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Sporadic clear cell renal cell carcinoma but not the papillary type is characterized by severely reduced frequency of primary cilia

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Renal cysts and clear cell renal cell carcinoma are common clinical manifestations of people with germ-line mutations of the von Hippel–Lindau tumor suppressor gene, *VHL*. Recent cell biological evidence suggests that the *VHL* gene product, pVHL, functions to maintain the primary cilium, a microtubule-based antenna-like structure whose functional integrity is believed to have an important role in cell-cycle control. As *VHL* mutations are common in sporadic clear cell renal cell carcinoma, but not papillary renal cell carcinoma, we asked whether there is an association between *VHL* status and primary cilia *in vivo*. *VHL* status was assessed in 20 cases of clear cell renal cell carcinoma and 9 cases of papillary renal cell carcinoma by DNA sequencing and by immunohistochemical staining for the hypoxia-inducible factor-α target gene products CA9 and GLUT-1. Of 20, 18 clear cell renal cell carcinomas, but only 1 of 9 papillary renal cell carcinomas, displayed evidence of *VHL* inactivation. In clear cell renal cell carcinoma the frequency of ciliated tumor cells ranged from 0 to 22% (median value $7.8 \pm 6.0\%$), whereas cilia frequency was significantly higher ($P < 0.0001$) in papillary renal cell carcinoma (range 12–83%, median value $43.3 \pm 21.3\%$). There was no correlation between Ki-67 staining and cilia frequency, suggesting that the observed differences between the tumor types in cilia frequency are not accounted for by differences in cellular proliferation rates and that primary cilia degeneration in sporadic clear cell renal cell carcinoma depends on *VHL* inactivation. We propose that the different ciliation status of clear cell and papillary renal cell carcinoma may contribute, at least in part, to the different biological behaviors of these tumor types.
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Primary cilia are specialized cellular organelles that project from the apical surface of almost all nondividing or interphase mammalian cells. They are thought to function in sensing various molecular and mechanical cues in the extracellular environment and to propagate signals into the cell. Defects in primary cilia structure and/or function have been causally linked to the development of various human diseases, including polycystic diseases of the kidney.[1]

Epithelial cells of the nephron have primary cilia, that protrude into the tubule lumen where they are believed to act in sensing the flow of urine.[1]

Disruption of the cilia structure or function causes uncontrolled kidney epithelial cell proliferation and cyst formation. At the core of a cilium lies the axoneme, which consists of a bundle of nine stable microtubule doublets $(9 + 0)$ that are nucleated from the basal body. The composition of proteins within the cilium and hence cilium structure and function is regulated by and dependent on microtubule motor-driven transport of specific cargo proteins and vesicles. Thus, proper regulation of microtubule stability and dynamics is central for the structural and functional integrity of the cilium.[1] For example, kidney-specific deletion of *Kif3a* coding, a subunit of the kinesin II motor protein responsible for retrograde transport in the cilium, results in loss of cilia and cyst formation in mice.[2]

Inactivating germ-line mutations of *VHL* represents the genetic hallmark of VHL disease, which, among other clinical manifestations predisposes affected individuals to develop renal cysts and clear

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cell renal cell carcinoma (ccRCC). The development of these kidney pathologies has been linked to the inactivation of *VHL*-dependent tumor-suppressive mechanisms. Prominent among these is pVHL's ability to act as an adapter protein of an E3 ubiquitin ligase complex that targets hypoxia-inducible factor-α (HIFα) subunits for ubiquitin-mediated degradation in the presence of oxygen.[3] But pVHL also binds and stabilizes microtubules by protecting them from depolymerization, which is a prerequisite for cilium formation.[4] Recent studies indicated that pVHL is necessary for the formation of primary cilia in cultured renal cell carcinoma cell lines.[5,6] It has been demonstrated that pVHL localizes to the primary cilium in human and mouse kidney cells and functions as an important part of a primary cilium-maintenance signaling network.[7] Taken together, these observations strongly suggest that pVHL function is a critical aspect in primary cilium maintenance.

