The role of von Hippel-Lindau tumor suppressor protein and hypoxia in renal clear cell carcinoma

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Sufan, Roxana I., Michael A. S. Jewett, and Michael Ohh. The role of von Hippel-Lindau tumor suppressor protein and hypoxia in renal clear cell carcinoma. Am J Physiol Renal Physiol 287: F1–F6, 2004; 10.1152/ajprenal.00424.2003.—The majority of kidney cancers are caused by the mutation of the von Hippel-Lindau (VHL) tumor suppressor gene. VHL protein (pVHL) is part of an E3 ubiquitin ligase complex called VEC that is composed of elongin B, elongin C, cullin 2, NEDD8, and Rbx1. VEC targets a hypoxia-inducible factor (HIF) transcription factor for ubiquitin-mediated destruction selectively in the presence of oxygen. In the absence of wild-type pVHL, as in VHL patients or in the majority of sporadic clear cell renal cell carcinomas, HIF-responsive genes are inappropriately activated even under normoxia. Recent insights into the molecular mechanisms regulating the function of pVHL, and thereby HIF, in the context of kidney cancer are the focus of this review.

hypoxia-inducible factor; renal cell carcinoma; E3 ubiquitin ligase

KIDNEY CANCER OF ALL TYPES is estimated to affect 32,000 Americans in 2004, with 12,000 deaths. Epithelial tumors comprise the majority of these cancers which are predominantly renal cell carcinomas (RCCs) (30). The extent or clinical stage of cancer at the time of diagnosis strongly influences survival. Tumors <4 cm in diameter are treated surgically by partial or complete (radical) nephrectomy (removal of the kidney) by either open surgery or less invasive techniques, including laparoscopic surgery (15). More than 90% of these patients with stage I disease survive 5 yr. The natural history of untreated small renal cancers is unknown, but some appear to grow very slowly (80). Less invasive therapies including radiofrequency ablation and cryotherapy are being employed (64). The survival of patients whose cancers have spread (metastatic) is <20% at 5 yr despite increasing knowledge of systemic therapy particularly immunotherapy (19).

Approximately 75% of RCCs are histologically of the clear cell type, 15% papillary (both types I and II), 5% chromophobe, with the remaining 5% consisting of a variety of tumors including oncocytoma (45). While the vast majority (96%) of these are sporadic or noninherited, there is a small proportion (4%) that are hereditary and, from these, the genetic basis and molecular pathways involved in the development of RCC are beginning to be defined (45). Hereditary kidney cancer can be subdivided into the following categories: type 1 hereditary papillary renal carcinoma, type 2 hereditary papillary renal carcinoma with liemymatoses RCC, Birt-Hogg-Dubé syndrome with chromophobe RCC and oncocytoma, and clear cell-RCC associated with the von Hippel-Lindau (VHL) disease (45). It is increasingly apparent that the survival as well as response to therapy differ among subtypes. Treatment planning may be determined by the histology of the primary tumor. The knowledge gained from the studies into the molecular basis of these kidney cancers may help to identify targets for therapy that will improve survival and possibly reduce morbidity even for localized tumors. In recent years, there have been major advances from the molecular study of VHL disease, which is the focus of this review.

VHL DISEASE

VHL disease (OMIM 193300) affects 1 in 36,000 individuals and is characterized by the presence of hypervascular tumors in multiple organs including the central nervous system (CNS; cerebellum and spinal cord), retina, pancreas, adrenal gland, endolymphatic sac of the inner ear, epididymus (male), broad ligament (female), and kidney (55). The kidney cancer in VHL patients is of the clear cell type, which is the most common form of kidney cancer and is the major cause of morbidity and mortality (55).

The inheritance of a defective copy of the VHL gene predisposes the individual to develop VHL disease. The tumors arise in VHL kindred when the remaining wild-type allele is mutated during their lifetime. Thus on the cellular level, VHL disease has an autosomal recessive pattern of inheritance. However, clinically, it is perceived as an autosomal dominant disease, because the likelihood of the second inactivating mutation to occur on the wild-type allele is virtually guaranteed (49). This loss or inactivation of the remaining wild-type VHL allele in susceptible renal tubular epithelial cells has been documented in early premalignant renal cysts in VHL patients (48). It is thought that the development of clear cell RCC arising from the renal cysts is due to mutations in other genetic loci. Thus VHL inactivation has been established as an early
and requisite step in RCC pathogenesis, and, in this regard, VHL is thought to play a “gatekeeper” role. In keeping with Knudson’s two-hit model, biallelic inactivation of the VHL gene is also observed in the majority of sporadic renal clear cell carcinomas and cerebellar hemangioblastomas (49).

