The Von Hippel-Lindau Tumor Suppressor Gene and Kidney Cancer

William G. Kaelin, Jr.
Howard Hughes Medical Institute, Dana-Farber Cancer Institute and Brigham and Women’s Hospital, Boston, Massachusetts

ABSTRACT
The von Hippel-Lindau tumor suppressor gene (VHL), which resides on chromosome 3p25, is mutated or silenced in >50% of sporadic clear cell renal cell carcinomas. Germ-line VHL mutations give rise to VHL disease, which is characterized by an increased risk of blood vessel tumors (hemangioblastomas) and renal cell carcinomas. In this setting, VHL inactivation gives rise to premalignant renal cysts. Additional genetic alterations are presumably required for conversion of these cysts to renal cell carcinomas. Restoration of VHL function in VHL−/− renal cell carcinomas is sufficient to inhibit tumorigenesis in vivo. On the basis of these and other data, VHL appears to be a critical gatekeeper with respect to the development of renal cell carcinoma. The VHL gene product, pVHL, is the substrate recognition module of an E3 ubiquitin ligase that targets the hypoxia-inducible factor (HIF) for destruction in the presence of oxygen. Hypoxic cells, or cells lacking pVHL, accumulate high levels of HIF, which activates the transcription of a variety of genes, including vascular endothelial growth factor, platelet-derived growth factor B, and transforming growth factor α. We have demonstrated that inhibition of HIF is necessary and sufficient for tumor suppression by pVHL in renal cell carcinoma nude mouse xenograft assays. This provides a rationale for treating VHL−/− renal cell carcinoma with inhibitors of HIF or its downstream targets. Genotype-phenotype correlations in VHL disease suggest, however, that pVHL has targets in addition to HIF. Elucidating these targets should provide a more complete picture of how pVHL suppresses tumor growth.

INTRODUCTION
The genes linked to hereditary cancers often, as first predicted by Comings (1) and Knudson et al., (2), play roles in their nonhereditary counterparts. For example, some hereditary colorectal cancers are due to germ-line APC mutations, and somatic APC mutations are common in nonhereditary (sporadic) colon cancers (3). The former observation establishes that APC mutations cause, and do not merely correlate with, the development of colorectal cancer. Interestingly, germ-line APC mutations also cause intestinal neoplasia in the mouse (4).

Individuals with von Hippel-Lindau (VHL) disease are at increased risk for developing clear cell carcinoma of the kidney, which is the most common histologic type of renal cancer (5, 6). Moreover, linkage studies performed in the 1980s correctly predicted that the VHL susceptibility gene would be located at chromosome 3p25, a region of the genome that is frequently deleted or altered in renal cell carcinoma. Together, these observations suggested that the VHL gene might play a pivotal role in renal cell carcinoma. The VHL gene was isolated in 1993 using a positional cloning strategy (7). With the gene in hand, it was possible to demonstrate that patients with VHL disease have typically inherited an inactive VHL allele from one of their parents (rare cases of VHL disease are due to de novo mutations, occasionally leading to VHL mosaicism). In short, patients with VHL disease are VHL+/− heterozygotes. The development of pathological features, such as premalignant renal cysts, is linked to loss of the remaining wild-type VHL allele in a susceptible cell (8, 9). It is presumed that additional mutations involving genes other than VHL are required for the conversion of such premalignant cysts to malignant tumors. In keeping with these considerations, biallelic VHL mutations (frequently involving point mutation of one allele and loss of the other) are common in sporadic renal cell carcinomas of the clear cell type (6). Moreover, VHL inactivation due to DNA hypermethylation has been documented in some renal cell carcinomas that lack VHL mutations (10). Restoring VHL function in VHL−/− renal cell carcinoma cells is sufficient to suppress their ability to form tumors in animals (11, 12). Hence, VHL is a tumor suppressor gene based on both genetic and functional criteria, and VHL inactivation appears to be a very early step in renal carcinogenesis, just as APC inactivation appears to be an early step in many colorectal cancers.