pVHL inactivation is not only restricted to the familial VHL cancer syndrome. In more than two-thirds of patients with sporadic ccRCC, which is the most frequent subtype of renal cancer, pVHL function is compromised by gene mutations or hypermethylation.[8] Like ccRCC, papillary renal cell carcinoma (pRCC) arises from tubular epithelial cells.[9] Morphologic, clinical, and genetic features clearly separate pRCC from ccRCC. Papillary tumors are characterized cytogenetically by trisomies/tetrasomies of chromosomes 7 and 17, and losses of chromosome Y, whereas chromosome 3p deletions and *VHL* mutations are common in ccRCC.[10] These findings raise the possibility that ccRCC may be characterized by the absence of primary cilia and as pRCC tumorigenesis follows a *VHL*-independent pathway, that pRCC might represent a disease that is not characterized by loss of primary cilia. To test this hypothesis, we analyzed and compared the frequencies of primary cilia in normal kidney tissue, 20 ccRCCs, and 9 pRCC specimens.

Materials and methods

Renal Cell Carcinoma Tissues

All formalin-fixed and paraffin-embedded renal tumors were from the archive of the Institute for Surgical Pathology, Zurich University Hospital, and were reviewed by one pathologist (HM). A total of 20 ccRCCs and 9 pRCCs were selected for this study and diagnosed according to the 2004 WHO (World Health Organization) classification.[11] Dominant architectural patterns were recorded for all ccRCCs. In total, 8 cases were dominantly solid/nested, 10 alveolar/acinar, and 2 tumors were mixed solid, alveolar, and papillary. This study was approved by the local commission of ethics (reference no. StV 38-2005).

Sequence Analysis

The *VHL* sequence analysis of 29 formalin-fixed and paraffin-embedded renal cell carcinomas was performed as described.[12] Sequencing was performed using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Rotkreuz, Switzerland) and forward or reverse primers that were applied for amplification of the *VHL* exons. Cycle sequencing products were analyzed using the AbiPrism 3100 Genetic Analyzer (Applied Biosystems).

Immunohistochemistry and Immunofluorescence

After dewaxing, rehydrating, and antigen retrieval for 10 min at 96°C in 0.1 M citrate buffer, renal cell carcinoma tissue sections (4 μm) were incubated using MIB-1 (1:20 dilution) (Dako, Glostrup, Denmark), mouse anti-CA9 M75 (1:200 dilution, gift of J Zavada, Institute of Molecular Genetics, Prague, Czech Republic), and rabbit anti-GLUT-1 AB1341 (1:1000 dilution) (Lucerna-Chem AG, Lucerne, Switzerland). Standard immunohistochemical detection of bound antibody was performed using biotin-conjugated goat F(ab′)2 anti-mouse immunoglobulin G (IgG; Jackson ImmunoResearch, West Grove, PA, USA) (1:500 dilution) or goat anti-rabbit IgG secondary antibodies followed by the ABC Vectastain and peroxidase substrate kits (Vector Laboratories, Burlingame, CA, USA) (1:200 dilution). Tumors were considered CA9 and GLUT-1 positive if membranous staining was found.

For immunofluorescence detection of primary cilia,[13] sections were incubated with mouse anti-acetylated tubulin (Sigma-Aldrich, Basel, Switzerland) (1:100 dilution) and goat anti-mouse secondary antibodies conjugated to Alexa 488 (Molecular Probes, Invitrogen, Basel, Switzerland) (1:1000 dilution). Nuclei were labeled with 2 μg/ml 4-6-diamidino-2-phenylindole (DAPI). Cilia counting was performed by focusing up and down on the microscope to capture cilia and nuclei that lay in different focal planes within the section. Pictures were obtained with a ×40 oil objective with samples mounted in an immersion medium. Optical sections were 0.2-μm thick and stacks were made encompassing a *Z*-plane depth of 2–4 μm. From each sample the end points were determined by focusing on the uppermost and lowermost visible primary cilia within the microscopy field.

Immunohistochemical and immunofluorescence images were obtained using a Zeiss Axioplan 2 microscope or an Olympus IX70 Delta Vision Spectris deconvolution microscope. Obtained stacks were deconvolved using SoftWoRx 3.3.4 (Applied Precision, Issaquah, WA, USA) and processed with Imaris 4.2 (Bitplane AG, Zurich, Switzerland) and Photoshop 7.0 (Adobe Systems Inc.).

Statistical Analysis

Mean and median values, contingency table analysis, and χ^2-tests for evaluating correlations between cilia frequencies and tumor subtypes were calculated using Statview Statistic Program (SAS, USA).

