VHL mutations and dysregulation of pVHL- and PTEN-controlled pathways in multilocular cystic renal cell carcinoma

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Multilocular cystic renal cell carcinoma is a rare renal cell carcinoma with an excellent prognosis. To clarify the relationship with typical clear cell renal cell carcinoma, we evaluated 15 cases of multilocular cystic renal cell carcinomas diagnosed according to the 2004 WHO classification. Von Hippel Lindau (*VHL*) gene mutations were determined by whole genome amplification and direct sequencing. Carbonic anhydrase 9 (CAIX), a hypoxia-inducible factor (HIF) target, paired box gene 2 (PAX2), cyclin-dependent kinase inhibitor p27 and glycogen synthase kinase 3-β (GSK3β) were immunohistochemically evaluated as members of the VHL protein (pVHL)- and phosphatase and tensin homolog (PTEN)-controlled pathways. *VHL* mutations were identified in 3 of 12 (25%) tumors. Inactivated GSK3β, decreased PTEN expression and PAX2 positivity were observed in the vast majority of the multilocular cystic renal cell carcinomas. Strong nuclear staining of p27 was seen in 14 of 15 cases. Compared with multilocular cystic renal cell carcinomas, expression frequencies of PAX2, p-GSK3β, PTEN and CAIX were similar in a set of low-grade, early-stage clear cell renal cell carcinomas, whereas only 30% had strong p27 positivity. These results are consistent with the hypothesis that multilocular cystic renal cell carcinomas are related at the molecular level with clear cell renal cell carcinomas. Maintenance of a strong subcellular p27 expression in all multilocular cystic renal cell carcinomas analyzed may in part explain the excellent prognosis of these tumor patients.

Multilocular cystic renal cell carcinoma is a rare renal carcinoma type showing characteristic cystic growth. It constitutes <2% of all renal cell carcinomas. According to the 2004 World Health Organization (WHO) classification of tumors, it is defined by morphologic criteria as a specific tumor type. Patients diagnosed with this tumor have an excellent outcome, as there are no reports of this tumor ever recurring or metastasizing to date.[1] Multilocular cystic renal cell carcinoma is potentially a subtype of clear cell renal cell carcinoma because the cysts are lined by single layers of clear cell epithelial cells. Recently, Halat *et al*[2] reported a high frequency of chromosomal loss of 3p similar to the one observed in clear cell renal cell carcinoma. As a chromosome 3p deletion is considered the hallmark of cytogenetic abnormality found in clear cell renal cell carcinoma, it was concluded that multilocular cystic renal cell carcinoma is a subtype of this tumor entity. However, chromosomal loss of the 3p arm has also been reported in some non-clear cell renal cell carcinomas and is a frequent finding in cancers of the lung, cervix, head and neck, esophagus and pancreas (http://www.progenetix.net/progenetix/). Importantly, tumor development in sporadic clear cell renal cancer is linked with inactivation of the von Hippel Lindau (*VHL*) gene by chromosome 3p loss in combination with mutation, or hypermethylation of the second *VHL* allele on the 3p arm. In multilocular cystic renal cell carcinomas, no data exist about the presence of *VHL* mutations, representing an additional hallmark of clear cell renal cell carcinoma.

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Alteration of pathways regulated by the von Hippel-Lindau protein (pVHL) due to mutation of its coding gene is the main characteristic molecular feature of all hereditary and most sporadic clear cell renal cell carcinomas. pVHL is a multifunctional protein that acts as an adapter for different molecular and subcellular complexes.[3] Recently, we comprehensively analyzed pVHL- and phosphatase and tensin homolog (PTEN)-controlled pathways in >800 clear cell renal cell carcinoma patients.[4] It was shown that the expression status of p27, PTEN, carbonic anhydrase 9 (CAIX) and phosphorylated ribosomal protein S6 is crucial for the biological behavior of sporadic clear cell renal cell carcinoma.

