Role of the VHL (von Hippel–Lindau) gene in renal cancer: a multifunctional tumour suppressor

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Abstract
The VHL (von Hippel–Lindau) tumour-suppressor gene is inactivated in VHL disease and in sporadic cases of CCRCC (clear-cell RCC (renal cell carcinoma)). pVHL (VHL protein) functions as part of an E3 ubiquitin ligase complex that targets proteins for proteasomal degradation. The best-characterized substrate is HIF-α (hypoxia-inducible factor-α). Loss of pVHL and subsequent up-regulation of HIF target genes has been attributed to the highly vascular nature of these neoplasms. However, pVHL does not just function as the executor of HIF-α. Additional functions of pVHL that may be important in preventing CCRCC tumorigenesis have been identified, including primary cilium maintenance, assembly of the extracellular matrix and roles in the stabilization of p53 and Jade-1 (gene for apoptosis and differentiation in epithelia). Current evidence indicates that pVHL probably requires additional co-operating signalling pathways for CCRCC initiation and tumorigenesis.

Introduction
The familial cancer syndrome, VHL (von Hippel–Lindau) disease, occurs as a consequence of inheriting a mutation in the VHL tumour-suppressor gene located on chromosome locus 3p25 [1]. VHL disease follows the classical Knudson two-hit model of tumorigenesis as inactivation of the remaining copy of the VHL gene in somatic cells leads to the development of highly vascular and benign tumours. The predominant tumour manifestations include haemangio-blastomas of the central nervous system and retina, renal cysts and CCRCC (clear-cell RCC (renal cell carcinoma)) in the kidney, phaeochromocytomas and pancreatic cysts and tumours [2].

The VHL tumour-suppressor gene is also frequently inactivated in sporadic cases of CCRCC. Several studies have recently shown that VHL genetic alterations (VHL gene mutations or hypermethylation of the promoter region) together with LOH (loss of heterozygosity) at the VHL locus are common genetic events in sporadic cases of CCRCC. The reported incidence of somatic VHL gene mutations varies from 17.9 to 71% with hypermethylation of the promoter region of the VHL gene occurring in 5.5–20.4% of sporadic cases of CCRCC [3–5]. LOH has also been reported in up to 98% of sporadic CCRCC cases [6]. CCRCC is the predominant subtype of RCC and there are approx. 210000 cases diagnosed worldwide each year (GLOBOCAN 2002; available at http://www-dep.iarc.fr).

RCC is an asymptomatic disease with approx. 25% of patients presenting with advanced disease. The presence of metastatic disease results in a median survival time of approx. 13 months [7]. Consequently, there is a need to decipher fully the functions of the commonly mutated VHL tumour-suppressor gene in CCRCC tumorigenesis and therefore the focus of this mini-review is on current knowledge of the multifunctional roles of pVHL (VHL protein).

pVHL’s role as an E3 ubiquitin ligase
The VHL gene produces two proteins: pVHL19 and pVHL19 (collectively referred to as pVHL). Internal translation at codon 54 of the VHL mRNA generates pVHL19. Reintroduction of either pVHL19 or pVHL19 into VHL-defective RCC cell lines suppresses their ability to form tumours in nude mice. pVHL therefore acts as a tumour suppressor. The best-characterized role of pVHL is as the substrate recognition component of an E3 ubiquitin ligase complex that also contains elongin C, elongin B, Cul-2 and Rbx1. This complex targets proteins for ubiquitin-mediated degradation by the 26S proteasome. pVHL contains two functional domains: the α domain binds to elongin C and the β domain acts as the substrate-docking interface for target proteins. Several substrates of this E3 ubiquitin ligase complex have been identified. These include aPKCα (atypical protein kinase Cα), VDUs (VHL-interacting deubiquitinating enzymes; VDU-1 and VDU-2), and two subunits of RNA polymerase II (Rpb1 and Rpb7). However, the best-characterized target of this complex is the α-subunit of the HIF (hypoxia-inducible factor) family members, HIF-1α, HIF-2α and HIF-3α (reviewed in [8]).

