Nanostring Technologies)
- Bioinformatics and Survival Statistical Analysis
- Gene Set Enrichment Pathway Analysis

# RESULTS

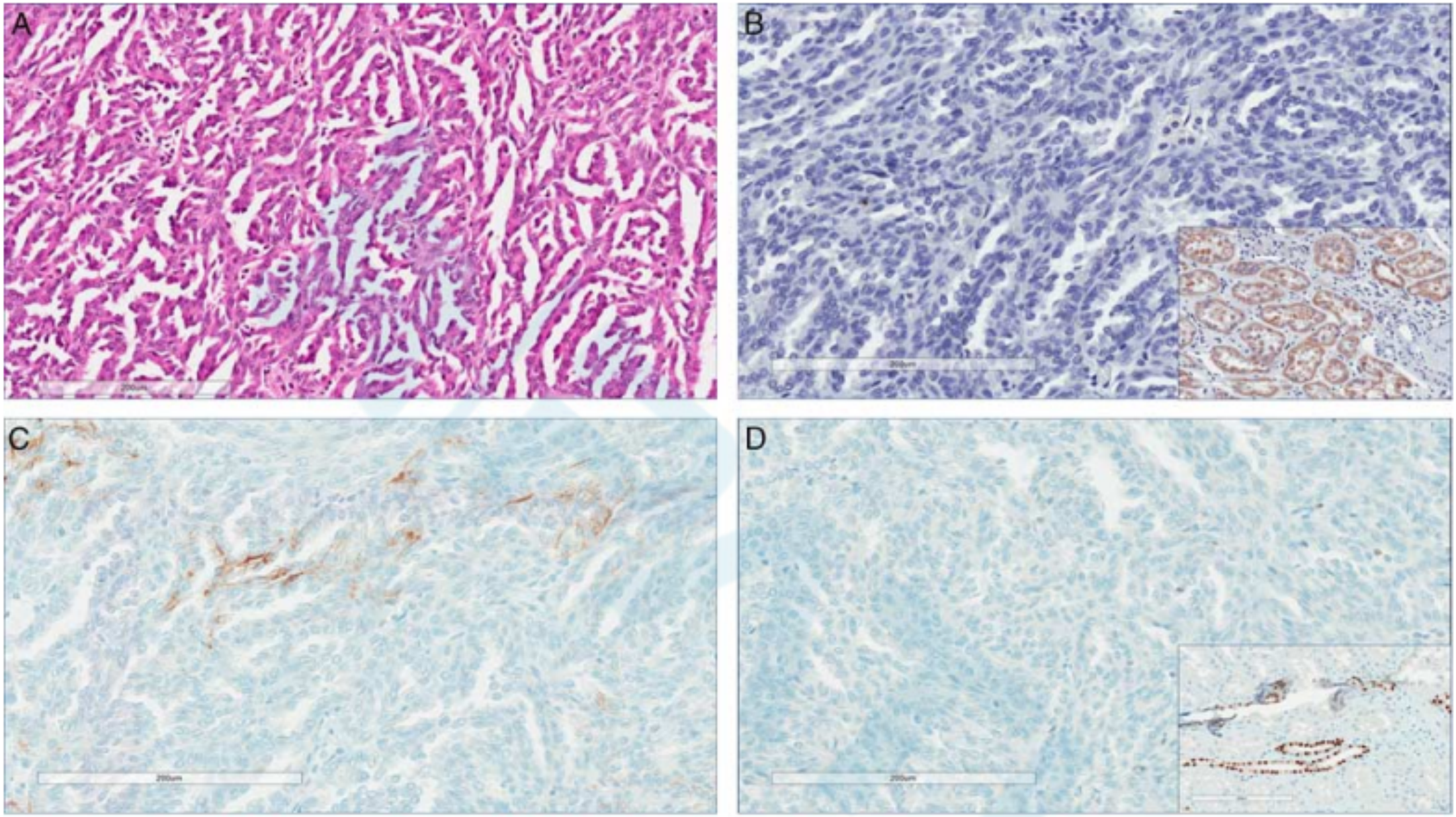
# PRCC Subtypes by Morphology and Correlation With Their IHC Profiles

- The Initial histologic subtype

| | | |
|---|---|---|
| PRCC1 | 17.5% | 19/108 |
| PRCC2 | 31.4% | 34/108 |
| OLG | 2.7% | 3/108 |
| NOS | 46.3% | 50/108 |

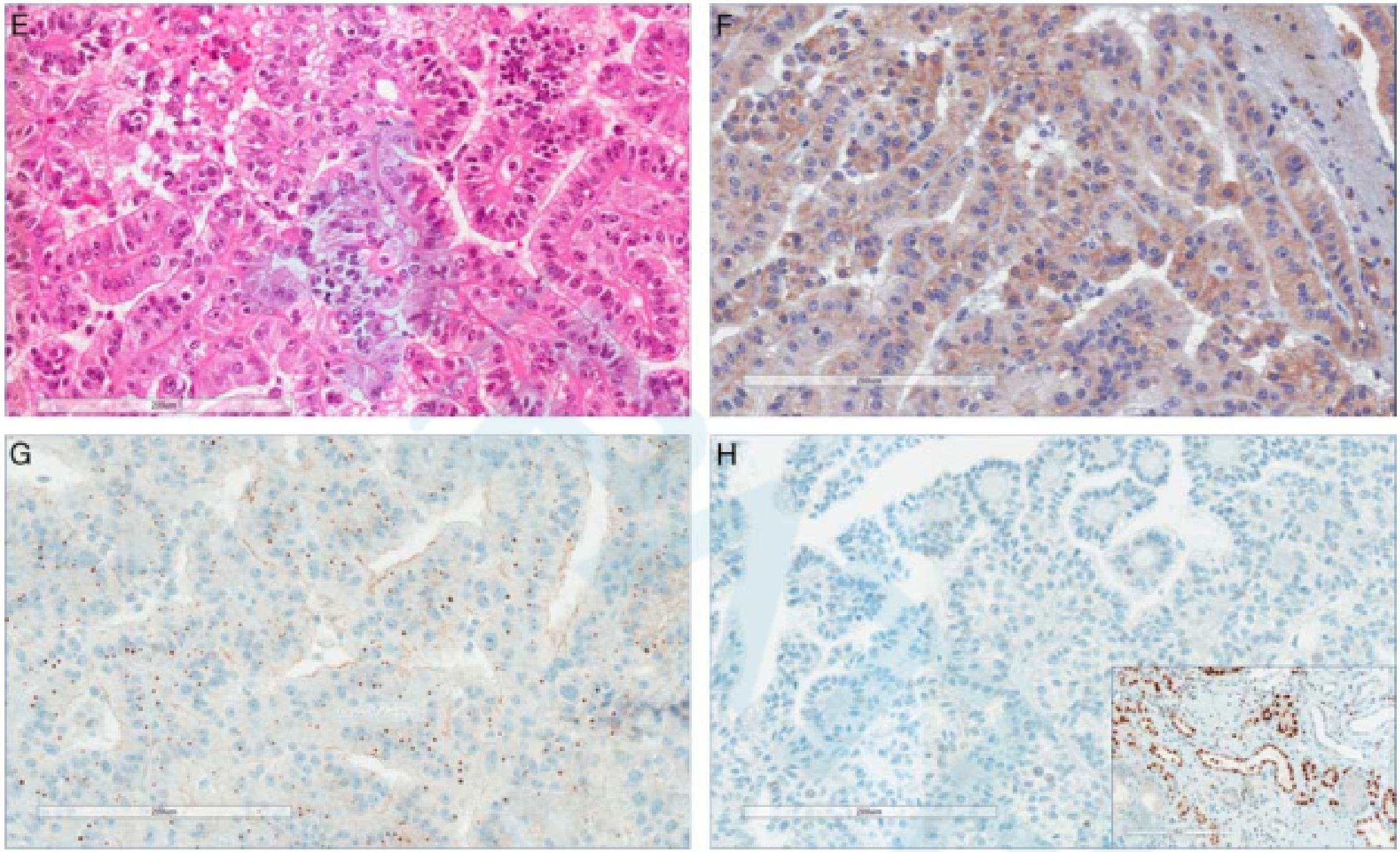
- The specific IHC profile was able to classify 49/50 PRCC NOS cases and resulted in reclassifying 3 of the histologically subtyped tumors.

- Only 1 case had an undetermined subtype with a mixed morphology and IHC profile between PRCC2 and PRCC3.




# PRCC1

A, hematoxylin and eosin stain
B, ABCC2 : negative stain with positive internal control (inset)
C, CA9 : negative (negative to patchy membranous staining)
D, GATA3 : negative stain  with positive internal control (inset)