In this study, we sequenced *VHL* and investigated the expression status of PTEN, phosphorylated (inactivated) glycogen synthase kinase 3-β (GSK3β) and paired box gene 2 (PAX2), all of which represent critical components for cyst formation[5–8] as well as of the tumor suppressor p27 and the hypoxia-inducible factor (HIF)-target CAIX in 15 multilocular cystic renal cell carcinomas. The results were compared with a control group of early-stage, low-grade clear cell renal cell carcinomas.

Materials and methods

Tumor Specimens

HE sections of 15 formalin-fixed and paraffin-embedded multilocular cystic renal cell carcinomas were reviewed by two pathologists (EC and HM). Areas with nodular aggregates containing clear cells in the cyst walls are shown in Figure 1a. All tumors were diagnosed with the Fuhrman differentiation grade 1. The control group consisted of 40 pT1 and pT2 clear cell renal cell carcinomas with low Fuhrman differentiation grade (1 and 2) from a recently published study.[4]

DNA Extraction and Whole Genome Amplification

For DNA extraction the marked HE slides were used to punch three tissue cylinders (diameter 0.6 mm) from each of the 15 paraffin blocks. Punched areas of one multilocular cystic renal cell carcinoma case are shown in Figure 1b. DNA was extracted using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Whole genome amplification of the yielded DNA was performed using the WGA kit (Sigma, St Louis, MO, USA). The quality of the amplified DNA was determined on a 1.5% agarose gel. DNA quantity was measured with the Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA).

VHL Sequencing

PCR and sequencing of *VHL* were performed as previously described,[9] with slight modifications. Only one PCR step with 40 cycles was carried out per exon. Exons 2 and 3 were amplified using forward primer 5′-ACCGGTGTGGCTCTTTAACA-3′, reverse primer 5′-TCCTGTACTTACCACAACAACC TT-3′ and forward primer 5′-GAGACCCTAGTCTGT CACTGAGG-3′, reverse primer 5′-TCATCAGTAC CATCAAAAGCTGA-3′, respectively. The obtained sequences were compared with the NCBI sequence AF010238 using NCBI's Blast 2 Sequences. *VHL* point mutations were validated by a second separate whole genome amplification and sequence analysis.

Immunohistochemistry

Paraffin sections (2.5 μm) were transferred to glass slides and treated using Ventana Benchmark XT (Tuscon, AZ, USA) or BOND-MAX (Leica Microsystems, Wetzlar, Germany) automated systems. Immunostainings were performed using mouse anti-CAIX (ab15086, 1:300 dilution; Abcam, Cambridge, UK), rabbit anti-phospho-Ser9 GSK3β (no. 9323, clone 5B3, 1:100 dilution; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-p27 (sc528, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-PTEN (M3627, 1:200 dilution; DAKO A/S, Glostrup, Denmark) and rabbit anti-PAX2 (71-6000, 1:100 dilution; Zymed Laboratories, South San Francisco, CA, USA). Nuclear (PAX2), nuclear and cytoplasmic (PTEN, p27), cytoplasmic (phospho-Ser 9 GSK3β) and membranous expression (CAIX) were defined positive if at least 5% of tumor cells showed weak (+1) or strong (+2) staining.

Statistical Analysis

Contingency table analysis and χ^2 tests were used for the analysis of statistical differences between protein expression patterns in multilocular cystic renal cell carcinomas and low-grade, early-stage clear cell renal cell carcinomas.

