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The VHL/HIF Axis in Clear Cell Renal Carcinoma

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Abstract

Inactivation of the VHL tumor suppressor protein (pVHL) is a common event in clear cell renal carcinoma, which is the most common form of kidney cancer. pVHL performs many functions, including serving as the substrate recognition module of an ubiquitin ligase complex that targets the alpha subunits of the heterodimeric HIF transcription factor for proteasomal degradation. Deregulation of HIF2 α appears to be a driving force in pVHL-defective clear cell renal carcinomas. In contrast, genetic and functional studies suggest that HIF1 α serves as a tumor suppressor and is a likely target of the 14q deletions that are characteristic of this tumor type. Drugs that inhibit HIF2 α , or its downstream targets such as VEGF, are in various stages of clinical testing. Indeed, clear cell renal carcinomas are exquisitely sensitive to VEGF deprivation and four VEGF inhibitors have now been approved for the treatment of this disease.

Keywords

Kidney Cancer; von-Hippel-Lindau; hypoxia; angiogenesis; VEGF

The VHL Tumor Suppressor

People who harbor a defective VHL tumor suppressor gene, which is located on chromosome 3p25, are predisposed to clear cell renal carcinoma, central nervous system hemangioblastomas, and pheochromocytomas (VHL disease) [1]. Tumor development in this setting caused by somatic inactivation of the remaining wild-type allele in a susceptible cell. In keeping with this knowledge, biallelic *VHL* inactivation, either due to somatic mutations or hypermethylation, is also common in sporadic clear cell renal carcinoma, which is the most common form of kidney cancer [2]. In many studies the frequency of *VHL* mutations in sporadic clear cell renal carcinoma is approximately 50%. This figure might move higher with the increased use of newer, more sensitive, sequencing methodologies. In this regard, some clear cell renal carcinomas that lack detectable *VHL* mutations or hypermethylation nonetheless display mRNA profiles consistent with *VHL* inactivation, suggesting that these tumors harbor genetic or epigenetic changes that directly target the *VHL* locus or indirectly compromise the function of the *VHL* gene product (pVHL) [3, 4].

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Studies of kidney cancers arising in VHL patients suggest that *VHL* inactivation in human kidneys leads to preneoplastic cysts but is not sufficient for malignant transformation [5, 6]. The latter appears to require the accumulation of additional genetic, and perhaps epigenetic, changes. Many non-random genomic abnormalities have been described in clear cell renal carcinoma including amplification of chromosome 5q and loss of 14q. These chromosomes are therefore suspected of harboring one or more oncoproteins and tumor suppressor proteins, respectively (see also below). In addition, genomic sequence analysis has revealed a number of genes that, similar to *VHL*, are recurrently mutated in clear cell renal carcinomas including *PBRM1*, *SETD2*, and *JARID1C* [7, 8]. Interestingly, *PBRM1* and *SETD2* reside on chromosome 3p and are therefore potentially codeleted with *VHL* in tumors that have sustained large losses of chromosome 3p. The *PBRM1* gene product, BAF180, is part of a chromatin remodeling complex that affects gene expression by repositioning nucleosomes [9, 10]. Loss of BAF180 blunts the induction of the canonical p53 target p21, which acts as a cyclin-dependent kinase inhibitor, in response to certain forms of stress. *SETD2* and *JARID1C* are a histone methylase and demethylase, respectively. Histone methylation marks are recognized by specific reader proteins that control chromatin structure and transcription [11, 12]. It is likely that *PBRM1*, *SETD2*, and *JARID1C* will operate in pathways that would otherwise constrain transformation driven by *VHL* loss. In this regard, acute *VHL* loss leads to senescence in many cell types [13, 14]. Conceivably this phenotype requires the action of a protein such as BAF180, *SETD2*, or *JARID1C*. Consistent with this idea, Yang and coworkers showed that pVHL inactivation leads to the induction of *JARID1C* and that *JARID1C* in this setting acts to block proliferation [15].