VHL orthologues have been identified in a variety of other species, including rodents, flies, worms, and fish (13–15). In humans, the VHL gene is ubiquitously expressed (16–19). This creates a paradox, because VHL mutations are tightly linked to only a handful of tumor types, including blood vessel tumors (hemangioblastomas) and adrenal gland tumors (pheochromocytomas), in addition to renal cell carcinoma (5, 6). Similar conundrums exist for other tumor suppressor genes, however (for example, the previously mentioned APC). VHL encodes two different protein isoforms, because two alternative in-frame ATGs can be used for translation initiation (20–22). Both isoforms are capable of suppressing renal cell carcinoma growth in vivo and will be referred to generically as pVHL. pVHL is found in multiple cellular compartments. It shuttles back and forth between the nucleus and cytoplasm and can also be found in association with the endoplasmic reticulum and mitochondria (12, 23–29).

The best understood function of pVHL relates to its ability...
to target specific proteins for destruction. pVHL forms stable complexes that contain other proteins called elongin B, elongin C, Cul2, and Rbx1 (30, 31). These complexes are capable of directing the covalent attachment of polyubiquitin tails to specific proteins, which serve as signals for such proteins to be degraded by the proteasome. Several pVHL targets have been identified, including the members of the hypoxia-inducible factor (HIF) α family (HIF-1α, HIF-2α, and HIF-3α), HIF-1α and HIF-2α, when bound to a HIF-β member (such as HIF-1β, also called ARNT), form a sequence-specific, DNA-binding transcription factor called HIF. Ordinarily, the HIF-α members are highly unstable except under low-oxygen conditions. In the presence of oxygen, these proteins become hydroxylated on conserved prolyl residues in a reaction catalyzed by members of the EGLN family (especially EGLN1; refs. 14, 32–34). pVHL recognizes the hydroxylated HIF-α species and orchestrates their destruction (35–37). In cells that lack pVHL, or when oxygen is limiting, HIF is free to accumulate and activates the transcription of a cadre of genes involved in short-term and long-term adaptation to hypoxia. Included among these genes are those that control glucose uptake and metabolism (such as the Glut1 glucose transporter and various glycolytic enzymes), extracellular pH (such as carbonic anhydrase IX), angiogenesis (such as vascular endothelial growth factor, VEGF), erythropoiesis (such as erythropoietin), and mitogenesis (such as transforming growth factor α, TGF-α, and platelet-derived growth factor B, PDGF-B). These considerations explain the frequent overproduction of HIF, as well as its downstream targets, in renal cell carcinoma.

pVHL binds to other cellular proteins, some of which may also be polyubiquitination targets or in some other way modulated by pVHL. These proteins include the atypical protein kinase C family members (38–41), Sp1 (42, 43), heteronuclear ribonucleoprotein hnRNP A2, (44), specific RNA Pol II subunits (Rpb1 and hsRPB7; refs. 45, 46), Jade-1 (47), Vdu1/2 (48, 49), fibronectin (50, 51), and proteins associated with microtubules (52). On the other hand, there is clear data that HIF is a critical downstream target with escape to the development of renal cell carcinoma. Renal cell carcinoma engineered to produce HIF-2α variants that escape recognition by pVHL (because the relevant prolyl hydroxylation sites have been eliminated) are inured to the tumor suppressor effects of pVHL as are cells that produce a decoy protein that binds to the HIF-binding site within pVHL (53–55). Conversely, elimination of HIF-2α in VHL−/− renal cell carcinoma is sufficient to suppress their ability to form tumors in vivo (54, 56). Hence, down-modulation of HIF-2α is both necessary and sufficient for pVHL to suppress tumor formation by renal cell carcinoma.