Results

Whole Genome Amplification

The yield of DNA after extraction from paraffin-embedded tissue varied between 0.22 and 2.18 μg. The starting amount of DNA for whole genome amplification was 75 ng. Figure 2 shows an example

Figure 1 HE section of a multilocular cystic renal cell carcinoma showing cysts and thin septae (**a**, middle panel). Septae lined by cuboid clear cells without a solid component (**a**, upper and lower panels). HE section of the same multilocular cystic renal cell carcinoma after punching (**b**, middle panel). Circular punched areas are highlighted by square boxes (overview in middle panel) and magnifications of two punched areas are shown (**b**, upper and lower panels). Sections were scanned using the NanoZoomer (zoom factor: ×40; Hamamatsu Photonics K.K., Hamamatsu City, Japan).
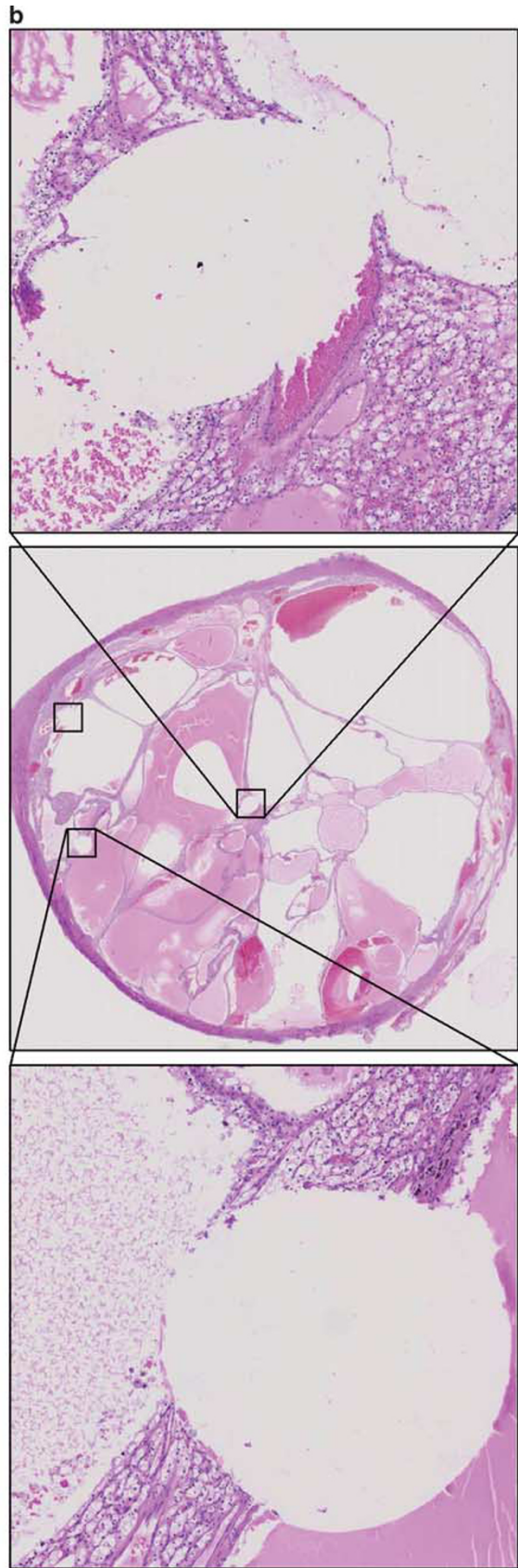
of four whole genome amplified samples on an agarose gel for quality control. The amounts of DNA after amplification and purification ranged between 0.87 and 8.68 μg, which is a 12- to 121-fold level of amplification.
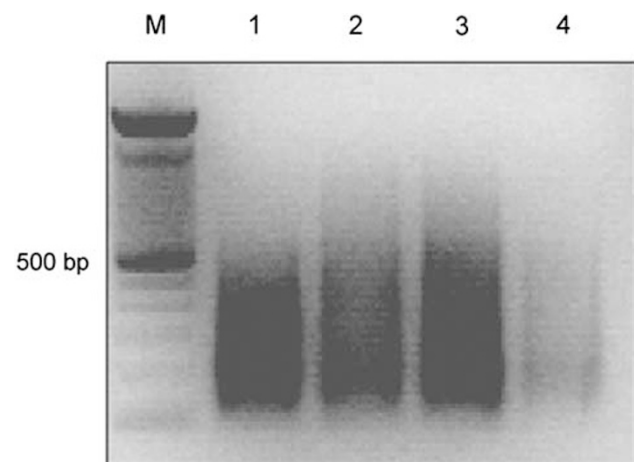
VHL Mutation Analysis