pVHL’s regulation of HIF
Regulation of the expression of HIF-α by the pVHL/E3 ubiquitin ligase complex is critical for the normal functioning...
Figure 1 | Simplified model of targeting HIF-α for degradation by the pVHL E3 ubiquitin ligase complex

(A) In normoxia, key proline residues of HIF-α are hydroxylated by PHDs (prolyl hydroxylase domain-containing proteins). This hydroxylation process enables recognition of HIF-α by the pVHL/E3 ubiquitin ligase complex. HIF-α is polyubiquitinated and subsequently degraded by the 26S proteasome. In addition, hydroxylation of an asparagine residue (N) in the C-terminal transactivation domain inhibits the association of HIF-α with its co-activators and prevents its transcriptional activity. (B) In normoxia, VHL gene mutations in the β or α domain can disrupt the binding of pVHL to HIF-α and/or to the other components of the E3 ubiquitin ligase complex. Stabilized HIF-α dimerizes with HIF-1β. Co-activators are recruited and the HIF complex can activate the transcription of target genes under normoxic conditions.

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of a cell. HIF is a heterodimeric transcription factor that mediates the cell’s response to hypoxia. HIF is composed of an α-subunit (HIF-α), which is oxygen-sensitive, and a β-subunit (HIF-1β), which is constitutively expressed. In normoxia, key proline residues within HIF-α are prolyl hydroxylated. This enables recognition of HIF-α by the pVHL/E3 ubiquitin ligase complex and leads to polyubiquitination and degradation of HIF-α. In hypoxia due to the lack of available oxygen, hydroxylation of the proline residues does not occur and pVHL does not recognize HIF-α. The HIF-α protein then translocates to the nucleus where it dimerizes with HIF-1β and activates the transcription of target genes. However, if loss of functional pVHL occurs due to genetic or epigenetic events in normoxia, HIF-α is not targeted for degradation and consequently is constitutively up-regulated (see Figure 1). This is a frequent occurrence in CCRCC (reviewed in [9]).

In humans, three HIF-α genes have been identified. HIF-1α and HIF-2α are well characterized and there have been over 100 genes identified downstream of HIF to date. Induction of these genes by HIF is critical in facilitating oxygen delivery and adaptation to oxygen deprivation. These genes function in stimulating angiogenesis [VEGF (vascular endothelial growth factor)], erythropoiesis (erythropoietin), glucose uptake and metabolism [GLUT (glucose transporter)-1 and GLUT-3], regulation of extracellular pH [CAIX (carbonic anhydrase 9)], matrix metabolism [MMPs (matrix metalloproteinases)], cell proliferation/survival [TGF-α (transforming growth factor-α)] and apoptosis (BNip3, which is a pro-apoptotic member of
of angiogenesis. However, down-regulation of HIF-2α sequence of up-regulation of HIF and hence the induction confined to regions important for HIF interaction. There are roles for pVHL. First, patient mutations in tumour growth, indicating the importance of HIF-2α could potentially confer a significant advantage on tumour the Bcl-2 family) (reviewed in [10]). Therefore stimulation of these targets by HIF following the loss of pVHL could potentially confer a significant advantage on tumour formation and progression.