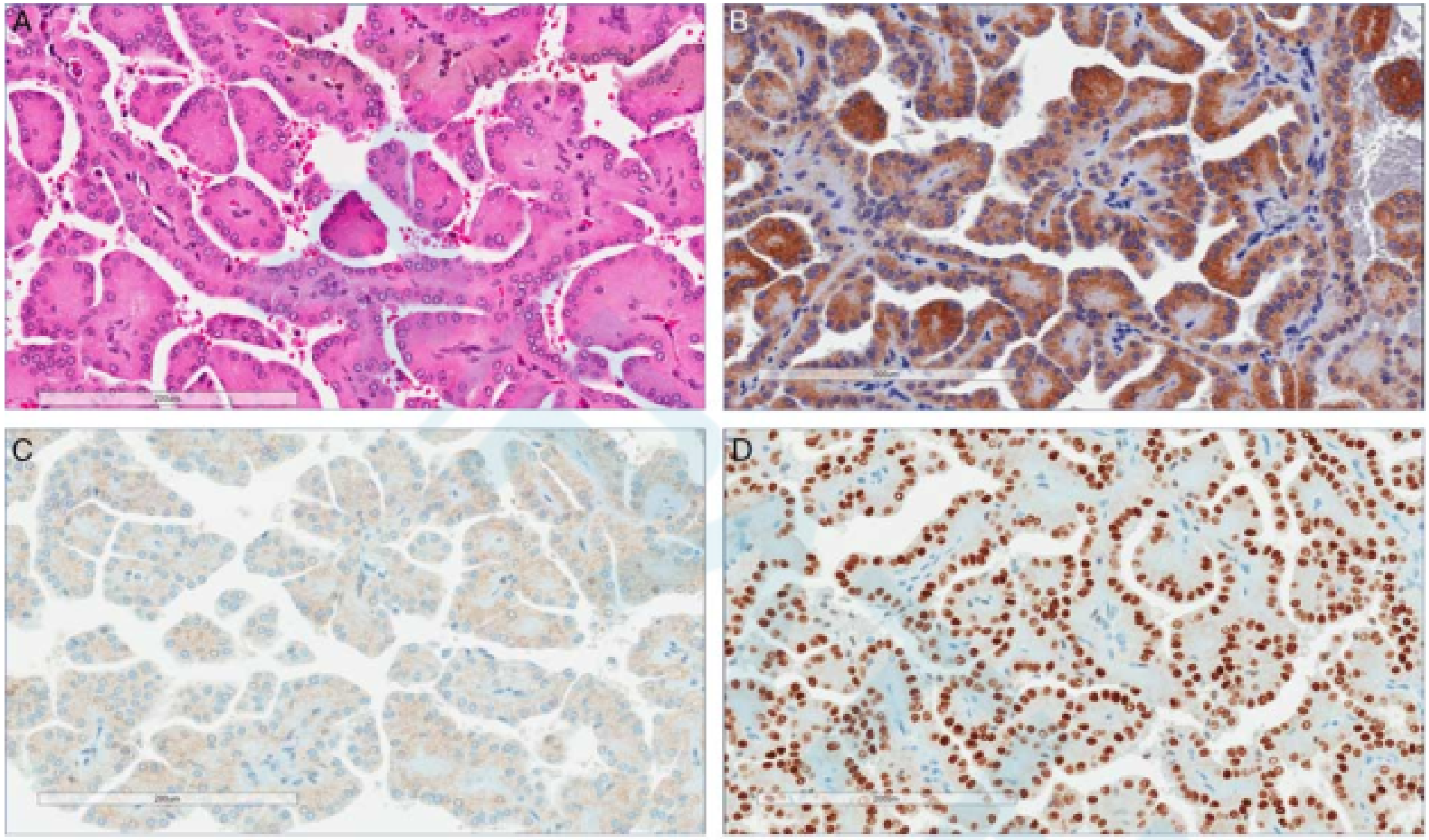



# PRCC2

F, ABCC2 : diffuse staining (similar to the surrounding renal tubules)

G,CA9 : perinuclear dot like staining

H, GATA3 : negative  with positive internal control (inset)

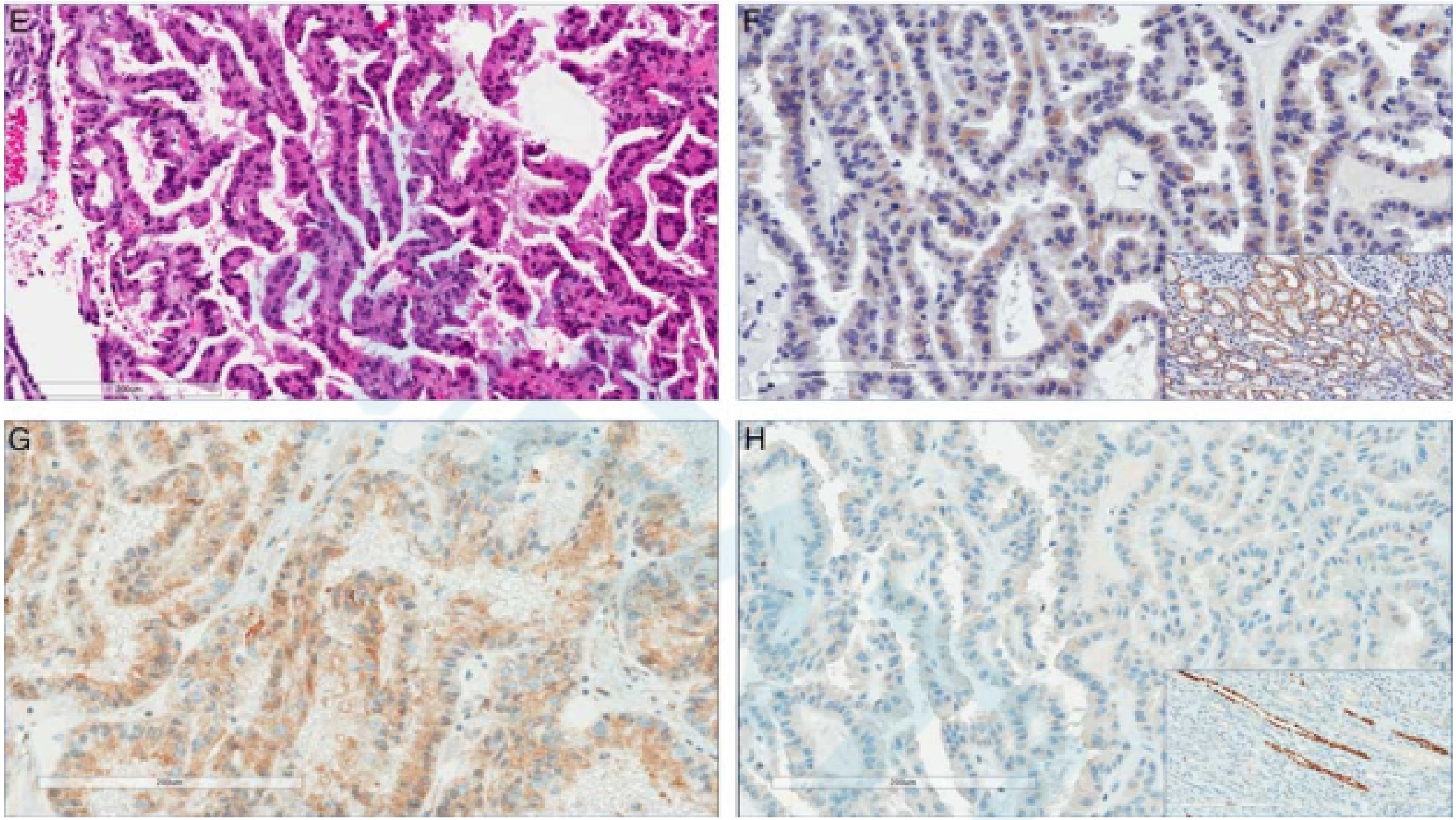



# PRCC4/OLG

B, ABCC2 : strong diffuse cytoplasmic staining.

C, CA9 : negative.

D, GATA3 : positive nuclear staining( specific to this particular subtype)