Evidence for a role of HIF in renal cell carcinoma has also come from studies of tuberous sclerosis (TSC), which is caused by inactivating mutations that affect either the TSC1 or TSC2 tumor suppressor genes (57). Some rodent models of TSC are characterized by the development of renal cysts and renal cell carcinomas (58, 59). For example, the Eker rat, which develops renal cell carcinomas, carries a mutant TSC2 allele (60). TSC1 and TSC2 form a complex that exhibits GTPase-activating protein activity and inhibits a protein called Rheb in response to a variety of extracellular signals, such as growth factor depletion or nutrient starvation (57). Rheb is an activator of the mTOR kinase, which regulates a number of processes linked to cell growth. Inactivation of the TSC complex, such as through mutation of TSC2 or activation of certain oncogenes that lie upstream of the TSC complex, therefore leads to inappropriate activation of mTOR. One consequence of dysregulated mTOR activity is overproduction of HIF, probably due to enhanced translation and possibly increased transcription (61–66).

These proof-of-concept data in VHL−/− renal cell carcinoma suggest that drugs that inhibit HIF or its downstream targets might be active against renal cell carcinoma. In general, transcription factors such as HIF have not proven to be highly tractable as drug targets. However, a number of agents have been reported to indirectly down-regulate HIF. For example, HIF levels, as described herein, are responsive to changes in mTOR, and several studies have shown that HIF can be downregulated with the mTOR inhibitor rapamycin (61, 63–65). HIF also requires folding by heat shock protein 90 and is downregulated by heat shock protein 90 inhibitors such as geldanamycin and 17-(allylamino)-17-demethoxygeldanamycin (67). A number of other agents down-regulate HIF via unknown mechanisms, including HDAC inhibitors, (68), topoisomerase I inhibitors (69, 70), thioredoxin-1 inhibitors (71, 72), and microtubule disrupters (73).

An alternative approach would be to use agents, alone or in combination, that target HIF-responsive gene products. For example, a variety of agents that inhibit VEGF or its receptors are currently being tested in renal cell carcinoma, including bevacinuzumab, PTK787, SU11248, and BAY 43–9006 (74–77). In a recent Phase II study, bezvacizumab was shown to delay progression in patients with metastatic renal cell carcinoma (77). One way to enhance the efficacy of VEGF antagonists might be to combine agents that block the HIF-VEGF pathway at multiple nodes (Fig. 1, vertical strategy). For example, one might conceivably combine a compound such as rapamycin, which down-regulates HIF levels through mTOR inhibition (as described herein), with agents that bind VEGF (such as bevacizumab) or small molecules that inhibit its receptor (such as PTK787).

Preclinical studies suggest that established, mature blood vessels have a diminished requirement for VEGF for survival relative to newly sprouting vessels due to auxiliary survival signals provided by surrounding pericytes and stroma (78, 79). For this reason, it might be useful to combine a VEGF antagonist with an inhibitor of the HIF-responsive pericyte growth factor PDGF-B (Fig. 1, horizontal strategy). Fortuitously, the VEGF and PDGF receptors share structural similarities and, for this reason, some small-molecule kinase domain-related inhibitors also inhibit the PDGF receptor. Examples of such molecules include BAY 43–9006 and SU11248. In time, agent(s) that target HIF-responsive angiogenic pathways might be combined with drugs that target HIF-responsive autocrine growth factors and their receptors. Several studies have suggested that one such growth factor is TGF-α, which is a ligand for the epidermal growth factor receptor (80, 81). Renal epithelial cells appear to be very sensitive to the mitogenic effects of TGF-α (81). Preclinical data implicate TGF-α and epidermal growth factor receptor in renal cyst formation (82, 83).

Some of these concepts can now be tested in mice that develop tumors due to pVHL inactivation. VHL+/− mice de-
velop hepatic blood vessel tumors over time due to stochastic loss of the remaining wild-type VHL allele (84). These lesions, as predicted, are characterized by increased levels of HIF and its downstream targets. Similar lesions are observed, with higher penetrance and shorter latency, after conditional inactivation of the mouse liver (using “floxed” VHL alleles and Cre recombinase; refs. 84, 85). To date VHL inactivation has not been linked to renal disease in the mouse, suggesting that the mouse kidney, in contrast to the human kidney, is not permissive for tumorigenesis after pVHL loss.