**VHL GENE**

Several lines of investigation have pointed to the presence of a renal carcinoma suppressor gene on the short arm of chromosome 3. First, it was noted that deletions of chromosome 3p are common in a variety of solid tumors, including renal carcinomas (36, 87). Furthermore, germline translocations of chromosome 3p to chromosomes 6, 8, or 11 were reported in kindreds with early-onset, bilateral, and multifocal clear cell RCCs (8, 35, 61). In an effort to locate the gene on chromosome 3p responsible for renal carcinoma, investigators studied the hereditary renal carcinoma associated with VHL disease, assuming that inactivation of the same gene causes sporadic renal carcinoma. Using genetic linkage analysis, Seizinger and colleagues (68) mapped the VHL gene to a 6- to 8-cM region of chromosome 3p25–26. The putative VHL locus was then narrowed to a 4-cM interval by multipoint linkage analysis, and the minimal genomic region commonly deleted among unrelated VHL kindreds was mapped by pulse-field gel electrophoresis to a more precise region within the 4-cM interval (66, 84). Based on this information, Latif and co-workers (41) cloned the VHL gene in 1993.

The VHL gene consists of three exons, and its promoter contains three positive regulatory regions. An Sp1 binding site (+1 to +11) significantly contributes to the transcriptional activation of VHL (86). The other two upstream positive regions (−49 to −19 and −114 to −91) interact with yet unidentified transcription factors (86). The VHL locus produces two transcripts that are translated into three proteins. The first VHL mRNA of ~4.5 kb contains exons 1–3 and is translated into two proteins due to an internal translational initiation start site at codon 54 (3, 26, 67). The larger product is a 213-amino acid protein of ~24–30 kDa (pVHL30), and the shorter is a 18- to 19-kDa isoform (pVHL19) of 160 amino acids. The second VHL mRNA contains exons 1 and 3 due to alternative splicing. Tumors that exclusively produce this exon 2-less transcript have been identified, which suggests that the protein product encoded by this alternatively spliced transcript is defective in tumor suppressor activity (16). Furthermore, VHL mRNA expression is ubiquitous and thus is not restricted to specific tissue types that have been associated with VHL disease (32, 65). In addition, the pVHL expression pattern in the fetal kidney suggests a role in normal renal tubular development and differentiation (32, 65).

**VHL CLASSIFICATION**

VHL disease has been classified into subcategories, depending on the patients’ likelihood of developing pheochromocytoma. Type 1 patients have a low risk of developing pheochromocytoma, but present with clear cell RCC. Type 2 patients have a high risk of developing pheochromocytoma, with type 2A patients having an additional low risk of developing clear cell RCC, whereas type 2B patients possess a high risk of clear cell RCC. Type 1, type 2A, and type 2B patients also develop the two cardinal features of VHL disease, namely, cerebellar and retinal hemangioblastomas. Type 2C patients only develop pheochromocytoma (49). Typically, the mutations associated with type 1 disease are deletions, microinsertions, and nonsense mutations, whereas type 2 patients often present with missense mutations (5, 88).

**pVHL PROTEIN**

The human VHL gene is translated into two wild-type VHL proteins: pVHL30 and the internally translated pVHL19 (3, 26, 67). Reconstitution of RCC cells with either pVHL30 or pVHL19 suppressed tumor development in a nude mouse xenograft assay (3, 17, 25, 67). Therefore, the functional significance of the NH2-terminal acidic domain of pVHL30 is currently unknown. However, VHL mutations associated with tumor development have been identified throughout the open reading frame, including the P25L mutation within the first 54 amino acids (10, 79, 88). The P25L mutation has been identified in patients with sporadic pheochromocytoma (79). This suggests that pVHL30 and pVHL19 do not have identical, overlapping roles in tumor suppression. Furthermore, pVHL30 and pVHL19 were shown to have differential subcellular localization profiles. While pVHL30 is found in the nuclear, cytosolic, and membrane [associated with endoplasmic reticulum (ER)] fractions, pVHL19 is found only in the nuclear and cytosolic fractions (9, 26, 47, 58). Although the functional significance of pVHL19 associating with ER is unclear, it may be related to the ability of pVHL30, but not pVHL19, to bind fibronectin and its requirement to promote the assembly of extracellular fibronectin matrix (13, 58).

**pVHL MULTIPROTEIN COMPLEX**

The VHL nucleotide sequence or the pVHL amino acid sequence was not homologous to proteins of known function; therefore, pVHL-associated proteins were sought with the supposition that these associated proteins might have known or identifiable functions. It is now known that pVHL is in a complex with elongin B, elongin C, cullin (Cul) 2, Rbx1 (also known as ROC1/Hrt1), and NEDD8, forming an E3 ubiquitin ligase complex called VEC (42, 55). Elongin C bridges pVHL to Cul2 (1, 75), Cul2 associates with elongin C, NEDD8, and Rbx1 (63). Rbx1 is thought to recognize a cognate E2, which is required for the E3 ligase function of VEC (59, 72, 77). Recently, it has been shown that NEDD8 covalently modifies Cul2, and this enhances ubiquitin ligase activity (56). pVHL consists of two functional domains: the α- and β-domains (75). The α-domain is required for binding elongin C to nucleate the VEC complex. The β-domain acts as a substrate-recognition/docking site. Disease-associated mutations in VHL kindred frequently map to the surface residues on both domains, suggesting that these domains are important for the tumor suppressor function of pVHL (75).