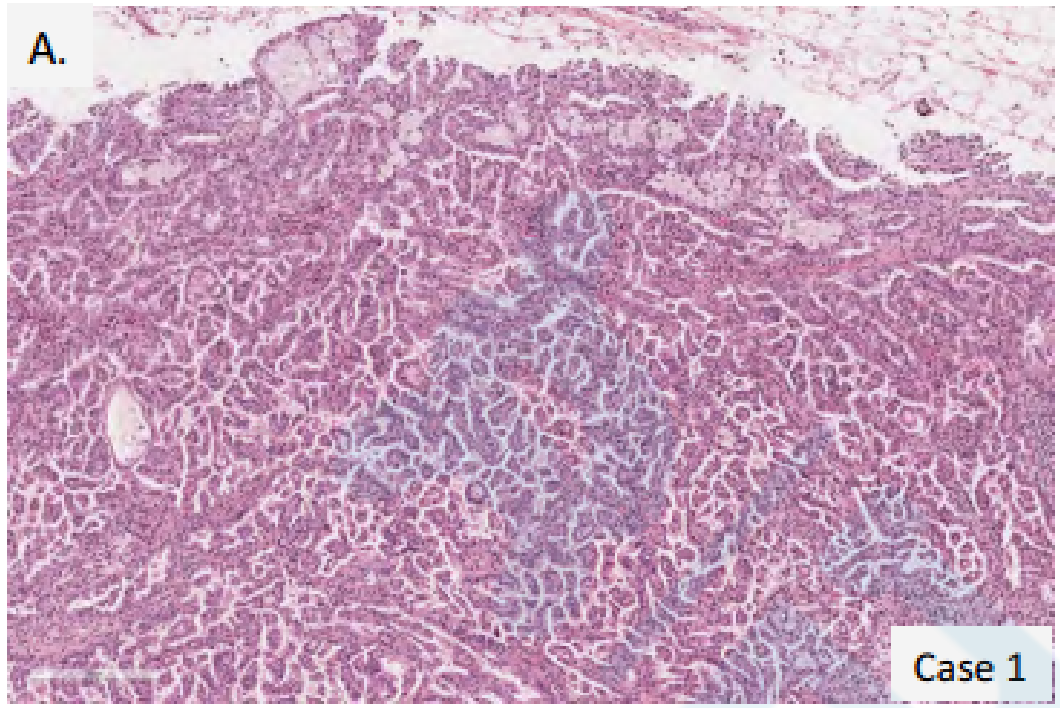


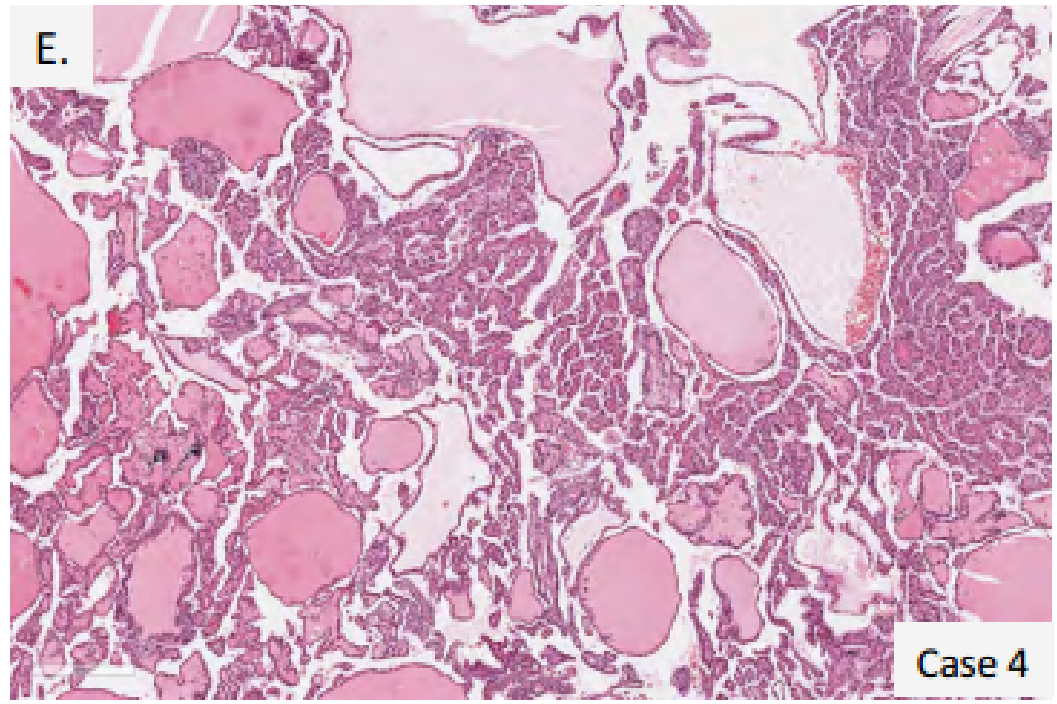

# PRCC3

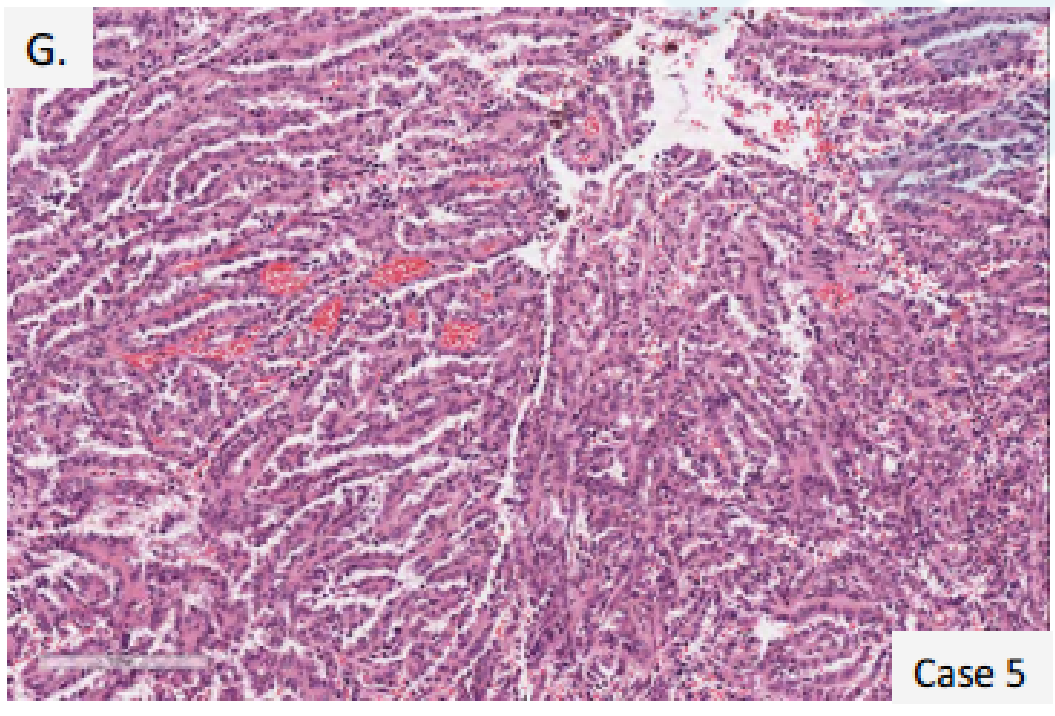
F, ABCC2 : moderate diffuse to patchy staining, weaker than the control normal renal tubules.
G,CA9 : negative (patchy membranous or unspecific cytoplasmic staining).
H, GATA3 : negative with positive internal control (inset).

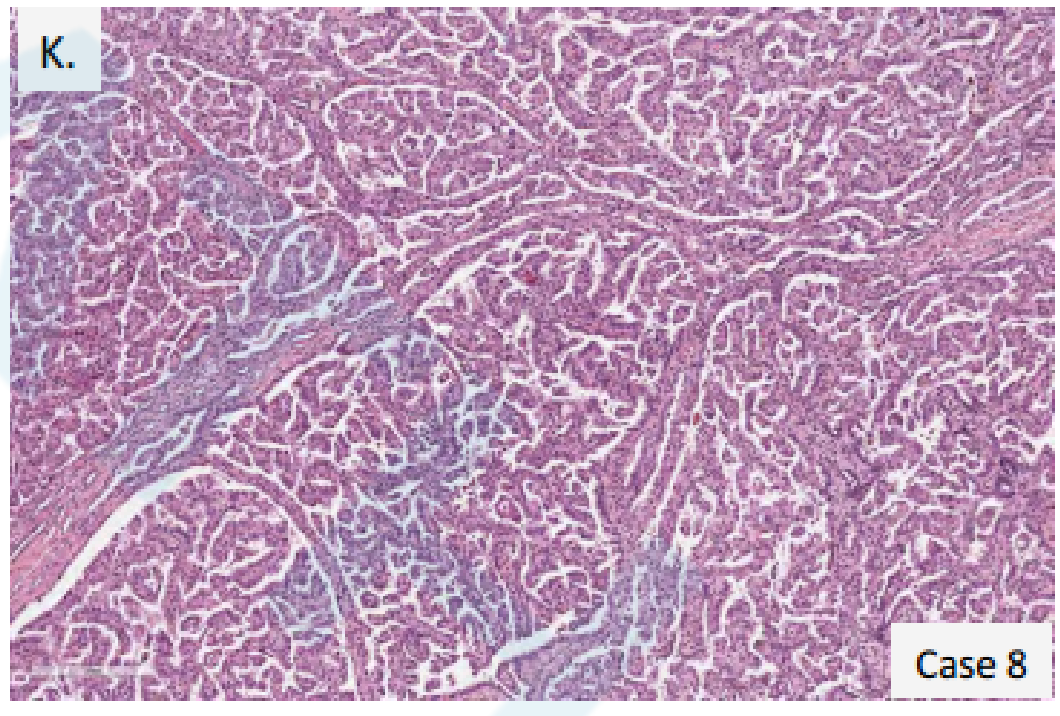
# PRCC3

- These tumors were mostly from the NOS group (65.8%) where tumors had mixed morphologic criteria between what is described for PRCC1 and PRCC2