Although the starting amount of DNA for the amplification was the same for all 15 samples, the quality and quantity of the PCR products of three samples were not sufficient for *VHL* sequence analysis. *VHL* mutations were identified in 3 of 12 (25%) analyzable multilocular cystic renal cell carcinoma patients. Two patients had missense

Figure 2 Agarose gel showing whole genome amplified DNA extracted from four multilocular cystic renal cell carcinomas. M: 100 bp ladder.

mutations in exon 1 (c.194C > T/ p.Ser65Leu) and in exon 2 (c.461C > T/p.Pro154Leu), respectively. Two frameshift mutations were identified in a third patient, one in exon 1 (c.201_203delCTCinsG/ p.Asn67LysfsX64) and the second in exon 3 (c.641_642delGA/p.XfsX).

Immunohistochemistry

Strong expression of CAIX and p27 was seen in the vast majority of the multilocular cystic renal cell carcinomas. In all, 80% of the tumors were PAX2 positive. In comparison with normal tubular epithelial cells in which PTEN was highly expressed and p-GSK3β was absent (data not shown), decreased PTEN expression and inactivated GSK3β was observed in 14 of 15 cases. The results of the immunohistochemical analysis are listed in Table 1. Examples of four multilocular cystic renal cell carcinomas with strong nuclear PAX2, cytoplasmic p-GSK3β, combined nuclear/cytoplasmic p27 and membranous CAIX positivity are shown in Figure 3. Besides p27 (nuclear and cytoplasmic) and PTEN (nuclear), no significant differences in CAIX, p-GSK3β, PAX2 and cytoplasmic PTEN expression frequencies were observed between multilocular cystic renal cell carcinomas and early-stage, low-grade clear cell renal cell carcinoma. The results are listed in Table 2.

Discussion

Our data demonstrate the existence of *VHL* mutations in multilocular cystic renal cell carcinoma. Furthermore, the downregulation of PTEN, the

Table 1 Expression of CAIX, p27, PTEN, PAX2, p-GSK3β and *VHL* mutation status in 15 multilocular cystic renal cell carcinomas

Tumor	CAIX	p27 (nuc)	p27(cyt)	PTEN (nuc)	PTEN (cyt)	PAX2	p-GSK3β	VHL
1	Negative	Strong	Strong	Negative	Strong	Negative	Strong	Wt
2	Strong	Strong	Weak	Weak	negative	Strong	Weak	Wt
3	Strong	Strong	Weak	Negative	Negative	Weak	Weak	Wt
4	Strong	Strong	Strong	Negative	Negative	Negative	Weak	Mutated
5	Negative	Strong	Strong	Weak	Weak	Negative	Weak	Wt
6	Strong	Strong	Weak	Negative	Weak	Strong	Negative	Wt
7	Strong	Strong	Weak	Weak	Weak	Strong	Weak	Wt
8	Strong	Strong	Strong	Negative	Weak	Strong	Weak	Wt
9	Strong	Strong	Strong	Negative	Weak	Weak	Weak	Mutated
10	Strong	Strong	Strong	Negative	Negative	Strong	Negative	Wt
11	Strong	Strong	Strong	Negative	Weak	Weak	Weak	Wt
12	Strong	Strong	Strong	Negative	Weak	Strong	Weak	Mutated
13	Strong	Strong	Weak	Negative	Weak	Weak	Weak	Wt
14	Strong	Weak	Weak	Weak	Weak	Strong	Weak	Wt
15	Strong	Strong	Strong	Weak	Weak	Weak	Weak	Wt
Staining intensity	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	
Negative	2 (13)	0	0	10 (67)	4 (27)	3 (20)	2 (13)	
Weak	0	1 (7)	6 (40)	5 (33)	10 (67)	5 (33)	12 (80)	
Strong	13 (87)	14 (93)	9 (60)	0	1 (7)	7 (47)	1 (7)	