Results

We utilized two different methods to characterize the *VHL* status of our cohort of renal cell carcinoma samples. First, direct *VHL* DNA sequencing revealed that 9 ccRCCs carried wild-type *VHL* alleles, 11 ccRCCs carried *VHL* 'loss-of-function'[12] (frameshift) mutations, and 9 pRCCs carried wild-type *VHL* alleles (Table 1), consistent with previous reports about the mutation frequency of *VHL* in these tumor types.[8,14]

CA9 as well as GLUT-1 expression is widely accepted as readout for pVHL loss and HIF stabilization.[15–17] To indirectly assess pVHL status, we utilized immunohistochemical staining for the HIFα target genes *CA9* and *GLUT1* (Table 1; Figure 1a). Strong membranous positivity of CA9 was accompanied by GLUT-1 expression in 10 of 11 ccRCCs with *VHL* mutation and 1 tumor was CA9 negative but showed GLUT-1 staining. Five of nine ccRCCs with wild-type *VHL* expressed both *CA9* and

GLUT-1 and two tumors were positive for CA9 but negative for GLUT-1, suggesting *VHL* inactivation without *VHL* mutation. Two tumors were CA9 and GLUT-1 negative. Only one pRCC showed moderate CA9 and weak GLUT-1 expression. Taken together, we conclude that 18 of 20 ccRCC samples, but only 1 of 9 pRCCs, are characterized by *VHL* mutation or inactivation.

We next assessed the frequency of primary cilia in normal kidney tubules, ccRCC, and pRCC by counting DAPI-stained cell nuclei and primary cilia marked by immunofluorescent staining using an antibody against acetylated tubulin (Table 1), a marker to study primary cilia in tumor cells.[13] On average 87.3% (median value) of epithelial cells in 10 normal tubules contained a primary cilium (Figure 1b). For each of the renal cell carcinoma tissues, the frequency of primary cilia was evaluated by analyzing 60 cells within an area that contained more than 90% tumor cells. Strikingly, ccRCC samples exhibited frequencies of primary cilia that were very low (median value $7.5 \pm 6.0\%$) compared to normal tubules (Table 1; Figure 1c). Cilia frequency was independent of the architectural pattern in ccRCC (nested, alveolar, solid, and papillary). Compared to ccRCC, pRCC exhibited significantly higher ($43.3 \pm 21.3\%$) frequencies of ciliated cells ($P < 0.0001$) (Figures 1d and 2). Thus, ccRCC is characterized by very low frequencies of

Table 1 Cilia frequency, histopathological and molecular parameters in clear cell and papillary renal cell carcinoma

Tumor subtype	Tumor grade (Thoenes)	Tumor stage	Ciliated cells, n (%)[a]	CA9	GLUT-1	VHL	Ki-67 LI[b]
Papillary	1	2	22 (36.7)	Negative	Negative	Wild type	Low
Papillary	1	1a	26 (43.3)	Negative	Negative	Wild type	High
Papillary	2	3	37 (61.7)	Negative	Negative	Wild type	Low
Papillary	2	1a	50 (83.3)	Positive	Positive	Wild type	Low
Papillary	1	1a	25 (41.7)	Negative	Negative	Wild type	Low
Papillary	1	3a	34 (56.7)	Negative	Negative	Wild type	High
Papillary	1	1	38 (63.3)	Negative	Negative	Wild type	Low
Papillary	2	3	7 (11.7)	Negative	Negative	Wild type	High
Papillary	1	1b	17 (28.3)	Negative	Negative	Wild type	Low
Clear cell	1	1b	10 (16.7)	Positive	Positive	NT288; 1 bp	Low
Clear cell	2	4	7 (11.7)	Positive	Positive	NT445; 1 bp	High
Clear cell	3	3a	2 (3.3)	Positive	Positive	NT462; 1 bp	Low
Clear cell	1	3b	13 (21.7)	Positive	Positive	NT499–510; 12 bp	Low
Clear cell	2	1b	5 (8.3)	Negative	Positive	NT424–439; 16 bp	Low
Clear cell	1	1b	11 (18.3)	Positive	Positive	NT397; 1 bp	Low
Clear cell	1	2	4 (6.7)	Positive	Positive	NT374–380; 7 bp	Low
Clear cell	1	1b	6 (8.3)	Positive	Positive	NT394; 1 bp	High
Clear cell	1	3a	6 (8.3)	Positive	Positive	NT299; 1 bp del	High
Clear cell	1	1a	2 (3.3)	Positive	Positive	NT501; 1 bp del	High
Clear cell	3	3	1 (1.7)	Positive	Positive	NT238–239; 2 bp del	High
Clear cell	1	1b	1 (1.7)	Positive	Negative	Wild type	High
Clear cell	2	1b	1 (1.7)	Negative	Negative	Wild type	Low
Clear cell	2	3	0	Positive	Negative	Wild type	High
Clear cell	2	1b	5 (8.3)	Positive	Positive	Wild type	Low
Clear cell	3	4	2 (3.3)	Negative	Negative	Wild type	Low
Clear cell	3	3a	7 (11.7)	Positive	Positive	Wild type	High
Clear cell	2	3a	2 (3.3)	Positive	Positive	Wild type	High
Clear cell	2	3	3 (5.0)	Positive	Positive	Wild type	High
Clear cell	2	1b	7 (11.7)	Positive	Positive	Wild type	Low