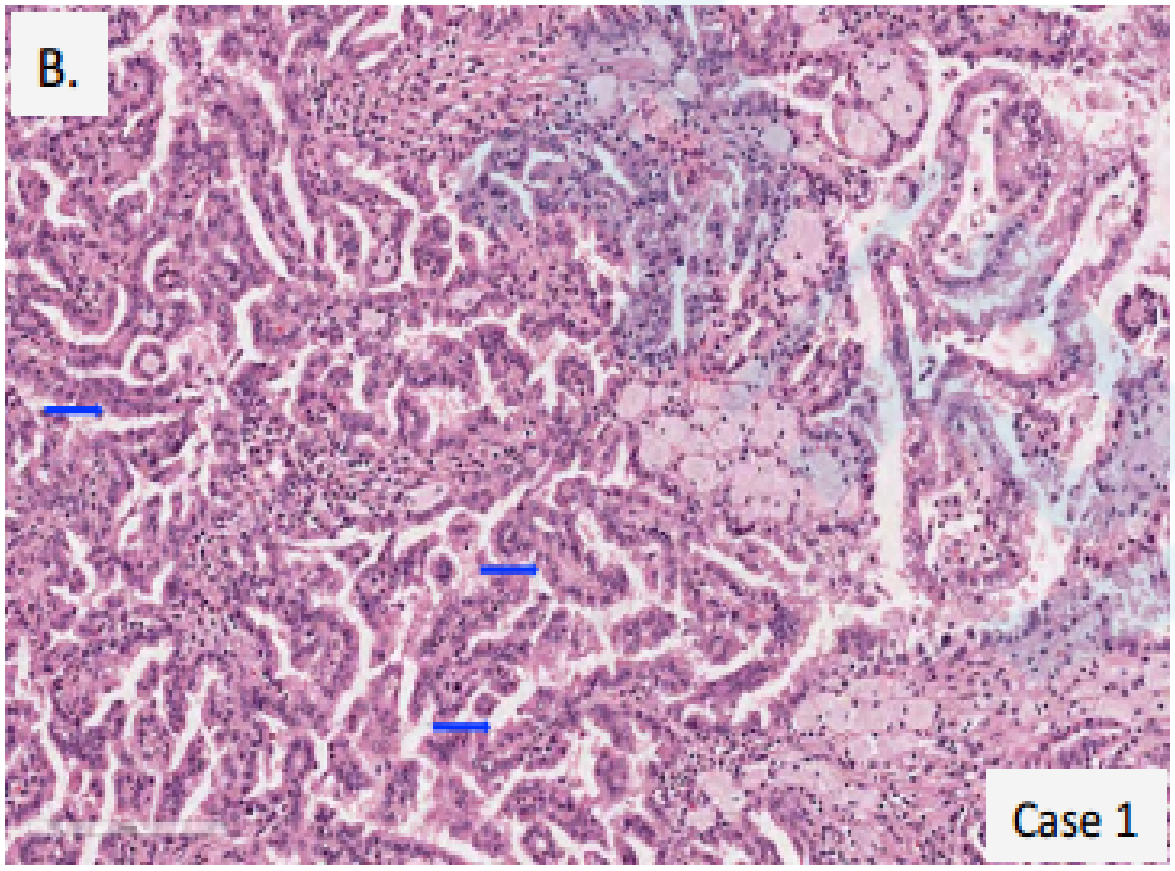



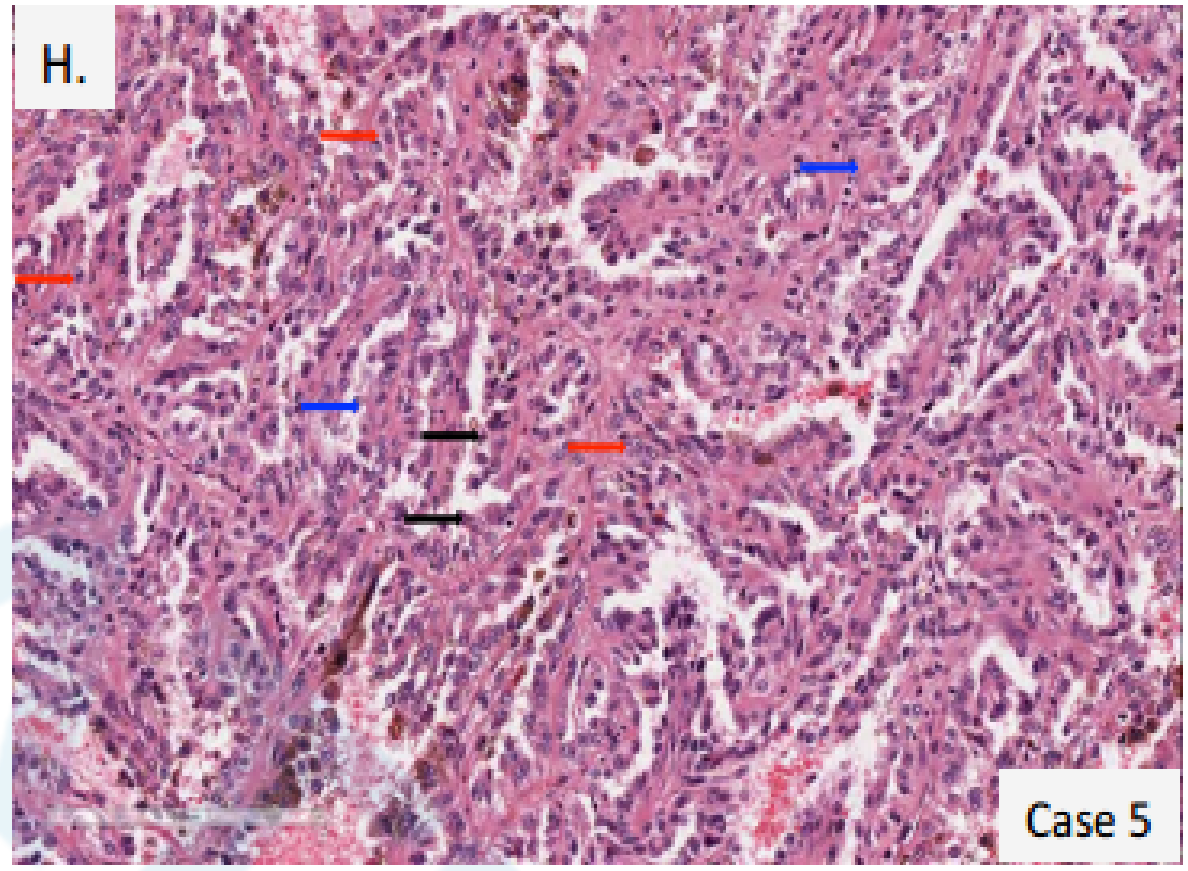




**Supplemental** Fig. 3: **PRCC3** tumors： On lower power magnification (A, E, G K) the tumors resemble the PRCC1 tumors
lack of prominent pseudostratification and smaller more basophilic cells.

# PRCC3



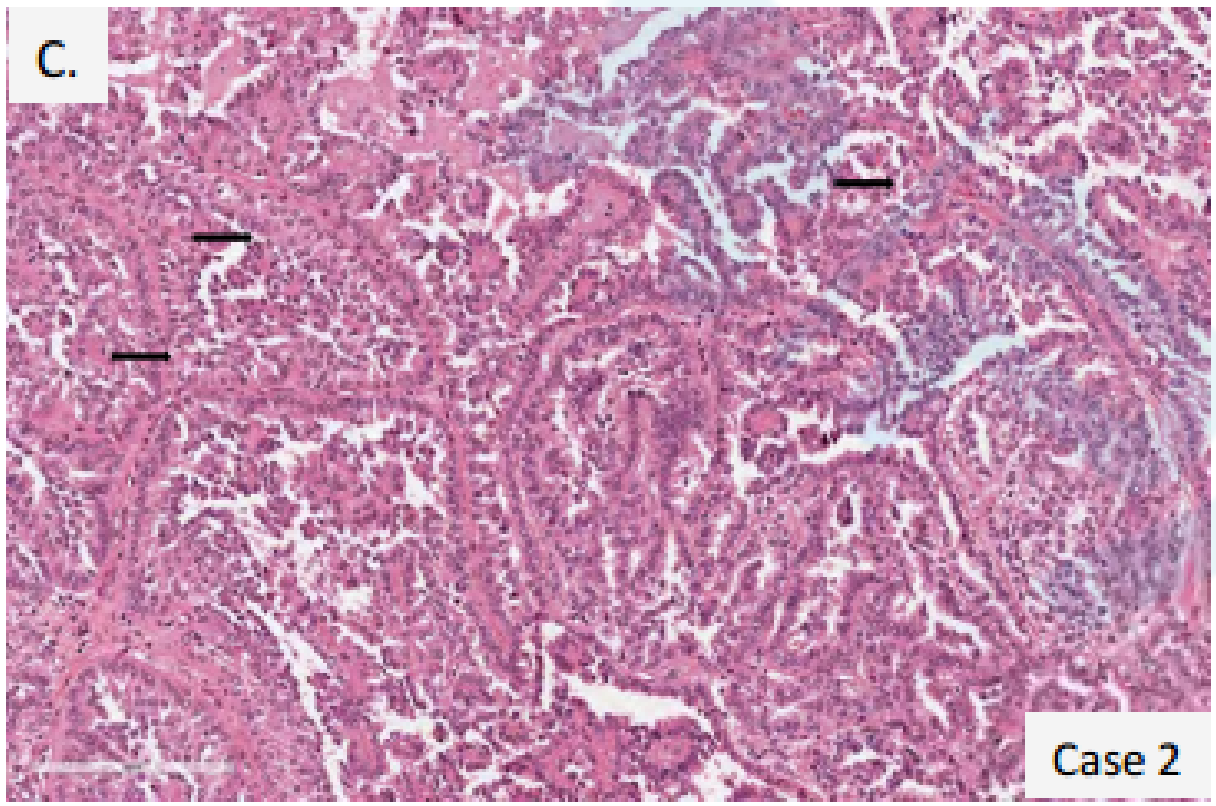

**Supplemental** Fig. 3: **PRCC3** tumors： On higher power magnification the tumors exhibit features that belong to the PRCC2 group

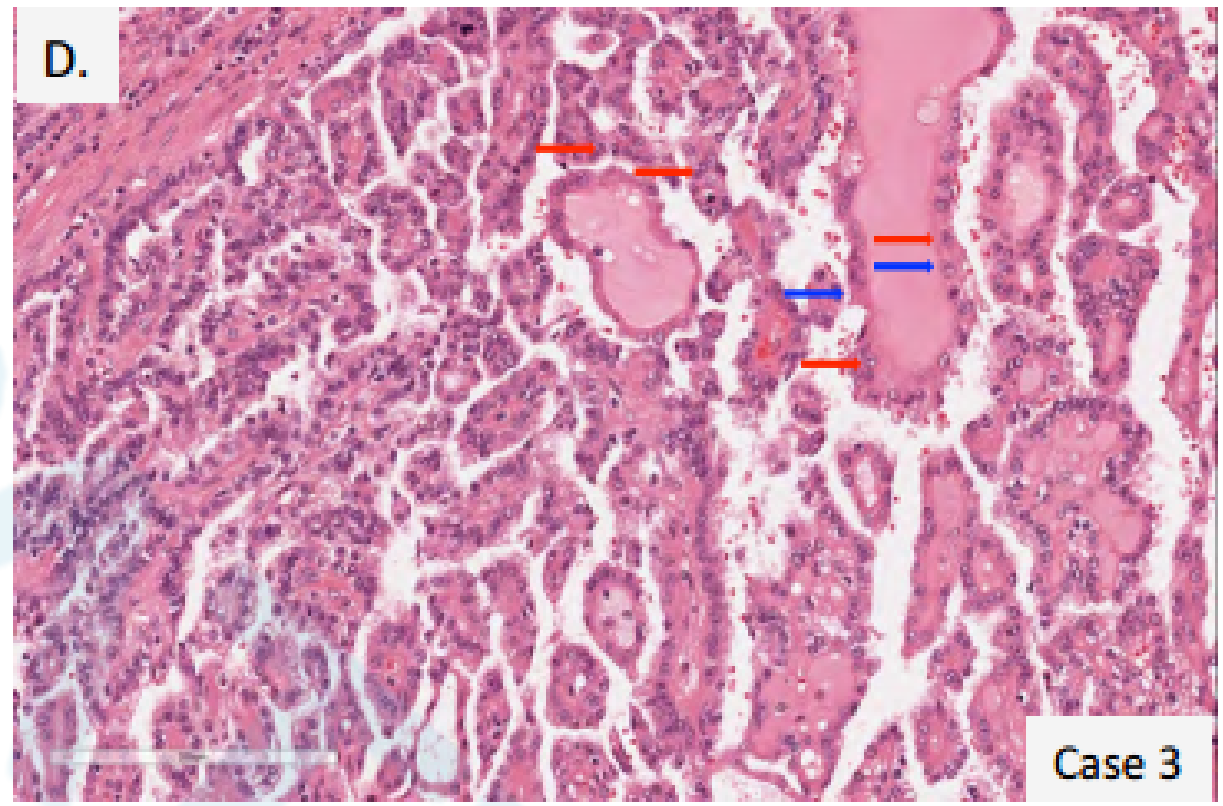
1.black arrows: focal pseudostratification