Several studies have questioned whether HIF-1α and HIF-2α contribute equally to renal tumorigenesis following loss of pVHL. Accumulating evidence suggests that HIF-2α is more oncogenic in the VHL-defective CCRCC setting. Analysis of patient renal tissue samples that lacked functional pVHL has shown that the majority had up-regulation of HIF proteins but there were a greater number that specifically up-regulated HIF-2α [11]. This phenomenon of bias towards HIF-2α is also observed in VHL-defective RCC cell lines where a number of these cell lines only express the HIF-2α isoform [12]. The pattern of expression of the HIF proteins also differs in renal cysts and overt CCRCCs from VHL disease patients. In the smallest cell foci where pVHL loss has occurred, staining for HIF-1α was observed. In contrast, expression of HIF-2α was only detected in cysts and in regions of overt CCRCC [13]. HIF-1α and HIF-2α have been reported to differ with respect to their ability to suppress tumour growth in vivo. In a wild-type pVHL CCRCC background, normoxic stabilization of HIF-1α failed to promote tumorigenesis in vivo [14,15]. In contrast, normoxic stabilization of HIF-2α was able to override tumour suppression by pVHL and promote tumorigenesis in vivo. Also, siRNA (small interfering RNA) knockdown of HIF-2α in VHL-defective cell lines suppressed tumour formation in vivo [15–17].

In pVHL-defective CCRCC cells, HIF-1α and HIF-2α also differ with respect to their transcriptional targets. A number of pro-tumorigenic genes encoding TGF-α, cyclin D1, VEGF and GLUT-1 are specifically up-regulated by HIF-2α. The pro-apoptotic gene encoding BNip3 is up-regulated by HIF-1α, but it is down-regulated specifically by HIF-2α [15,18]. This may indicate that development of overt CCRCC requires a shift from expression of HIF-1α to HIF-2α. HIF-2α may exert its influence on renal cell growth by activating the TGF-α/EGFR (epidermal growth factor receptor) pathway [19]. In contrast with other tumour types, higher HIF-1α expression in CCRCC patients was associated with increased survival times [20]. These results indicate that HIF, in particular HIF-2α, plays a pathogenic role in pVHL-defective CCRCC. In support of this, in primary murine embryonic stem cells, replacement of HIF-1α expression with HIF-2α by homologous recombination led to increased tumour growth, indicating the importance of HIF-2α [21].

The highly vascular nature of CCRCC may be a consequence of up-regulation of HIF and hence the induction of angiogenesis. However, down-regulation of HIF-2α alone does not alter the characteristics associated with pVHL loss. This indicates that deregulation of HIF alone cannot fully explain the initiation of renal carcinogenesis [19,22].

Several lines of evidence now suggest HIF-independent roles for pVHL. First, patient mutations in VHL are not confined to regions important for HIF interaction. There have been approx. 30 studies to date that have screened for the presence of VHL gene mutations in sporadic CCRCC. The mutations are located throughout all three exons of the gene but are rare within the first 54 codons (see Figure 2). The importance of multiple domains suggests that there may be more than one key target involved in the tumour formation. pVHL has been shown to interact with a number of additional proteins, several of which may contribute to the tumorigenic phenotype.

**Alternative functions of pVHL**

Several alternative functions of pVHL have been identified that are independent of its role as the substrate-recognition component of an E3 ubiquitin ligase. These include, among others, regulation of the ECM (extracellular matrix), microtubule stability and primary cilium maintenance, regulation of E-cadherin and stabilization of p53 and Jade-1 (gene for apoptosis and differentiation in epithelia). Failure of pVHL to control these functions may also contribute to tumour progression and metastasis.

**pVHL, the ECM and MMPs**

pVHL has a critical role in the regulation of the ECM. All VHL disease types have impaired ECM assembly capabilities and sporadic CCRCC cases also show reduced fibronectin staining, which highlights the importance of pVHL for this process [23,24]. The precise mechanisms of how pVHL regulates the assembly of the ECM are yet to be fully elucidated; however, it appears to be independent of HIF. Recent studies have reported that pVHL interacts directly with COL4A2 (collagen IV α2 chain) and indirectly with fibronectin [25–27]. The interaction of pVHL with collagen IV is dependent on hydroxylation of the unfolded COL4A2. pVHL has been shown to interact specifically with the N-terminal tail of COL4A2, which protrudes into the cytosol from the endoplasmic reticulum and this direct interaction is important for the proper assembly of the ECM. In addition, it is proposed that fibronectin interacts directly with COL4A2, which also binds to pVHL [26]. The association of pVHL with these ECM components is crucial to the proper assembly of the ECM, and loss of these interactions can result in a failure to assemble an intact ECM.

pVHL has also been linked to the regulation of MMPs, particularly MMP-2, MMP-9 and MT1-MMP (membrane type 1-MMP) [28–30]. MT1-MMP expression is mediated by HIF-2α [29]. Therefore loss of pVHL up-regulates the expression of MMPs and may promote ECM degradation and possibly an increase in tumour invasiveness and progression.