cyt, cytoplasmic; nuc, nuclear; Wt, wild type.
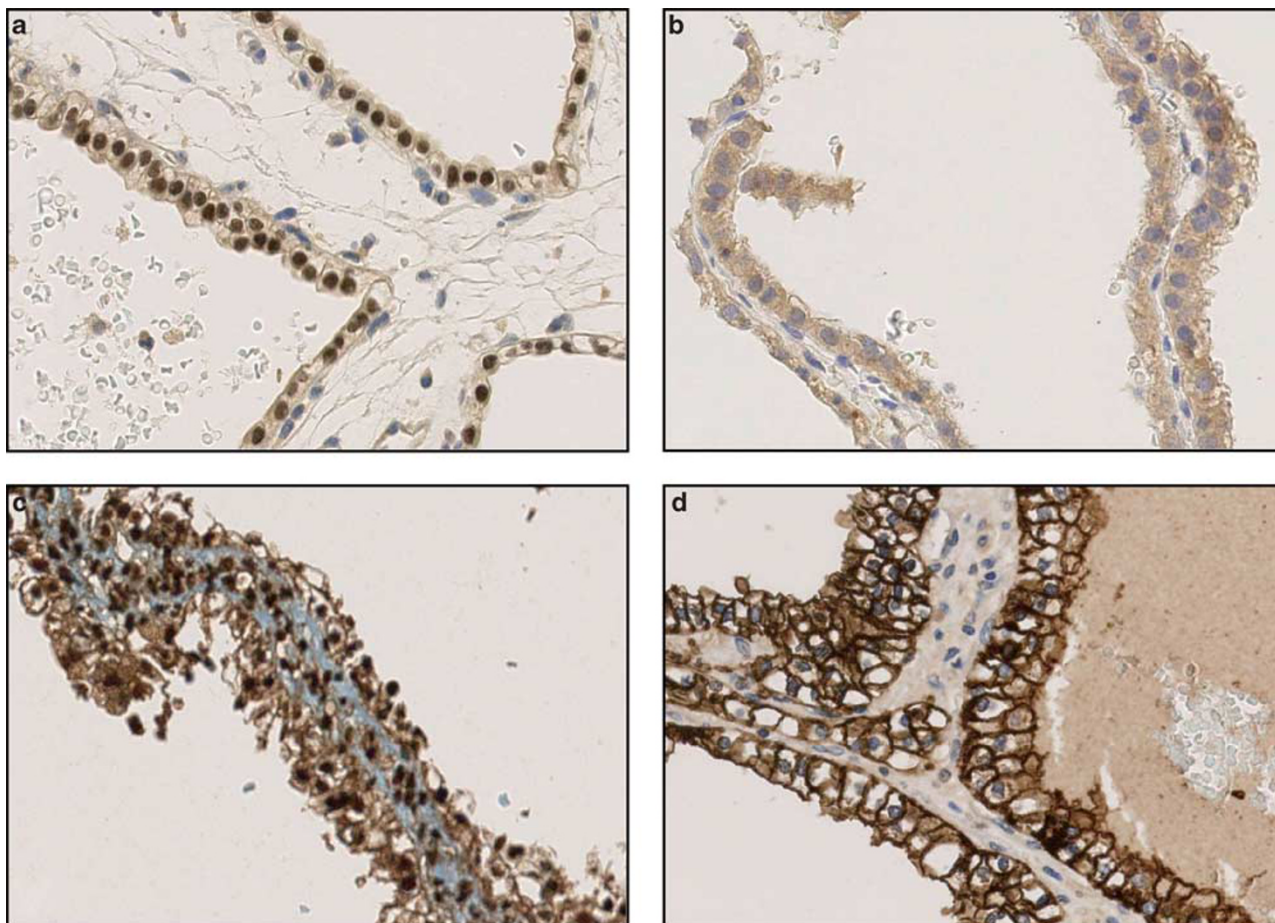
Figure 3 Scanned examples of immunohistochemically stained multilocular cystic renal cell carcinoma. Nuclear PAX2 (**a**), cytoplasmic p-GSK3β (**b**), combined nuclear and cytoplasmic p27 (**c**) and membranous CAIX (**d**).