2.blue arrows: larger cells with moderate amount of easinophilic cytoplasm

3.red arrows: cells with prominent nucleolus consistent with an ISUP nucleolar grade 3

# PRCC3

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1.black arrows: focal pseudostratification

2.blue arrows: larger cells with moderate amount of easinophilic cytoplasm

3.red arrows: cells with prominent nucleolus consistent with an ISUP nucleolar grade 3

These tumors in the current classification would be classified as **PRCC NOS**

**TABLE 1.** Morphological Characteristics of the 4 PRCC Subtypes

| Features | PRCC1 | PRCC2 | PRCC3 | PRCC4/OLG |
|---|---|---|---|---|
| Cytoplasmic quantity | Scant, occasionally moderate | Abundant | Moderate | Abundant |
| Cytoplasmic color | Basophilic or eosinophilic or clearing | Eosinophilic or clearing | Eosinophilic, or clearing | Oncocytic eosinophilic |
| Cell size | Small to intermediate | Large | Intermediate | Large |
| Nucleolar prominence at ×10 | Inconspicuous, rarely prominent | Very prominent | Often prominent | Inconspicuous, rarely prominent |
| % nucleolar prominence at ×10 | If present <5 | 30-100 | 10-70 | If present <5 |
| Nuclear pseudostratification (presence or absence) | Absent | Mostly present, occasionally absent | Mostly absent, occasionally present | Absent. Linear. Nuclei arranged away from base of the cells |
| Nuclear size | Small | Large | Small to intermediate | Intermediate |
| Nuclear shape | Elongated oval (angulations and grooves) or round | Mostly round | Round or elongated | Round |
| Chromatin (open or closed) | Closed or open | Open vesicular nuclei, rarely focal areas with closed chromatin | Open, rarely closed | Open |
| ISUP nucleolar grade | 1-2, very rarely focal 3 | Mostly 3 | Mostly 3 | 1-2 |
| Foamy macrophages | Present or absent | Present or absent | Present or absent | Absent |
| ABCC2 IHC | Negative | Strong diffuse positive | Weaker patchy positive | Strong diffuse positive |
| CA9 IHC | Negative | Positive Golgi pattern (perinuclear dot) | Negative | Negative |
| GATA3 IHC | Negative | Negative | Negative | Positive |

ISUP indicates International Society of Urological Pathology.

# Molecular Classification of the Different PRCC Subtypes

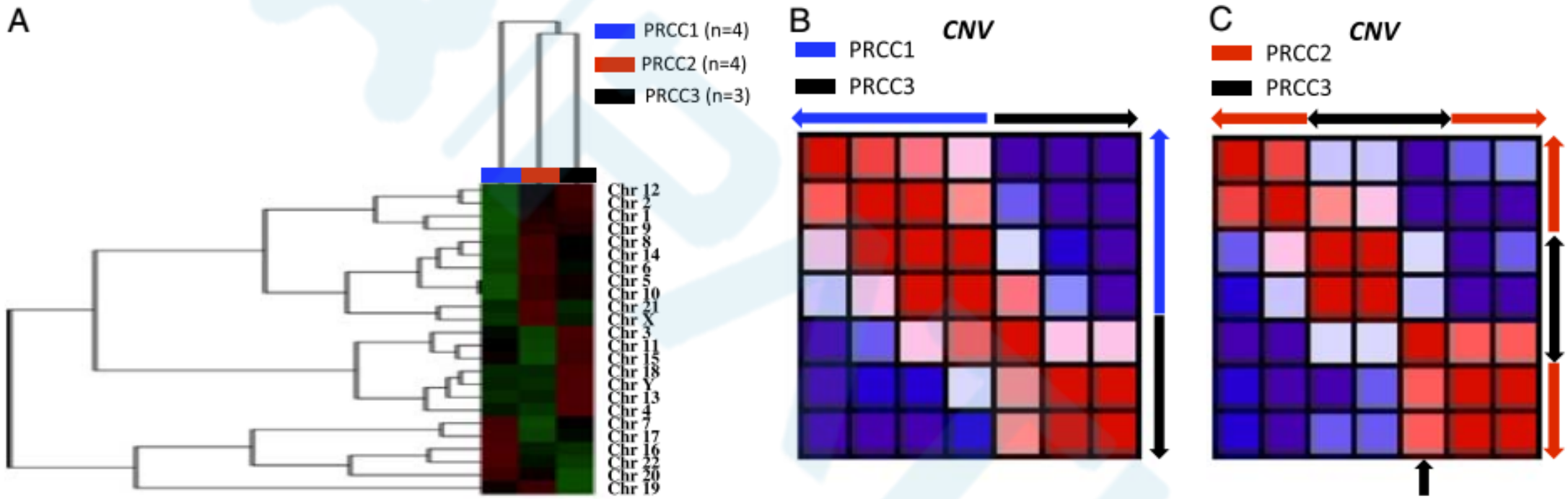
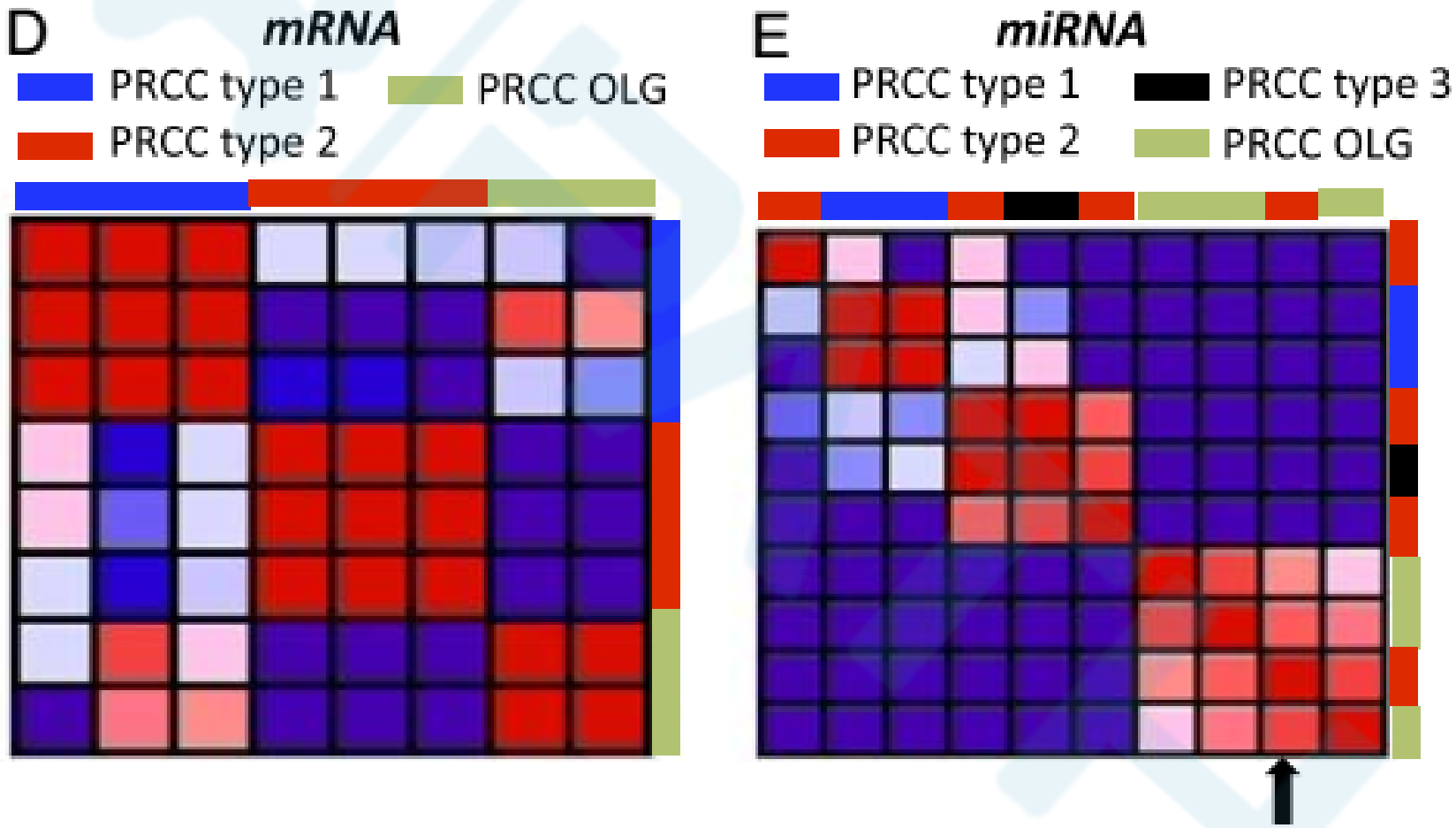



FIGURE 3. Molecular clustering analysis of the PRCC subtypes.
A, CNV clustering analysis of the PRCC1, PRCC2, and PRCC3 showing distinct chromosomal CNV profiles for each group.
B, CNV clustering analysis： PRCC1 clearly distinct from PRCC3.
C, CNV clustering analysis： some degree of overlap between PRCC2 and PRCC3 (overlapping case indicated by an arrow).

# Molecular Classification of the Different PRCC Subtypes




Clustering analysis of miRNA expression profiles
D, PRCC4/OLG have a distinct molecular cluster.
E, PRCC4/OLG and PRCC3 to be distinct from PRCC1 while having minimal overlap with PRCC2 (overlapping case indicated by an arrow).