The *VHL* gene encodes two different protein by virtue of alternative, in-frame, start codons [16]. For simplicity both proteins are referred to generically as pVHL because they behave similarly in many biochemical and cell-based assays. pVHL is a multifunctional protein. The pVHL function that has been most thoroughly studied, and most clearly linked to kidney carcinogenesis, relates to its role in polyubiquitination. Specifically, pVHL is the substrate recognition subunit of a ubiquitin ligase complex that also contains elongin B, elongin C, Cul2, and Rbx1 [16]. Under well-oxygenated conditions this complex binds directly to the alpha subunit of the heterodimeric transcription factor HIF (hypoxia-inducible factor) and targets it for proteasomal degradation. Under low oxygen conditions (or in cells with defective pVHL) HIF α escapes recognition by pVHL, dimerizes with HIF β , and transcriptionally activates 100–200 genes, many of which are believed to promote adaptation to a low oxygen environment (see also below) [17]. pVHL contains two hot-spots for missense mutations, called the alpha domain and the beta domain. The alpha domain is critical for binding to elongin C and hence the remainder of the ubiquitin conjugating machinery while the beta domain binds directly to HIF α [18]. The risk of developing kidney cancer associated with different germline *VHL* alleles correlates well with the degree to which their protein products are impaired with respect to HIF regulation [19–21]. Moreover, all *VHL* mutations detected in hereditary and sporadic clear cell carcinomas severely compromise pVHL's ability to suppress HIF. This, together with the preclinical studies outlined below, underscores the importance of HIF in pVHL-defective kidney cancers.

The HIF Transcription Factor

There are three HIF α family members (HIF1 α , HIF2 α , HIF3 α) and two HIF β family members (HIF1 β and HIF2 β) [22]. HIF β is often referred to as ARNT (aryl hydrocarbon receptor nuclear translocator). HIF1 α is ubiquitously expressed and is the canonical HIF α family member. The expression of HIF2 α is more restricted.

The HIF proteins are members of the basic helix-loop-helix PAS family of DNA-binding transcription factors and recognize the core sequence 5'-RCGTG-3' where R = purine. Both HIF1 α and HIF2 α have two dedicated transcriptional activation domains [the N-terminal transactivation domain (NTAD) and C-terminal transactivation domain (CTAD)] and can activate transcription when bound to DNA [22]. HIF3 α undergoes extensive mRNA splicing and many of the resulting splice variants lack a transactivation domain and can competitively inhibit transcriptional activation by transactivation-competent HIF α protein isoforms [23–26].

Recognition of HIF α by pVHL requires that HIF α be hydroxylated on one (or both) of two conserved prolyl residues within the NTAD by members of the EglN (also called PHD) family of prolyl hydroxylases [27–32]. This posttranslational modification is oxygen-dependent, thereby coupling HIF α ubiquitination by pVHL (and hence stability) to oxygen availability. It appears that HIF1 α but not HIF2 α , can also be recognized by at least one hydroxylation-insensitive ubiquitin ligase complex that does contain pVHL [33–36].

HIF α can also be hydroxylated on a conserved asparaginyl residue within the CTAD by the asparaginyl hydroxylase FIH1, which results in impaired CTAD activity [37–39]. The asparaginyl hydroxylation reaction, like the prolyl hydroxylation reaction, requires molecular oxygen although the oxygen K_m for FIH1 is below the oxygen K_m for the EglN family members [40]. Thus, FIH1 remains active at intermediate levels of hypoxia that are sufficient to partially stabilize HIF α and thereby tunes the hypoxic response [41, 42]. Of note, the HIF2 α CTAD is relatively resistant to FIH1 relative to the HIF1 α CTAD [43, 44]. Different HIF target genes display different sensitivities to FIH1 inhibition, presumably reflecting their differential dependencies on NTAD vs CTAD activity and/or on HIF1 α vs HIF2 α (see also below).

Some HIF target genes are induced by HIF in a wide variety of cells and tissues while others are more restricted. A striking example of the latter is erythropoietin, which in adults is largely confined to dedicated cells in the kidney. Additional layers of complexity stem from the facts that the sets of genes regulated by HIF1 α and HIF2 α are overlapping, but not identical, and the relative contributions of the two paralogs to the control of specific HIF target genes can differ in different cellular contexts [45]. For example, many genes linked to glycolysis are driven primarily by HIF1 α [46] while HIF2 α is the primary regulator of the abovementioned erythropoietin, the stem cell factor Oct4 [47], and, at least in kidney cancer cells, Cyclin D1 [48–51]. VEGF is primarily regulated by HIF2 α in pVHL-defective renal carcinoma cells but by HIF1 α in breast cancer cells [45]. Differential control of genes by HIF1 α and HIF2 α presumably reflects a variety of factors including differential engagement with cis-acting non-HIF transcription factors.