**Influence of pVHL on the primary cilium and microtubules**

The primary cilium (microtubule-based structure) is important for sensing signals in the extracellular environment and has been proposed to be a negative regulator of cell proliferation. Loss of cilia function in the kidney leads to excessive proliferation of tubular epithelial cells, formation of fluid-filled cysts and kidney failure (reviewed in [31]).
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Figure 2 | Distribution of VHL gene mutations identified in sporadic CCRCC

The frequencies of sporadic VHL gene mutations identified at a particular codon were plotted. The lower part of the schematic diagram illustrates the location of the major protein-binding sites of pVHL. Within exon 1, (GXEEX)_8 represents eight copies of an acidic pentameric repeat Gly-Xaa-Glu-Glu-Xaa sequence. Thirty published sporadic VHL gene mutation studies were examined and the data corresponding specifically to CCRCC were used for this analysis. Studies examining occupational exposure to trichloroethylene were not included. Mutations mentioned in more than one study were only represented once. In certain cases, codon numbering needed to be changed to GenBank sequence L15409 with codon number 1 (ATG) at nucleotide 214 representing the initiator codon. hsRPB7, human RNA polymerase II subunit seven; VBP-1, VHL-binding protein 1. Owing to space limitations, all references for this work cannot be included; further details can be obtained from S.L.M. on request.

![VHL Gene Mutations in Sporadic CCRCC](image)

Regulation of the primary cilia has been linked to pVHL’s effect on the stabilization of microtubules at the cell periphery [32,33]. pVHL controls the orientation of microtubules and can interact with the Par-3–Par-6–aPKC complex, which is a component of intraflagellar transport complexes (important for maintenance of the primary cilium) [34].

pVHL has been localized to the primary cilium in mouse and human proximal tubule epithelial cells and in mouse distal tubules [35]. Analysis of renal cysts from VHL disease patients and CCRCC cell lines (devoid of functional pVHL) demonstrated that these cell types lacked primary cilium, implicating that pVHL may have an important role in the regulation of primary cilium [34,36,37]. The smallest disease foci observed in VHL disease display loss of VHL; however, only a limited number of these lesions develop into cysts [13]. This indicates that a second co-operating event may be required for renal cells to lose their primary cilia and progress to renal cysts.

Current evidence proposes that pVHL functions co-operatively with GSK3β (glycogen synthase kinase 3β) in regulating primary cilium maintenance [34]. GSK3β has previously been identified as being essential for microtubule dynamics (reviewed in [38]). It has been suggested that when GSK3β is active in a cell, it phosphorylates pVHL and inhibits its ability to regulate microtubules [39]. Cilia are maintained by GSK3β independent of pVHL. However, when GSK3β is inactivated, pVHL is active and can regulate the microtubules and primary cilium independent of GSK3β. Of note, GSK3β has been shown to be inactivated in a number of cysts from VHL disease patients. This suggests that loss of both
Figure 3 | Summary of the multifunctional roles of pVHL in CCRCC

pVHL acts as a component of an E3 ubiquitin ligase to target HIF-α for degradation and thereby prevent its transcriptional activity. In addition, pVHL can stabilize p53 and Jade-1. pVHL also stabilizes microtubules, which is important for primary cilium maintenance and may prevent renal cyst formation. pVHL also regulates ECM assembly, which is critical for maintaining tissue integrity and inhibits invasion and angiogenesis. Arrows represent positive effects and ‘⊥’ represents inhibitory (negative effects).