# Clinical Characteristics and Survival Analysis Among the Subtype Categories

**TABLE 2.** Patient Demographics and Tumor Characteristics Between PRCC Subtypes

| Variables | PRCC1 | PRCC2 | PRCC3 | PRCC4/ OLG | *P* |
|---|---|---|---|---|---|
| Age (mean age [SD]) (y) | 60 (11.4) | 64 (12.8) | 65 (10.6) | 62 (16.5) | 0.311 (1-way ANOVA) |
| Sex (n [%]) | | | | | |
| M | 19 (70.4) | 24 (64.9) | 33 (86.8) | 2 (33.3) | 0.022* ($\chi^2$) |
| F | 8 (29.6) | 13 (35.1) | 5 (13.2) | 4 (66.7) | |
| Size (cm) | | | | | |
| Mean | 3.4 | 4.5 | 3.96 | 1.6 | 0.027* (Kruskal-Wallis) |
| Range | 0.5-12.5 | 0.6-18 | 1-14 | 0.65-3.10 | |
| Median | 3 | 3 | 3 | 1.55 | |
| Stage (n [%]) | | | | | |
| I | 24 (88.9) | 23 (62.2) | 27 (71.1) | 6 (100) | 0.018* (1-way ANOVA) |
| II | 2 (7.4) | 2 (5.4) | 3 (7.9) | — | |
| III | 1 (3.7) | 9 (24.3) | 2 (5.3) | — | |
| IV | 0 | 3 (8.1) | 5 (13.2) | — | |
| Laterality (n [%]) | | | | | |
| Right | 12 (44.4) | 20 (54.1) | 19 (50) | 2 (33.3) | 0.863 ($\chi^2$) |
| Left | 13 (48.1) | 15 (40.5) | 18 (47.4) | 4 (66.7) | |
| NS | 2 (7.4) | 2 (5.4) | 1 (2.6) | — | |

*Statistically significant ($\leq 0.05$).
ANOVA indicates analysis of variance; NS, not specified.

# Clinical Characteristics and Survival Analysis Among the Subtype Categories

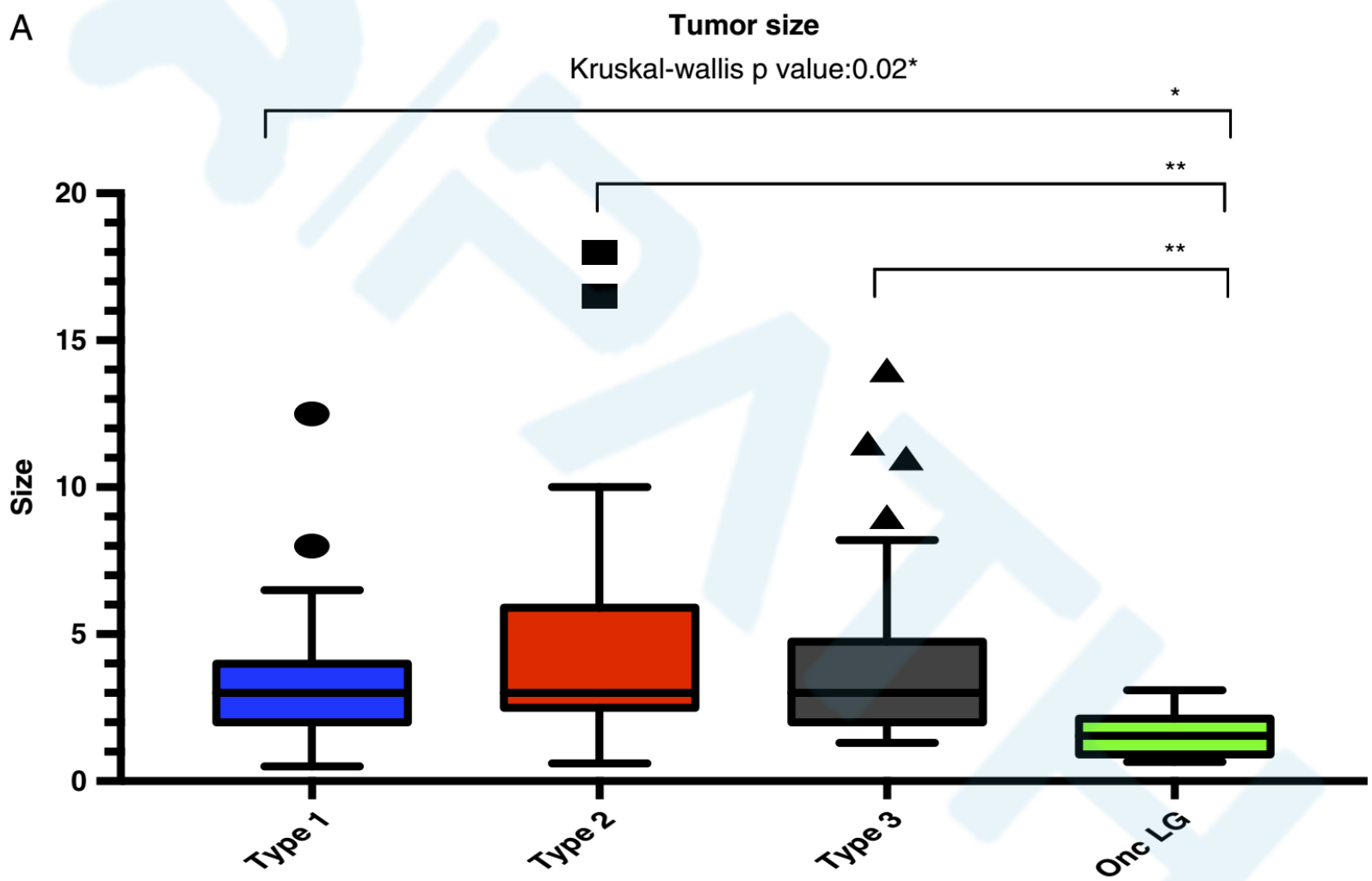



FIGURE 4. A, Tumor sizes: Only the PRCC4/OLG is significantly smaller than the other subtypes.

# Clinical Characteristics and Survival Analysis Among the Subtype Categories

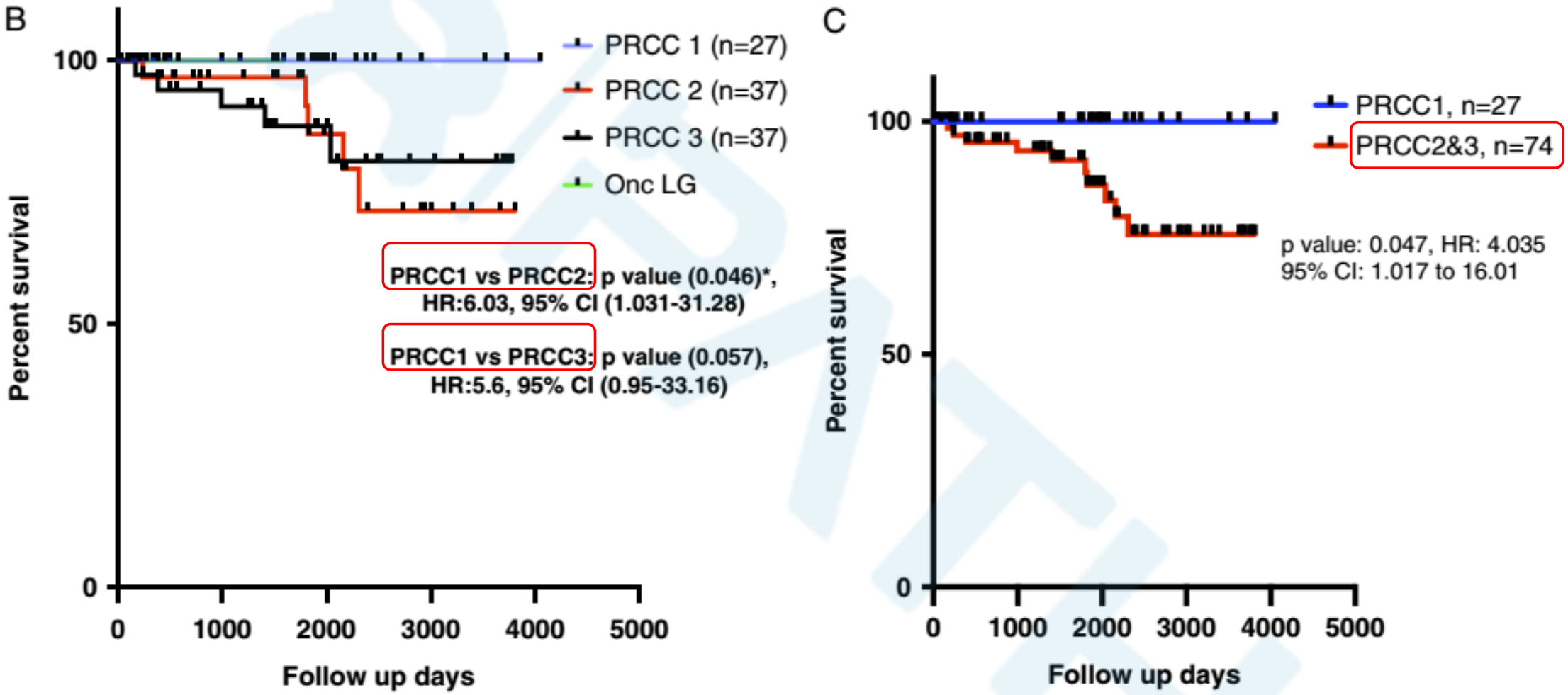



FIGURE 4. B and C, Univariate survival analysis with DFS (disease-free survival) shown on Kaplan-Meier curves.

B, DFS of all 4 PRCC subtypes (There were no disease recurrence events in the PRCC4/OLG and PRCC1 subgroups)

C, DFS of PRCC1 versus PRCC2 and PRCC3.

# Clinical Characteristics and Survival Analysis Among the Subtype Categories

**TABLE 3.** Multivariate Survival Analysis Between the 4 PRCC Subtypes (Cox Regression)

| Variable | Hazard Ratio | 95% CI | *P* |
|---|---|---|---|
| Multivariate analysis (n = 107) | | | |
| PRCC subtype | 6.34 | 1.25-32.2 | **0.026** |
| Size | 1.32 | 1.07-1.64 | **0.010** |
| Stage | | | |
| Stage I/II (n = 87) | 671179.3 | 2.3172E68−1.94E+79 | 0.876 |
| Stage IIII/IV (n = 20) | | | |

CI indicates confidence interval.
Bold indicates $P < 0.05$.