inactivation of GSK3β and the upregulation of PAX2 were identified as indicators for the dysregulation of the pVHL and PTEN-controlled pathways. With the exception of p27, the expression frequencies of the remaining proteins were comparable between multilocular cystic renal cell carcinoma and clear cell renal cell carcinoma and confirm the close relationship.

Cystic lesions are found not only in multilocular cystic renal cell carcinoma but also in cystic nephroma, extensively cystic clear cell renal cell carcinoma, tubulocystic carcinoma, mixed epithelial and stroma tumor,[10] clear cell papillary renal cell carcinoma[11] and tubulocystic carcinoma.[12] Cystic nephroma, mixed epithelial and stroma tumor as well as clear cell papillary renal cell carcinoma also have clear cells lining the septum. Given the broad range of tumors with clear cells in the kidney, the histogenetic relationship of multilocular clear cell renal cell carcinoma to one of the cystic renal tumor entities with clear cells requires molecular analyses. Little is known about the molecular genetic characteristics of multilocular cystic renal cell carcinoma. Halat *et al*[2] recently identified chromosome 3p deletions in 14 of 19

(74%) multilocular cystic renal cell carcinomas. Loss of the chromosome 3p is a consistent finding in clear cell renal cell carcinoma and is considered one of the primary events in the pathogenesis of this tumor type.[1] Inactivation of *VHL* is mostly because of mutation of the second *VHL* allele, located at chromosome 3p25, and is specific for sporadic clear cell renal cell carcinoma and for tumors associated with the von Hippel Lindau syndrome. *VHL* mutations have never been described in multilocular cystic renal cell carcinoma.

In this study, we performed direct sequencing of *VHL* for mutations on samples from multilocular cystic renal cell carcinoma, and *VHL* mutations were identified in three cases. We used a whole genome amplification step, because the yield of DNA extracted from the tumor cells in the cyst walls was frequently insufficient. The frequency of validated *VHL* mutations was 25%, which is somewhat below the mutation rates reported in clear cell renal cell carcinoma (reviewed in Gossage *et al*[13]). The two missense mutations, c.194C>T and c.461C>T, have been described in both sporadic and hereditary clear cell renal cell carcinomas.[14,15] Possible effects of the first point mutation on pVHL binding partners

Table 2 Expression of CAIX, p27, PTEN, PAX2 and p-GSK3β in multilocular cystic and clear cell renal cell carcinomas (low-grade, early-stage)

	MCRCC N (%)	CCRCC N (%)	P-*value*
CAIX			
Negative	2 (13)	3 (7.5)	
Weak	0	2 (5)	NS
Strong	13 (87)	35 (87.5)	
p27 (nuc)			
Negative	0	2 (5)	
Weak	1 (7)	25 (62.5)	0.0003
Strong	14 (93)	13 (32.5)	
p27 (cyt)			
Negative	0	9 (22.5)	
Weak	6 (40)	20 (50)	<0.05
Strong	9 (60)	11 (27.5)	
PTEN (nuc)			
Negative	10 (67)	7 (17.5)	
Weak	5 (33)	26 (65)	0.014
Strong	0	7 (17.5)	
PTEN (cyt)			
Negative	4 (27)	8 (20)	
Weak	10 (67)	28 (70)	NS
Strong	1 (7)	4 (10)	
PAX2			
Negative	3 (20)	8 (20)	
Weak	5 (33)	11 (27.5)	NS
Strong	7 (47)	21 (52.5)	
p-GSK3β			
Negative	2 (13)	5 (12.5)	
Weak	12 (80)	30 (75)	NS
Strong	1 (7)	5 (12.5)	

cyt, cytoplasmic; NS, not significant; nuc, nuclear.