HIF2 α is a Renal Oncoprotein

Multiple lines of evidence underscore the importance of HIF, and particularly HIF2 α , in pVHL-defective clear cell renal carcinoma. For example, all *VHL*^{−/−} clear cell renal carcinoma lines examined to date express HIF2 α whereas many do not express HIF1 α [52–54] (see also below). Elimination of HIF2 α in *VHL*^{−/−} clear cell lines can, like restoration of pVHL function itself, suppress their ability to form tumors in nude mouse [55, 56]. Conversely, overproduction of HIF2 α , but not HIF1 α , can override pVHL's tumor suppressor activity in such xenograft assays [48, 57, 58]. HIF2 α appears to be both necessary and sufficient for much of the pathology that has been described in genetically engineered mouse models in which *VHL* has been inactivated in specific tissues [33, 59–62]. Interestingly, the appearance of HIF2 α in preneoplastic renal lesions in VHL patients

heralds incipient transformation [5] and HIF2 α single nucleotide polymorphisms (SNPs) have been linked to the risk of developing kidney cancer in the general population [63].

The importance of HIF2 α in the pathogenesis of *VHL*^{-/-} clear cell renal carcinomas might stem, at least partly, from its ability to escape from proteins such as FIH1 that would be predicted to blunt HIF1 α activity in cells lacking pVHL. In addition, it is possible that some genes that are preferentially activated by HIF2 α relative to HIF1 α are particularly oncogenic.

HIF1 α is a Renal Tumor Suppressor

HIF1 α resides on chromosome 14q, which as noted above is frequently deleted in clear cell renal carcinomas [52]. Loss of 14q in this setting is associated with a poor prognosis [64]. Many clear cell renal carcinoma lines harbor focal, homozygous, *HIF1 α* deletions leading to absent protein production [52]. In other cases alternative mRNA splicing around deleted *HIF1 α* exonic sequences leads to the production of aberrant HIF1 α isoforms [52]. Reintroduction of wild-type HIF1 α , but not these aberrant HIF1 α species, in clear cell renal carcinoma lines that lack endogenous, wild-type, HIF1 α , suppresses their proliferation in vitro and in vivo [48, 52]. Conversely, shRNA-mediated downregulation of HIF1 α in clear cell renal carcinoma lines that retain endogenous, wild-type, HIF1 α , stimulates their proliferation in vitro and in vivo [52, 53].

Consistent with these observations, many clear cell renal carcinomas produce no, or very low, levels of HIF1 α and 14q deleted tumors display a transcriptional signature indicative of decreased HIF1 α activity [52–54] [65]. Clear cell renal carcinoma tumors, in contrast to lines, however, frequently appear to retain a wild-type *HIF1 α* allele [52]. Although the failure to document biallelic *HIF1 α* inactivation in tumors could be technical (for example, due to difficulties stemming from the contamination of tumor DNA with host DNA) it raises the possibility that *HIF1 α* haploinsufficiency is sufficient to promote tumorigenesis in vivo and that reduction to nullizygosity occurs during the generation and propagation of cell lines. It should also be borne in mind, however, that cell lines are frequently established from metastatic lesions. It is possible that reduction to nullizygosity is a late event in renal carcinoma progression and therefore underrepresented in primary tumors, especially in patients without metastases who have undergone a nephrectomy with curative intent.

Although rare, intragenic *HIF1 α* mutations, including missense mutations, have been described in clear renal carcinoma tumors and, when tested, these mutations compromise HIF1 α 's ability to suppress proliferation when reintroduced into clear cell renal carcinoma lines that lack endogenous, wild-type, HIF1 α [8, 52, 66]. Collectively, these observations support that wild-type HIF1 α acts as a renal carcinoma suppressor and is one of the relevant targets of the 14q deletions that are typical of this tumor (Fig 1). Although HIF1 α is often thought of as an oncoprotein there is precedence for it acting as a tumor suppressor. For example, loss of HIF1 α scores as a tumor suppressor in teratomas formed by embryonic stem cells [67], in astrocytes grown orthotopically in nude mice [68], and in some leukemia models [69–71].