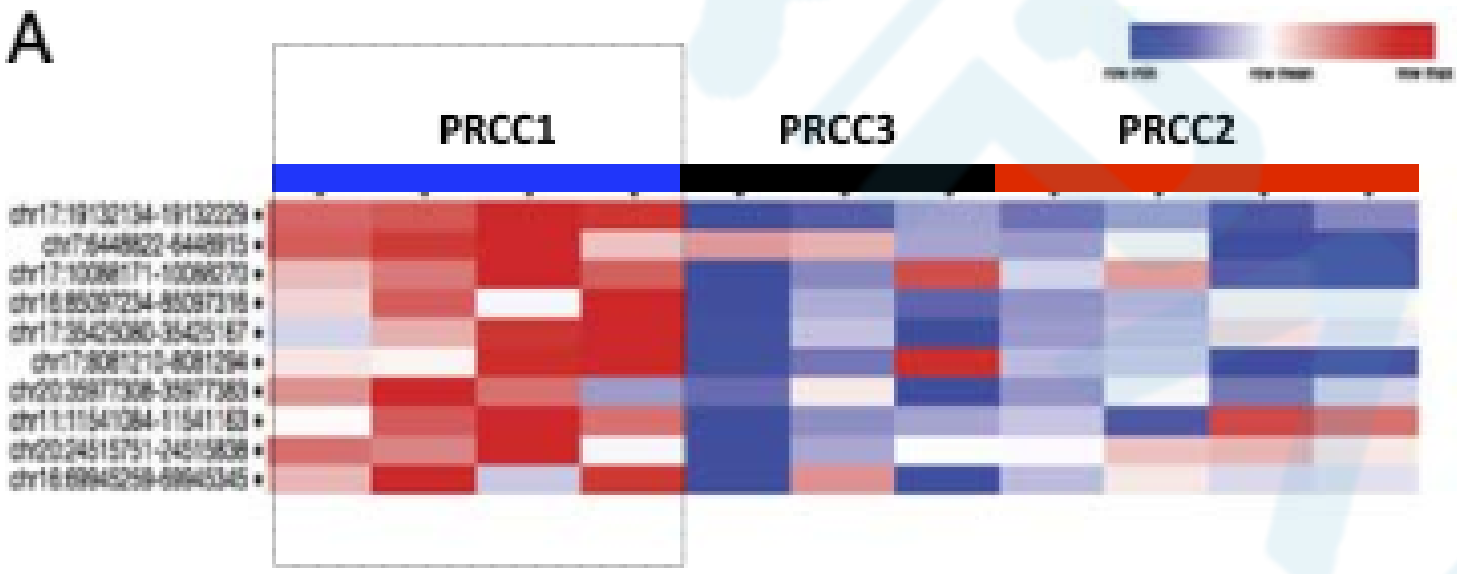
PRCC subtyping with the current IHC panel was significant on multivariate analysis when adjusting for tumor size and stage

(P = 0.025; hazard ratio, 6; 95% confidence interval, 1.25-32.2)

# Biological Pathways Enriched in Different PRCC Subtypes

- We next performed bioinformatics analysis to shed more light on the distinct biological pathways associated with each PRCC subtype.
  - Gene set enrichment analysis (GSEA)

# Biological Pathways Enriched in Different PRCC Subtypes




B

***CNV***

- WNT signaling pathway -Chr 7 & CH11**
- MET activation -Chr 7**
- NOTCH signaling pathway -Chr 17*
- DNA damage bypass related pathways - Chr 16*

***miRNA***

- WNT signaling pathway
- MET activation
- NOTCH signaling pathway

**PRCC 1**

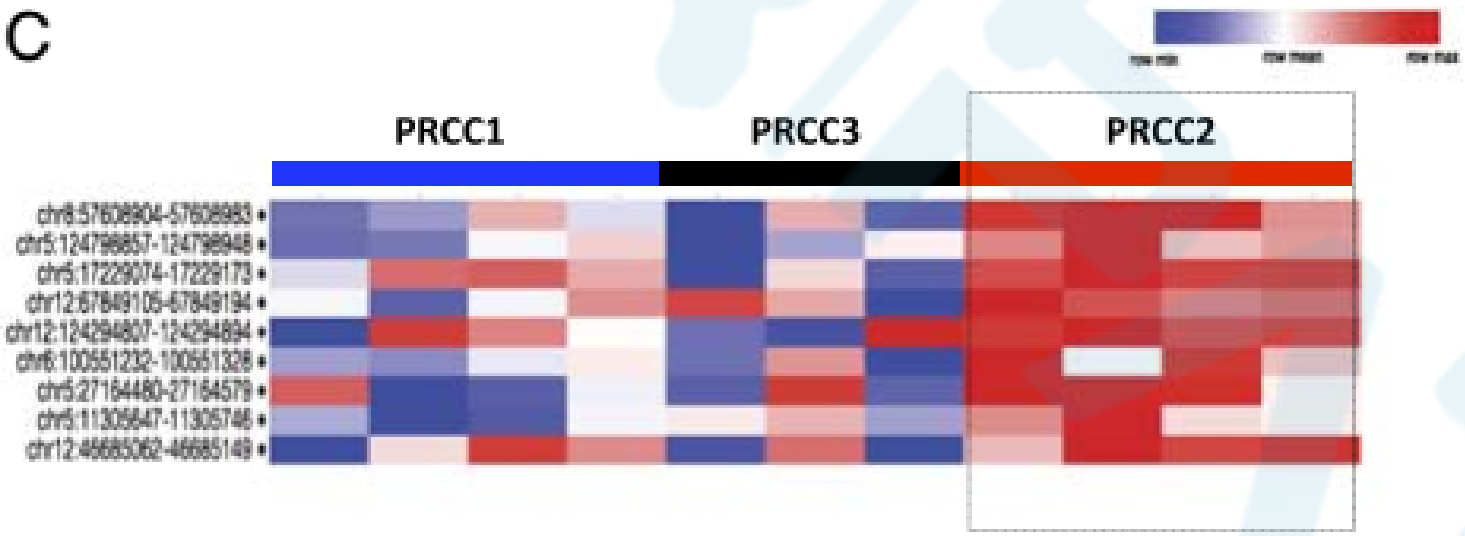
A and B, PRCC1.

A, Chromosomal regions that are significantly enriched in PRCC1 compared with the other types (Chr 7, 17, 16, and 20) analyzed with comparative marker selection testing.

B, GSEA of differentially expressed chromosomal regions (CNVs) and miRNAs correspond to the WNT, MET, NOTCH, and DNA damage bypass pathways.

# Biological Pathways Enriched in Different PRCC Subtypes

C




D

***CNV***

- Metastasis enhancing pathways-Chr 5&8
  - Adherens junctions interactions***
  - Gap junction degradation**
  - DDC mediated attractive signaling
- Cell cycle-Chr5
  - Meiosis & meiotic recombination

***miRNA***

- Metastasis enhancing pathways
  - FGFR pathways
- Cell cycle
  - G2 phase

**PRCC 2**

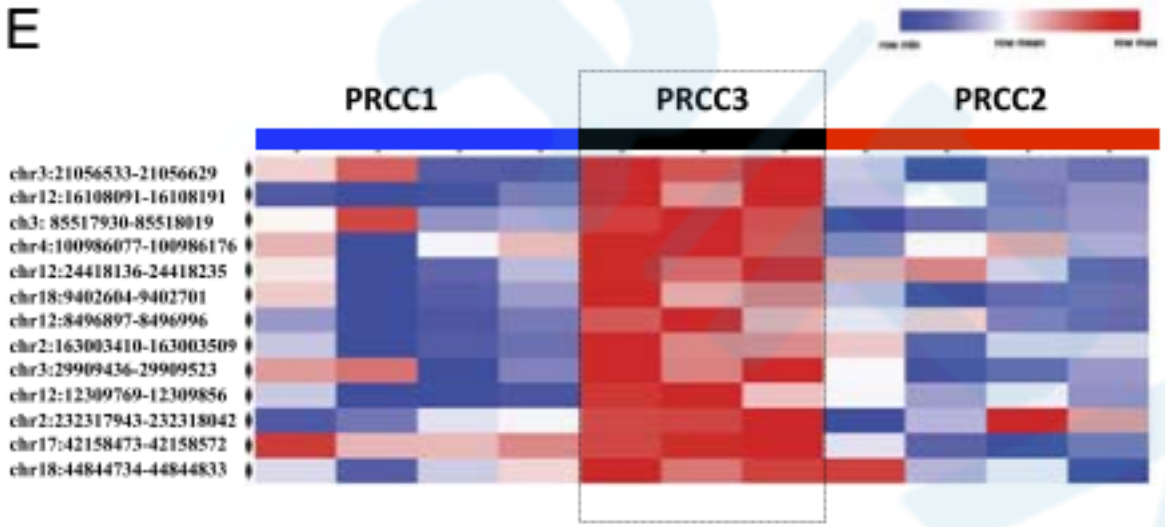
C and D, PRCC2.

C, Chromosomal regions that are significantly enriched in PRCC2 compared with the other types (Chr 5, 8, and 12).

D, GSEA of differentially expressed CNVs and miRNAs correspond to a number of metastasis enhancing and cell cycle pathways.