Pathways may be required to disrupt primary cilia, thereby leading to renal cyst formation [35] (reviewed in [40]). Hence loss of specific additional co-operating pathways may be required in addition to pVHL loss for renal carcinogenesis to occur.

pVHL, cell junctions and E-cadherin

pVHL is important for the assembly of intercellular junctions. pVHL-defective RCC cell lines display abnormal intercellular adherens junction and tight junction structures and have alterations in cell polarity [41]. pVHL has also been identified as a regulator of expression of E-cadherin. Loss of expression of E-cadherin is a hallmark of the epithelial–mesenchymal transition and is associated with loss of the cell–cell adhesion capabilities, which can promote tumour progression. VHL-defective RCC cell lines and patient tissue samples display loss of E-cadherin expression in a HIF-α-dependent manner [42–44]. Current evidence proposes that loss of pVHL results in the stabilization of HIF-α, which leads to the transactivation of E-cadherin repressors that act on the E2 boxes present in the promoter of E-cadherin [43]. At present, it is unclear if these events are dependent on...
either HIF-1α or HIF-2α or both. The E-cadherin repressors involved may include SIP1 (Smad-interacting protein 1), Snail, TCF-3 (transcription factor 3) or δEF1. Knockdown of E-cadherin in human RCC cells resulted in an increase in the invasive capabilities of these cells. Therefore it can be envisioned that loss of pVHL and subsequent loss of E-cadherin could be critical for CCRCC development and progression [42–44].

pVHL and p53

p53 has recently been identified as interacting with pVHL and this interaction enables the stabilization of p53 by suppressing Mdm2 (murine double minute 2)-mediated ubiquitination. Stabilization of p53 resulted in increased p53 transcriptional activity. Loss of pVHL prevents this stabilization [45]. However, this result is in contrast with a CCRCC cell line study that showed that pVHL had a negligible effect on the expression of p53 in these cells [46]. Therefore more research is required to elucidate fully the link between p53 and pVHL.

pVHL and Jade-1

In addition to stabilizing microtubules and p53, pVHL also interacts with and stabilizes Jade-1. Jade-1 is a novel protein identified by yeast two-hybrid analysis as a pVHL-interacting protein [47]. Mutations of the VHL gene disrupt this process and may be associated with an increase in renal cancer risk [48]. Jade-1 has been proposed to act as a renal tumour suppressor as it inhibits renal cancer cell growth and tumour formation in nude mice. The relationship between Jade-1 and pVHL is yet to be elucidated fully, but may have important implications for renal cancer development [49].

Mouse models of pVHL inactivation

Homozygous knockout of VHL (VHL−/−) is embryonic-lethal. Cavernous liver haemangiomas develop in adult mice that possess a germline loss of one VHL allele (VHL+/−). This type of angioma is a rare manifestation of VHL disease. Many of the classical VHL disease tumour manifestations were rarely observed in these animals, e.g. renal cysts. An adult mouse model of VHL-associated CCRCC was recently developed by conditional inactivation of VHL in renal proximal tubule cells. Renal cysts developed in approx. 20% of the mutant mice, but CCRCC was not observed. This was dependent on the presence of intact HIF signalling. This implies that the development of CCRCC requires inactivation of pVHL and additional genetic events in co-operating signalling pathways (reviewed in [50]).

Conclusions

pVHL is a multifunctional tumour-suppressor protein. It has crucial roles in the cell including targeting proteins, such as HIF, for ubiquitin-mediated degradation. In addition, alternative functions of pVHL that are independent of HIF have been identified and other interacting proteins are yet to be fully characterized (see Figure 3). It is clear that loss of pVHL can result in the activation of cellular pathways that are strongly associated with both tumour initiation and progression. However, current evidence suggests that other deregulating events are likely to co-operate with pVHL in the development of a tumour. Further research is essential in order to understand fully the tumour-suppressive capabilities of pVHL and its critical targets in renal cancer.

References


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