Quantitative and Qualitative Differences between HIF1 α and HIF2 α

Several differences between HIF1 α and HIF2 α might ultimately account for their opposing effects with respect to clear cell renal carcinogenesis. First, the HIF1 α CTAD would theoretically be silenced in *VHL*^{-/-} cells by FIH1, for the reasons outlined above, unless those cells were profoundly hypoxic. If true, the transcriptionally crippled HIF1 α might act as a dominant-negative by competitively displacing HIF2 α , which is largely insensitive to FIH1, from specific HIF target genes (Fig 2). Indeed, some HIF target genes are

paradoxically increased when HIF1 α is downregulated in *VHL*^{-/-} renal carcinoma cells [48]. Structure-function studies indicate that both the HIF2 α NTAD and HIF2 α CTAD contribute to its ability to promote renal carcinoma growth [44].

Secondly, it is possible that some HIF target genes that are regulated primarily by HIF1 α suppress renal carcinoma growth and/or that some HIF target genes that are primarily regulated by HIF2 α promote renal carcinoma growth (Fig 3). In this regard, several genes that are regulated by HIF1 α in pVHL-defective clear cell renal carcinomas are known, or suspected, or acting as tumor suppressors (at least in some contexts) including BNIP3, REDD1, TXNIP, and ZAC1 [52]. Interestingly, ZAC1 maps to chromosome 6q23, which is frequently deleted in VHL-associated renal cancers, hemangioblastomas, and pheochromocytomas [72, 73].

Finally, it is possible that the relevant differences between HIF1 α and HIF2 α relate to their ability to engage collateral signaling pathways, either due to differential regulation of HIF target genes or due to non-canonical HIF function (reviewed in [74])(Fig 4). For example, in some systems HIF2 α enhances c-Myc activity while HIF1 α suppresses c-Myc activity. A number of non-mutually exclusive mechanisms have been put forth to explain how HIF2 α potentiates c-Myc activity. At the same time, HIF1 α enhances, and HIF2 α suppresses, p53 function. Accordingly, clear cell renal carcinoma cells that lack HIF1 α exhibit reduced sensitivity to genotoxic agents [75, 76].

Targeting HIF2 α

The considerations suggest that targeting HIF α , and particularly HIF2 α , would be useful to the treatment of clear cell renal carcinoma. Unfortunately, DNA-binding transcription factors, with the exception of the steroid hormone receptors, have traditionally been difficult to inhibit with drug-like small organic molecules. Proof of concept experiments suggest that it might be possible, in time, to target HIF2 α with DNA-binding polyamides that bind in a sequence-specific manner to HREs if the bioavailability of such agents can be improved [77–80]. Semenza and coworkers showed that the small molecule acriflavine binds directly to HIF1 α and HIF2 α and interferes with their ability to dimerize with HIF1 β [81]. siRNA targeting HIF2 α might one day be an option if robust methods for systemic siRNA delivery become available.

Two groups screened for drugs that, at least indirectly, inhibit HIF2 α in *VHL*^{-/-} renal carcinoma cells [82–85]. Iliopoulos and coworkers identified molecules that promote IRP1's ability to inhibit HIF2 α translation in an mTOR-independent manner [82]. Subsequent studies revealed that the endogenous anti-inflammatory cytokine 15-deoxy-Delta(12,14)-prostaglandin J(2) behaved in a similar manner [86]. The Molecular Targets Laboratory at the National Cancer Institute identified a number of natural products as potential HIF2 α agonists [83]. The specificity of these chemicals remains to be established.