# Biological Pathways Enriched in Different PRCC Subtypes

E




F

***CNV***

- Loss of Function of TGFBR1 in Cancer*
- SMAD2/3 Phosphorylation Motif Mutants in Cancer**
- Loss of Function of SMAD2/3 &4 in Cancer**
- TGFBR1 KD Mutants in Cancer*
- Immunosuppression in tumor microenvironment
  - SIRP family interactions*
  - Synthesis, secretion, and inactivation of GLP-1*
  - Dectin-2 family *
  - Severe congenital neutropenia type 4 (G6PC3)*

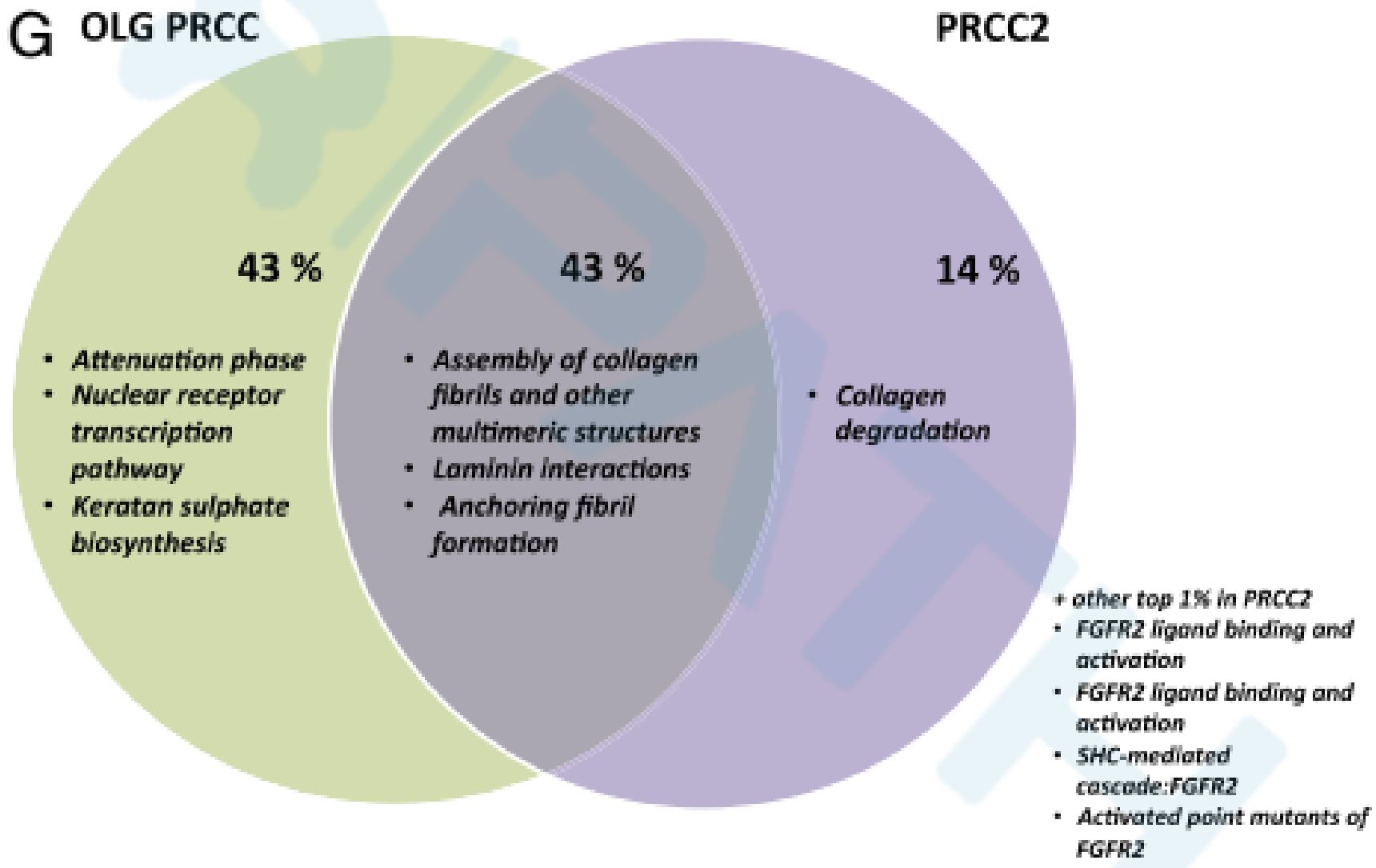
**PRCC3**

E and F, PRCC3.

E, Chromosomal regions that are significantly enriched in PRCC3 compared with the other types (Chr 3, 4, 12, 18, and 2).

F, GSEA of upregulated CNVs corresponds to TGF β in cancer and downstream pathways.

# Biological Pathways Enriched in Different PRCC Subtypes




**G, PRCC4/OLG versus PRCC2，**
significant pathway **overlap** between PRCC4/OLG and PRCC2 (43%).
GSEA of miRNA data

# DISCUSSION

- The results identified 2 additional classes of PRCC (other than the classic PRCC1 and PRCC2) that are associated with distinct clinical behavior and unique molecular pathways.
- Our findings are consistent with other studies regarding PRCC being a heterogenous disease with multiple molecular signatures

# DISCUSSION

- Among our promising new biomarkers, ABCC2 was effective in our earlier analysis in separating the PRCC NOS group into statistically significant prognostic groups.
- ABCC2 is a human drug/renal transporter, which is innate to the renal tubules. It is additionally known to be involved in chemotherapy resistance through drug efflux, where it mediates transport of chemicals and drugs out of the cells.

# DISCUSSION

- PRCC2 exhibited perinuclear dot like Golgi pattern of CA9 staining. CA9 is normally located at the cell membrane,thus this perhaps presents an abnormal segregation of the protein at the Golgi.

- Accumulation of drugs in perinuclear vesicles is also a described feature of tumors containing high levels of drug transporters as ABCC2 and is thought to be an added feature contributing to their drug resistance.

# DISCUSSION

- PRCC3 ——TGFβ (and downstream) pathways
- TGFβ dysregulation is involved in multiple aspects of tumor pathogenesis
  - epithelial to mesenchymal transition
  - tumor proliferation
  - alterations to the tumor microenvironment.

# DISCUSSION

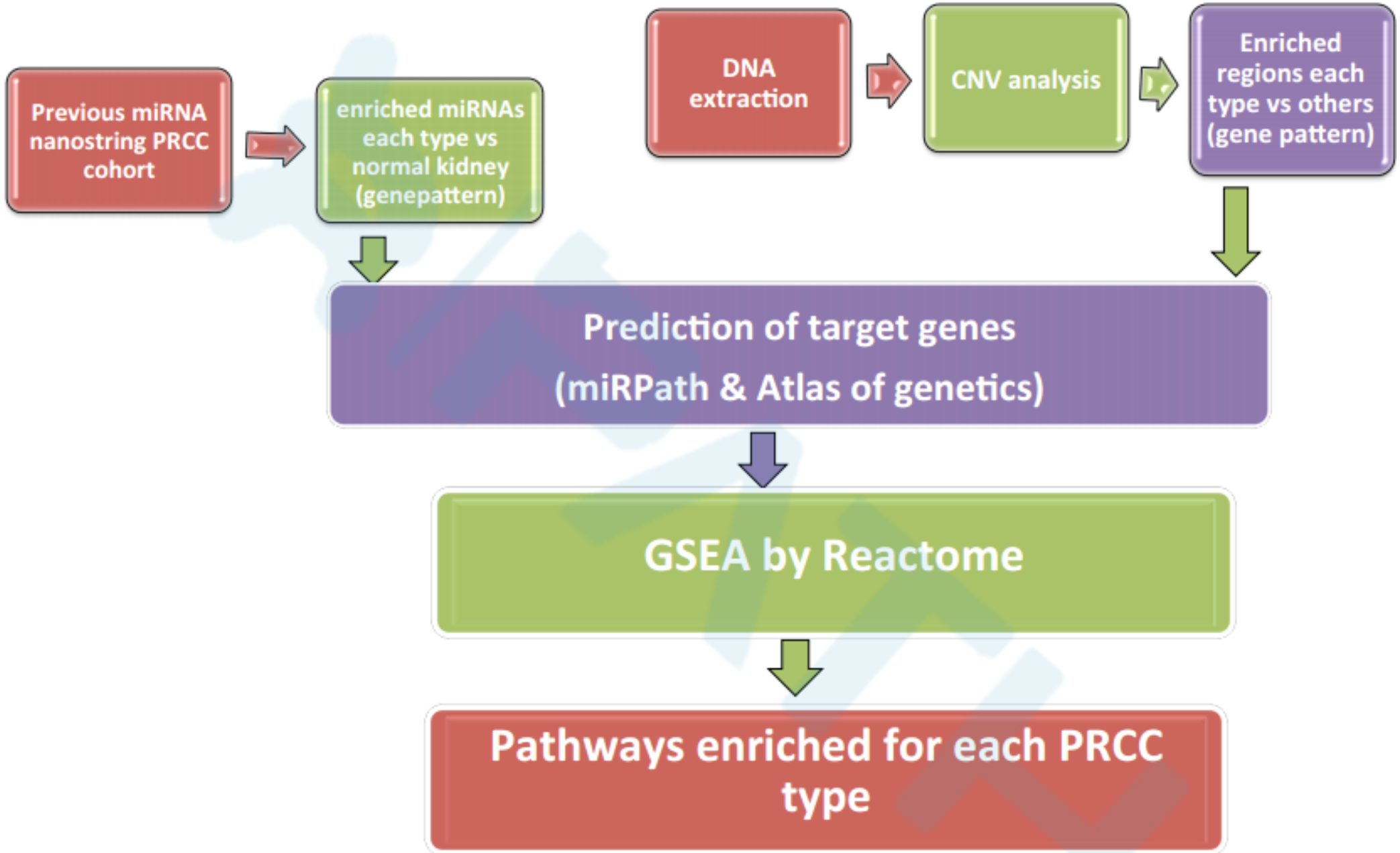
- About 1/3 of the NOS cases were further stratified into either PRCC1 or PRCC2 with immunostaining, while the other 2/3 of the NOS belonged to the PRCC3 group.
- Generally the NOS group (47% of the PRCC cohort) showed variable nucleolar prominence, even within the same case.
- Thus the morphology and grading alone had very low sensitivity and specificity in accurately stratifying these cases.

# CONCLUSION

- We provide evidence that our newly described PRCC subtype PRCC3 and PRCC4/OLG are distinct tumors with unique clinical and molecular profiles.
- The 4 PRCC subtypes have different clinical characteristics and hence there is great value in properly stratifying them.
- Given their overlapping histologic features, IHC appears to be critical for accurate subtyping.

# THANK YOU

**Supplemental Fig. 5**




Supplemental figure 5: Schematic representation of the chromosomal number variation （CNV）and miRNAs dependent
Process： gene set enrichment analysis (GSEA)