**pVHL FUNCTION**

Clues of potential substrate(s) recognized by pVHL came from phenotypic observations of VHL disease-associated tumors, which are hypervascular (55). This condition has been attributed to the inappropriate overproduction of angiogenic peptides, such as VEGF (42, 55). In addition, pVHL-defective cells were shown to overproduce numerous hypoxia-inducible transcripts even in the presence of oxygen (17, 24, 37, 53, 83).
Reconstitution of pVHL-defective cells with functional pVHL corrected the cell’s ability to regulate, or more precisely downregulate, the expression of hypoxia-inducible genes in the presence of oxygen (17, 24, 46, 71, 76). Furthermore, the conditional VHL knockout in the liver of mice resulted in severe steatosis and foci of excessive vascularization within the hepatic parenchyma (18). In accordance, hypoxia-inducible mRNAs were markedly increased (18). Thus the conditional VHL knockout mouse model underscored the importance of pVHL in regulating hypoxia-inducible genes.

Hypoxia-inducible factor (HIF) is the major transcription factor that transactivates a myriad of hypoxia-inducible genes, including the aforementioned VEGF and GLUT1 (23, 69, 70, 81, 89). There are three identified HIFs (HIF-1, HIF-2, and HIF-3) in humans (22, 82). HIF is a heterodimeric transcriptional complex consisting of an α- and a β-subunit (23, 69, 70, 81, 89). While the β-subunit, also known as aryl hydrocarbon receptor nuclear translocator, is abundantly expressed independently of Po2, the α-subunit is labile under normal Po2 (69, 70). Specifically, the α-subunit is ubiquitinated on a stretch of residues within the oxygen-dependent degradation (ODD) domain and consequently targeted for degradation via the 26S proteasome (23, 74). Under hypoxia, HIF-α is stabilized and binds to the common HIF-β-subunit to form an active HIF complex, which binds to the hypoxia-responsive elements (HREs) within the promoter/enhancer of hypoxia-inducible genes (4, 12). Thus HIF regulation occurs at the level of the α-subunit.

It is now known that pVHL via its β-domain binds the HIF-α subunit for oxygen-dependent ubiquitination (7, 53, 57, 78). In the presence of oxygen, HIF-α is hydroxylated on a conserved proline residue at position 564 (number according to HIF-1α) within the ODD domain by prolyl hydroxylase domain-containing enzymes (PHDs) (27, 29). P564 hydroxylation is both necessary and sufficient for binding of HIF-α ODD to pVHL (27, 29). Thus ubiquitin-mediated destruction of HIF-α only occurs in the presence of oxygen (see Fig. 1). Accordingly, under hypoxia, HIF-α is not prolyl hydroxylated and thus escapes recognition by pVHL. The now stable HIF-α dimerizes with HIF-β to bind HREs to trigger transcriptional activation of numerous hypoxia-inducible genes (see Fig. 1). Recently, it has been shown that in the presence of oxygen, a conserved COOH-terminal asparagine residue at position 803 on HIF-1α is also hydroxylated by the factor-inhibiting HIF-1 (FIH1) enzyme (39, 40, 50). Unlike prolyl hydroxylation, which induces pVHL binding to HIF-α, aspariginyl hydroxylation prevents the recruitment of the p300/CBP transcriptional coactivators to HIF-α. Thus aspariginyl hydroxylation of HIF-1α attenuates the transcription of HIF-target genes (see Fig. 1) (11, 14). This would suggest that there are, at a minimum, two mechanisms that govern proper triggering of hypoxia-induced gene expression: 1) oxygen-dependent ubiquitination of HIF-α via VEC and 2) inhibition of p300/CBP binding to HIF-α under normoxia. This underscores the importance of oxygen homeostasis in fundamental biological processes from the level of gene expression to development.