Many other compounds are also known to indirectly downregulate HIF α including mTOR inhibitors, HSP90 inhibitors, and HDAC inhibitors [87]. Indeed, two rapamycin-like mTOR inhibitors (“rapalogs”), everolimus and temsirolimus, have been approved for the treatment of kidney cancer based on randomized clinical trial data [88, 89]. In principal mTOR inhibitors would downregulate HIF levels in tumor cells and growth factor signaling (for example, VEGF signaling) in supporting cells (for example, endothelial cells) although preclinical models have underscored the importance of the former [90].

Unfortunately, the clinical activity of mTOR inhibitors in the treatment of kidney cancer is rather modest. This might reflect the fact that rapalogs primarily inhibit mTOR in the so-called TORC1 complex and have less activity against mTOR present in the TORC2 complex

[91]. Inhibition of TORC1 preferentially affects HIF1 α relative to HIF2 α [92] and can also paradoxically increase upstream receptor tyrosine kinase signaling due to a loss of TORC1-dependent feedback pathways [93, 94]. Preclinical models suggest that newer, ATP-competitive, mTOR inhibitors, which inhibit both TORC1 and TORC2, suppress HIF2 α levels and renal carcinogenesis more effectively than do rapalogs [95].

Targeting HIF2 α -Responsive Gene Products

HIF transcriptionally activates several hundred genes. As described above, some of these genes are regulated by HIF in a wide variety of tissues while others are regulated by HIF in very specific cells and tissues. Moreover, some HIF targets are preferentially controlled by HIF1 α while others are preferentially controlled by HIF2 α . A number of the HIF target genes that are upregulated in pVHL-defective renal carcinoma cells, including cells that produce exclusively HIF2 α , are suspected or known to promote tumorigenesis. This description below focuses on HIF targets for which preclinical and/or clinical data support a role in renal carcinogenesis.

VEGF

Renal carcinomas are highly angiogenic and overproduce the HIF-responsive growth factor VEGF. Indeed, renal carcinomas have the highest levels of VEGF amongst epithelial neoplasms. The induction of VEGF consequent to pVHL loss would be predicted to diminish the selection pressure to activate alternative angiogenic factors during tumor progression. In contrast, a variety of angiogenic factors, in addition to VEGF, are likely at work in other solid tumors. Consistent with this idea, kidney cancer is the solid tumor where VEGF inhibitors have consistently demonstrated single agent activity in terms of tumor regressions and disease stabilization. Indeed 4 drugs, including the VEGF neutralizing antibody Bevacizumab and the VEGF receptor antagonists Sunitinib, Sorafenib, and Pazopanib, have been approved for this indication based on positive, randomized, clinical trial data [96–100]. Nonetheless, these drugs are not curative. A number of second generation VEGF receptor antagonists that exhibit enhanced specificity and/or potency are currently undergoing clinical trials and it is hoped that these drugs will exhibit greater clinical activity, either alone or in combinations, than do the existing agents. There is already a hint, however, that there the degree with which VEGF signaling can be safely suppressed in man will have limits. For example, combinations of VEGF antagonists have been associated with microangiopathic hemolytic anemia, presumable reflecting on-target endothelial cell damage [101–103].

PDGF

In addition to VEGF, HIF also regulates PDGF B (hereafter called PDGF), which supports vasculature pericytes. Newly sprouting blood vessels that lack pericytes are hypersensitive to VEGF withdrawal [104–106], which probably explains the almost universal sensitivity of rapidly growing tumor xenografts in mice to VEGF antagonists. Once vessels mature and are properly invested with pericytes, however, they become less dependent on VEGF for survival [104–106]. It is thus potentially fortuitous that most of the available VEGF receptor antagonists also inhibit the PDGF receptor. Whether PDGFR blockade does, however, truly contribute to the activity of such VEGF receptor antagonists remains an open question. Moreover, inhibition of PDGF itself has produced significant clinical responses in kidney cancer [107, 108].