[a]Number of cilia in 60 tumor cells. [b]Cutoff for high and low Ki-67 labeling index (LI) is 7%.
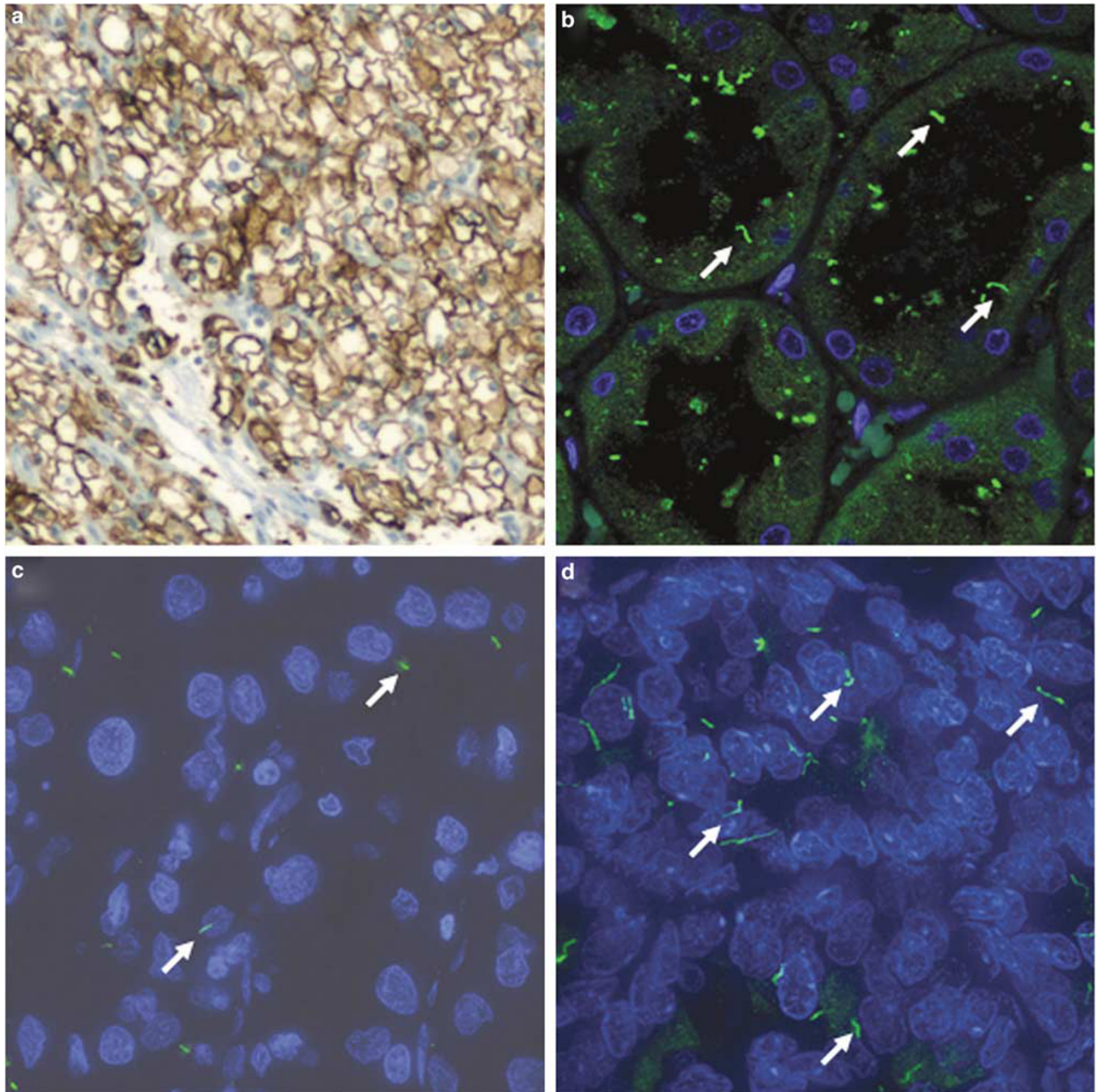
Figure 1 (**a**) Example of an immunohistochemically stained renal tumor showing membranous GLUT-1 expression in a clear cell renal cell carcinoma. (**b–d**) Ciliated tumor cells (white arrows) stained with antibody against acetylated tubulin and DAPI in tubular epithelial cells of normal kidney (magnification ×40) (**b**), one papillary renal cell carcinoma (×40) (**c**), and one clear cell renal cell carcinoma (×40) with the highest number of primary cilia (**d**).