are unknown; however, on the second mutation, Hansen *et al*[16] showed that exon 2 mutants may exhibit an impaired degradation of HIF-α as these pVHL mutants display defects in substrate binding. The two frameshift mutations found in one patient were validated by two separate whole genome amplifications. In several studies, clear cell renal cell carcinoma cases with two or more *VHL* mutations have been described.[17–19] It is conceivable that in this tumor both parental *VHL* alleles were affected, whereas chromosome 3p was retained.

Amplifying whole genome DNA extracted from only a few thousand micro-dissected and formalin-fixed tumor cells is accompanied by the high risk of producing artificial mutations.[20,21] As a consequence, several whole tissue sections would be required for a micro-dissection to yield sufficient amounts of DNA to reliably perform a PCR. To decrease the risk of PCR artifacts and to avoid wasting of valuable multilocular cystic renal cell carcinoma tissue, we punched directly in the cyst walls using a hollow needle with a diameter of 0.6 mm. We cannot exclude that some of the tissue biopsies were contaminated with non-tumor cells,

thus preventing a higher *VHL* mutation rate more similar to that observed in clear cell renal cell carcinoma. This is supported by our observation that 13 of 15 multilocular cystic renal cell carcinoma showed a strong expression of CAIX, a HIF target that is considerably upregulated because of loss of the pVHL function.[22] The existence of *VHL* mutations is a clear indicator that multilocular cystic renal cell carcinoma is a subtype of clear cell renal cell carcinoma.

Loss of the pVHL function has an important role in the initiation of clear cell renal cell carcinoma. However, additional signaling pathways are required for further tumor progression. It is unclear why multilocular cystic renal cell carcinoma is associated with such an excellent prognosis. Recently, novel mechanisms of tumor initiation have been identified in clear cell renal cell carcinoma because of the hereditary von Hippel-Lindau syndrome. A model for a cyst-dependent and a cyst-independent pathway was proposed[23] and extended to sporadic clear cell renal cell carcinoma. The disruption of the maintenance of the primary cilia in the renal tubules due to combined inactivation of pVHL and GSK3β is an important step for the cyst-dependent progression pathway.[7] Recent findings strongly suggest that additional deregulating factors are required to develop cysts. In mice, combined deletion of the two tumor-suppressor genes, *VHL* and *PTEN*, caused benign genital tract tumors with regions of cyst adenoma that were histologically identical to lesions found in VHL patients.[24] pVHL and PTEN also cooperatively suppress kidney cyst formation.[5] Combined conditional inactivation of *VHL* and *PTEN* in the mouse kidney elicits cyst formation. Interestingly, in two-thirds of our multilocular cystic renal cell carcinomas, nuclear staining of PTEN was absent, whereas only 17.5% of the low-grade, early-stage clear cell renal cell carcinomas were PTEN negative. These data suggest the dysregulation of pVHL, GSK3β and, probably, nuclear import of PTEN as common molecular mechanisms that are important to produce the phenotype characteristic for multilocular cystic renal cell carcinoma.

It is important to note that the risk of metastasis is proportional to the number of carcinoma cells present. Multilocular cystic renal cell carcinomas contain rather small numbers of carcinoma cells (most of their volumes are spaces filled with fluid), whereas clear cell renal cell carcinomas are composed of significantly more tumor cells.