EGFR and c-Met

Kidney cancers frequently overproduce TGF α and its receptor, EGFR [109–116]. TGF α is a HIF-target [117] and pVHL loss enhances EGFR translation and delays EGFR

internalization [118–120]. In mouse models blocking EGFR signaling decreases renal carcinoma growth in vivo [121]. Unfortunately, however, EGFR inhibitors have so far been largely ineffective for the treatment of kidney cancer in man [122–125]. This apparent disconnect might suggest that the EGFR dependence exhibited by renal carcinoma cell lines, whether grown on plastic or in immunocompromised mice, only developed after the cell lines were initiated and propagated ex vivo. A second possibility is that the failure to achieve a clinical benefit in man reflects an inability to achieve adequate EGFR inhibition (pharmacodynamic failure). Yet a third possibility is suggested by recent studies of acquired and de novo resistance of other forms of cancer to EGFR inhibitors. In these settings some cases of resistance have been linked to collateral signals provided by activated c-Met [126–128]. c-Met is on a large kidney cancer amplicon located on chromosome 7 and is also activated upon pVHL loss by a variety of mechanisms [129–131]. Interestingly, pVHL-defective cells are more dependent on c-Met than are their pVHL-proficient counterparts [132]. Human tumor xenograft studies performed in mice frequently underestimate the importance of c-Met because the mouse ligand for c-Met, HGF, does not efficiently engage the human c-Met protein [133]. These considerations, together with the role of c-Met in angiogenesis, warrant an exploration of c-Met as a target in clear cell renal carcinoma.

Cyclin D1

In pVHL-defective clear cell renal carcinomas, but in no other cell type examined to date, HIF induces (rather than represses) Cyclin D1 [48–51]. Moreover, pVHL-defective renal carcinoma cells are hypersensitive to one of Cyclin D1's catalytic partners, Cdk6 [132]. Finally, many clear cell renal carcinomas have sustained deletions of the Cyclin D/Cdk inhibitor p16/Ink4A [3, 134–136]. A relatively promiscuous kinase inhibitor that inhibits multiple Cdk family members did not display significant activity against kidney cancer, possibly because off-target toxicities prevented effective dosing [137]. It would be of interest to test new agents that exhibit greater specificity for Cdk6 and its paralog Cdk4.

Histone Demethylases

HIF induces the expression of a number of JmjC-containing histone demethylases [138–143]. Under hypoxic conditions this presumably provides a form of compensation because catalysis by these enzymes requires oxygen. In pVHL-defective tumor cells, however, this might contribute to altered gene expression and transformation. The identification of histone methylase (SETD2) and demethylase (UTX, JARID1C) mutations in renal cancer underscore that deregulated histone methylation can contribute to renal carcinogenesis. It remains to be seen which, if any, HIF-responsive demethylase are important for the genesis and maintenance of pVHL-defective clear cell carcinomas. In one study shRNA-mediated downregulation of JMJD1A led to modest decrease in renal carcinoma growth [138].

Conclusions

Inactivation of the VHL tumor protein is a signature lesion in clear cell renal carcinoma and leads to the deregulation of HIF α . HIF2 α acts as an oncoprotein in pVHL-defective clear cell renal carcinoma whereas HIF1 α acts as a tumors suppressor in this context and appears to be a target of the 14q deletions that are frequently observed in this tumor type. This knowledge has provided a foundation for the development of drugs that inhibit HIF or selected HIF targets for the treatment of clear cell renal carcinoma.

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Fig 1. Role of pVHL, HIF1 α , and HIF2 α in Clear Cell Carcinoma

Loss of pVHL leads to the accumulation of both HIF1 α and HIF2 α , which have opposing effects on clear cell carcinogenesis. This leads to selection pressure to downregulate HIF1 α .

Fig 2. Quantitative differences between HIF1 α and HIF2 α related to activation of HIF target genes

HIF1 α is more sensitive than HIF2 α to inhibition by FIH1. Competitive displacement of HIF2 α by HIF1 α in a well-oxygenated pVHL-defective cell might therefore decrease the transcription of certain genes that are responsive to HIF α CTAD function.

Fig 3. Qualitative differences between HIF1 α and HIF2 α related to activation of HIF target genes

The genesets regulated by HIF1 α and HIF2 α overlap but are not entirely congruent. The former might be biased toward renal carcinoma suppressors and the latter to renal carcinoma oncoproteins.

Fig 4. Qualitative differences between HIF1 α and HIF2 α with respect to non-canonical HIF functions

HIF1 α and HIF2 α have opposing actions in some systems with respect to collateral signaling pathways involving cancer-relevant proteins such as c-Myc and p53.