primary cilia compared to normal tubules and to pRCC.

Because primary cilia formation occurs in the quiescent phase of the cell cycle (G_0) and not in mitotically active cells,[18] we compared the frequency of expression of the cell proliferation marker Ki-67, which is expressed in late G_1-, S-, G_2-, and M-phases of the cell cycle but not in nonproliferating G_0, with the frequency of ciliated cells in our renal cell carcinoma tissues (Table 1). We used the same cutoff (7%) for high and low Ki-67 labeling index (LI) as previously described.[12] We observed no correlation between Ki-67 and cilia frequencies within or between groups of pRCC and ccRCC, suggesting that the ciliation frequency differences between pRCC and ccRCC do not arise because of differences in proliferation rates of these tumor types.

Discussion

We demonstrate that cilia degeneration is common in sporadic ccRCC. It seems obvious that genetic
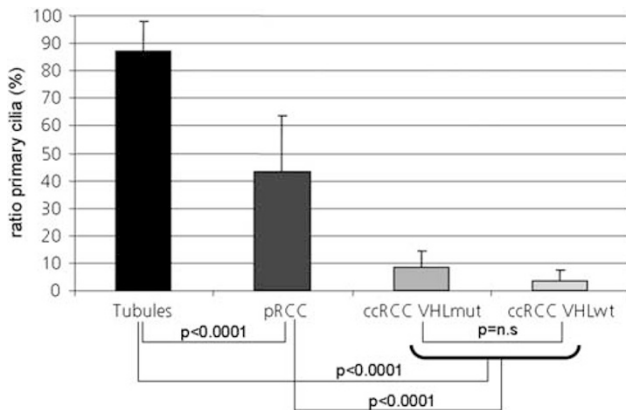
Figure 2 Cilia frequencies (median values) in normal epithelial cells of renal tubules, papillary renal cell carcinoma, and clear cell renal cell carcinoma with and without *VHL* mutations.

alterations affecting cilia stabilization and maintenance have a minor role in the majority of pRCC. This is supported by the fact that cytogenetic differences between pRCC and ccRCC are strikingly different.[19,20] In contrast to ccRCC, 3p loss[19] and *VHL* mutations[14] are rare in pRCC. Thus, the association between *VHL* inactivation and loss of cilia strongly argues for a characteristic feature of ccRCC and not of pRCC.

Besides mutations, additional molecular mechanisms may contribute to pVHL inactivation and consequently to HIFα stabilization in ccRCC carrying wild-type *VHL* alleles. Hypermethylation of the *VHL* promotor may lead to gene silencing and occurs in about 20% of ccRCCs.[8,21] As the *VHL* methylation status was not analyzed in our samples, possible hypermethylation in some ccRCCs with wild-type *VHL* cannot be excluded. E2-EPF ubiquitin carrier protein (UCP) associates with and targets pVHL for ubiquitin-mediated proteolysis in tumor cells.[22] High expression of UCP supports HIF-1α stabilization in human liver, colon, and breast tumors. Whether UCP expression influences the VHL–HIF pathway in ccRCC still remains to be elucidated.