On the molecular level, we have previously demonstrated that the expression status of p27, PTEN, CAIX, phospho-S6 as well as PAX2 characterizes a subgroup of sporadic clear cell renal cell carcinoma with a better prognosis.[4] In contrast to multilocular cystic renal cell carcinoma patients, who have an extremely good prognosis after surgery,[25] a substantial fraction of clear cell renal cell carcinoma patients die because of metastases.

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This implies that pathways, including those mediated by EGFR,[26,27] with the tumor-progressing potential of clear cell renal cell carcinoma are kept under control in multilocular cystic renal cell carcinoma. One of these pathways appears to be regulated by p27, as almost all multilocular cystic renal cell carcinomas expressed this tumor suppressor in both the nucleus and the cytoplasm. The dominant presence of p27 in multilocular cystic renal cell carcinoma indicates a negatively controlling role on pathways that are important in different malignant tumors in which the antagonists of p27, such as AKT,[28] SKP2[29] and SRC,[30] are highly active. The abundant expression of p27 in multilocular cystic renal cell carcinoma may partly explain the high rate of surgical cure of these patients. A recent study that demonstrated nuclear and cytoplasmic p27 expression primarily in low-grade and early-stage clear cell renal cell carcinomas supports this hypothesis.[4]

It was shown that clear cell renal cell carcinomas with strong expression of CAIX have a favorable outcome.[4,31] Furthermore, Patard *et al*[32] was able to divide patients with clear cell renal cell carcinoma into three prognostic groups. Patients with a poor prognosis had no *VHL* mutations and low CAIX levels, whereas, those with either a *VHL* mutation or high CAIX expression had an intermediate prognosis. Finally, patients who had *VHL* mutations and a high CAIX expression had a good prognosis. It is therefore tempting to speculate that the latter group of clear cell renal cell carcinoma is molecularly closely related to multilocular cystic renal cell carcinoma.

Nuclear positivity of PAX2 was seen in the majority of the multilocular cystic renal cell carcinoma specimens. PAX2 is a member of the PAX gene family of developmental transcription factors and is known to be involved in cyst formation. Patients with polycystic kidney disease and juvenile cystic kidneys abundantly express PAX2 in cystic and hyperproliferative epithelial cells.[6,8] In addition, it was shown that a reduced *PAX2* gene dosage significantly inhibited renal cyst growth in mice.[33] In renal cancer cell lines, loss of the pVHL function leads to a strong reactivation of the *PAX2* gene[34] that is normally silenced in the mature epithelium of the glomeruli, the proximal and distal tubules of the kidney. Furthermore, in clear cell renal cell carcinoma, PAX2 expression mainly occurs in low-grade tumors and significantly correlates with a better outcome.[4,34] These data suggest that PAX2 is a pVHL-dependent factor that contributes to cyst formation in multilocular cystic renal cell carcinoma.

In summary, the results of our study suggest that cyst formation in multilocular cystic renal cell carcinoma is caused by molecular mechanisms similar or even identical to those seen in *VHL*-associated clear cell renal cell carcinoma. As PAX2, p27 as well as pVHL-dependent CAIX expression correlate with low differentiation grade and/or a favorable outcome in clear cell renal cell carcinoma, these proteins may represent potential markers of the benign behavior of multilocular cystic renal cell carcinoma. The combined deregulation of the pVHL/HIF axis and PTEN alone appear to be important for multilocular cystic renal cell carcinoma development but not sufficient to drive metastatic processes. The abundance of p27 is a characteristic feature of multilocular cystic renal cell carcinoma and potentially implies that this protein acts as a metastasis suppressor. Our observations, together with the recently published finding of frequent chromosome 3p deletions,[2] further support the concept that multilocular cystic renal cell carcinoma is a subtype of clear cell renal cell carcinoma. The term 'multilocular cystic clear cell renal cell carcinoma' would better reflect its relationship with clear cell renal cell carcinoma.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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