HIF IN VHL DISEASE

The significance of HIF in the pathogenesis of VHL disease is underscored by the finding that most VHL mutations occur on the surface residues of either the α- or β-domain (75). These pVHL mutants when tested failed either to bind HIF-α (i.e., β-domain mutation) or to form an E3 ubiquitin ligase complex (i.e., α domain mutation) (6, 7, 21, 31, 57). These failures ultimately resulted in the stabilization of HIF-α and subsequent accumulation of HIF target proteins (6, 7, 21, 31, 57). Moreover, synthetic peptides specifically designed to block the interaction between the β-domain of pVHL and HIF-α (or other cellular proteins that bind to the pVHL β-domain) suppressed the tumor suppressor function of pVHL (51). These pVHL mutations affecting HIF regulation were predominantly associated with the development of hemangioblastoma and clear cell RCC but not pheochromocytoma (6, 21). For example, pVHL mutants associated with type 2C-VHL disease (i.e., pheochromocytoma only) were shown to have “normal” E3 ubiquitin ligase activity and retained proper HIF function (6, 21). However, these mutants were incapable of binding and regulating the assembly of fibronectin (6, 21). It remains to be tested whether the P25L mutation, which is associated with sporadic pheochromocytoma, similarly results in wild-type E3 activity and faulty fibronectin assembly.

OTHER pVHL TARGETS

In addition to recognizing HIF-α subunits for ubiquitination, pVHL also targets the following proteins for ubiquitination: a typical protein kinase C, the VHL-interacting deubiquitinating enzymes (VDU), the seventh subunit of RNA polymerase II (Rpb7), and the large subunit of RNA polymerase II (Rbp1). However, not every protein bound by pVHL is subjected to
polyubiquitylation. These include the SP1 transcription factor, the pVHL-associated KRAB-A domain-containing protein (VHL-Ak) transcription repressor, microtubules, and fibronectin (8, 20, 21, 38, 43, 44, 54, 58, 60). Thus pVHL is thought to play a role in transcription, cytoskeletal organization, and extracellular matrix assembly involving ubiquitin-dependent and -independent mechanisms.

pVHL also downregulates metalloproteinases, such as MMP1, and upregulates MMP inhibitors (TIMPs) (34). Lack of functional pVHL has been associated with overproduction of carbonic anhydrases 9 and 12 (CA9, CA12), which are involved in the acidification of the microenvironment, favoring the growth and invasive properties of tumor cells (28, 52, 73). In addition, renal carcinoma cells devoid of functional pVHL downregulate the cyclin-dependent kinase inhibitor p27, up-regulate cyclin D1, and fail to exit the cell cycle on serum withdrawal (2, 33, 62, 85).

Whether the above additionally ascribed functions of pVHL are directly involved in the tumor suppressor function of pVHL remains to be determined. Moreover, elucidating the significance of pVHL interaction with these pVHL-associated proteins might pave the way for our understanding of the pathophysiology behind the various subtypes of VHL disease.

SUMMARY

The development of VHL disease and most sporadic clear cell RCCs is due to the biallelic inactivation of the VHL gene. The hypervascularity of clear cell RCC and other VHL-associated tumors has been attributed to the overexpression of HIF target genes, such as VEGF. pVHL is the substrate-specifying component of the VEC E3 ubiquitin ligase complex and promotes polyubiquitination of HIF-α. The ubiquitin-tagged HIF-α is then targeted for destruction via the 26S proteasome. Under normal PO2, HIF-α becomes hydroxylated at the conserved proline residue within the ODD domain, which is required for binding by pVHL. Under hypoxia, unhydroxylated HIF-α escapes recognition by pVHL and dimerizes with the constitutively expressed HIF-β to form the active HIF transcription factor. Subsequently, p300/CBP coactivators are recruited to the HIF complex to transactivate HRE-containing hypoxia-inducible genes.

VHL and sporadic clear cell RCC patients often have mutations in the α- or β-domains of pVHL. Mutations in the α-domain prevent pVHL association with the rest of the VEC complex, whereas β-domain mutations prevent HIF-α substrate recognition. In either case, HIF-α is not tagged with polyubiquitin for degradation, resulting in the triggering of an untimely hypoxic response. This likely accounts for the hypervascular nature of VHL disease-associated tumors. Moreover, an explanation for the hypervascularity of solid tumors in the context of intact functional pVHL can be proposed. In general, as tumors grow, the diffusional capacity of oxygen is surpassed, thereby creating solid tumors with increasingly reduced level of oxygen toward the tumor core. Thus tumor hypoxia is a common feature of solid tumors, and this likely accounts for the increased level of HIF-α and underscores the importance of HIF in the growth and eventual spread of tumors. Clinically relevant is the fact that tumor hypoxia represents a poor prognosis and resistance to conventional anticancer therapies. The clear cell RCCs are indeed recalcitrant to chemo- and radiotherapies, and to date this represents the leading cause of death in VHL patients.

Further molecular characterization of pVHL targets and delineation of the pathways governing the cellular response to hypoxia will undoubtedly aid in understanding the development of renal clear cell carcinoma and other pVHL-associated tumors. Lessons learned from these studies will undoubtedly provide new therapeutic approaches to combat kidney cancer and other solid tumors.

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REGULATION OF HIF BY pVHL IN KIDNEY CANCER

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