No *VHL* mutations were found in our pRCC samples, suggesting that other, VHL-independent, pathways contribute to the development of this tumor subtype. In contrast to ccRCC, in most of the pRCCs, the decrease of primary cilia was relatively mildly that supports pVHL's important role in maintaining the primary cilia structure in this tumor subtype. Interestingly, the pRCC with the highest cilia frequency showed moderate CA9 and weak GLUT-1 positivity. Hypermethylation of the *VHL* promoter or transcription factor(s) other than HIF might be responsible for the activation of the two genes in this tumor. It is of note that in another pRCC, although being CA9 and GLUT-1 negative, cilia numbers were similar to those seen in ccRCC. As *VHL* was not affected in this tumor mutations or deregulation of another, yet unknown, genetic factor might lead to the disruption of primary cilia.

A number of proteins localized to the primary cilia are associated with various hereditary human cystic kidney disorders. These include the autosomal dominant and recessive forms of polycystic kidney disease and several other cystic kidney diseases, such as nephrophthisis, oral–facial–digital syndrome type 1, and Bardet–Biedl syndrome.[23] As primary cilia harbor pVHL[7] and renal cysts are present in about 60% of VHL patients,[24] this syndrome highly likely represents a ciliopathy. In fact, compared to normal tubular cells, pVHL-defective epithelial cells lining cysts of VHL patients display a reduced frequency of cilia.[7] Kidneys isolated from VHL patients typically contain hundreds of *VHL* mutant and, consequently, HIFα activated preneoplastic lesions, the majority of which are single cells.[7,16] It appears that only a few of these single cell lesions progress further to cysts and about half of them (21–45%)[24] are destined to form tumors. This observation suggests that loss of pVHL is necessary but not sufficient for promoting cyst formation in the setting of VHL disease. Recently it has been shown that in renal cysts, glycogen synthase kinase (GSK)3β that regulates pVHL-mediated microtubule stabilization by phosphorylation,[25] is subjected to inhibitory phosphorylation through the PI(3)K signaling pathway. Importantly, these cysts exhibit reduced frequencies of primary cilia.[7] Thus, in VHL patients combined *VHL* mutation and GSK3β inactivation is required to disrupt primary cilia, which in turn cause renal cyst formation and possibly ccRCC.

Patients with sporadic ccRCC also frequently have intratumoral cysts.[26] Interestingly, 5 of 20 (25%) of our ccRCCs displayed detectable phospho-serine 9-GSK3β (data not shown). Therefore, our findings suggest that cilia degeneration driven by VHL dysfunction and GSK3β inactivation is also an important mechanism of renal carcinogenesis in a significant fraction of non-VHL patients. It also indicates that in a larger subset of sporadic ccRCC, the inactivation of proteins other than GSK3β is required to destabilize primary cilia.

The reduction in primary ciliation could be the result, rather than the cause of the renal carcinogenesis and therefore be reflective of its morphology. For example, pRCC typically has papillary or tubular structures that are lined with tumor cells with apical cell membrane where cilia are found. In contrast, ccRCC is frequently composed of nests or solid sheets of tumor cells with less apical cell membranes. We have therefore looked at whether the number of cilia correlates with the architectural patterns (solid/nested, alveolar/acinar, and papillary). Importantly, a majority of ccRCC showed an acinar or alveolar pattern with apical cell membranes. There was no correlation between the number of cilia and the architectural pattern. We conclude that cilia degeneration is definitely driven by pVHL dysfunction and is not just a reflection of morphology.

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In conclusion, this is the first study demonstrating that the frequencies of primary cilia differ significantly between sporadic clear cell and papillary subtypes of renal cell carcinoma *in vivo*. Low numbers of ciliated tumor cells correlated with the upregulation of HIF targets *CA9* and *GLUT-1* in ccRCC. In contrast, high cilia frequencies were found in the papillary subtype in which functioning pVHL exists. Our results suggest that loss of primary cilia may contribute to the development of sporadic ccRCC that is characterized by loss of pVHL function. In contrast, pRCC can progress without dismantling their primary cilia. This difference may explain at least in part the different biological behavior and phenotype of ccRCC and pRCC.

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Disclosure/conflict of interest

The authors declare that there is no conflict of interest.

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