Not all renal cell carcinomas are linked to VHL loss, and there are some data that suggest that VHL status is an independent prognosticator in this disorder (86). It will be important to ask whether pVHL status affects treatment outcomes as new agents directed against HIF or its downstream targets are ultimately tested for the treatment of renal cell carcinoma of humans. Direct sequencing of the VHL gene in archival tumor specimens is possible as is determination of VHL methylation status. Direct sequencing is facilitated by the fact that the VHL contains only 3 exons, and the entire open reading frame spans only 639 nucleotides. An alternative approach would be to rely on immunohistochemical markers that are likely to reflect pVHL status, such as HIF-2α, carbonic anhydrase IX, and Glut1. These markers should be diffusely positive in pVHL-defective tumors but absent or focally positive (due to localized areas of hypoxia or anoxia) in tumors with intact pVHL (9, 53, 87–91).

The earliest recognized genetic change in chronic myelogenous leukemia and gastrointestinal stromal cell tumor leads to activation of c-Abl and c-Kit, respectively. The remarkable activity of imatinib mesylate, which inhibits these two oncogenic kinases, against these two disorders supports the development of anticancer drugs based on early genetic changes that are causally connected to cell transformation (92). Inactivation of pVHL is a common and apparently early step in renal carcinogenesis. Although our knowledge of the functions of pVHL is still incomplete, studies conducted thus far highlight its role as an inhibitor of HIF, and dysregulation of HIF is likely to contribute to the development of renal cell carcinoma. Therefore, drugs that inhibit HIF or its downstream targets warrant testing in this disease.

OPEN DISCUSSION

Dr. Tim Eisen: Did you discover the possibility of an environmental impact or do you think these cancers are purely genetically inherited?

Dr. William G. Kaelin: I certainly don’t discount the environment. There is the question of the second hit that has to occur, and I don’t know whether it is random via mutation or perhaps some environmental carcinogen. Many studies have shown that you can link certain VHL mutations to certain occupational exposures. One thing that I didn’t talk about is an interesting syndrome called “Chuvash” polycythemia in the former Soviet Union. These patients are homozygous not heterozygous for a mutated VHL allele. It’s probably a hypomorphic allele, meaning that there is not a complete loss of function. Interestingly, these people develop polycythemia and have overproduction of HIF, but they don’t develop tumors. One explanation for this would be that HIF is not the only driver, even if it is a principal driver, and maybe some other functions are left intact. Another possibility, however, is that because this is a highly genetically inbred population, there could be a modifier gene that is suppressing tumor growth or, again, there may be some environmental factors that are peculiar to living in the Chuvash region of the Soviet Union.

Dr. James Yang: What do you see in this pathway as the dominant, proliferation driver? Do you believe the cyclin D1 data or do you think TGF-α is important?

Dr. Kaelin: I think that TGF-α is a potent renal epithelial mitogen. Among the epithelial cell types tested renal epithelia was the most sensitive to mitogenic effects of TGF-α. Furthermore, there is at least one mouse model where overproduction of TGF-α in the kidney causes renal cysts. When cyclin D1 comes up on the arrays, it behaves like a HIF target, but I can’t tell you yet whether the cyclin D1 is simply an indirect readout of this pathway or not. I don’t know what the molecular link is between VHL and cyclin D1.

Dr. W. Marston Linehan: Do you think that in 5 years we will say to ourselves that these other components of the VHL pathway are equally or more important than HIF?

Dr. Kaelin: I think until we learn a little bit more, we have to focus on these pathways. The related question you might have asked is, “Assuming that VHL inactivation is not sufficient to cause a full-blown renal cancer, is dealing with the VHL pathway going to be sufficient?” VHL is the earliest recognizable driver in renal carcinoma, so I am hopeful that drugs that block this pathway are going to have an effect even given the complexity of renal cell carcinoma.
Dr. Othon Iliopoulos: There are several animal models that have shown that overexpression of HIF in certain organs may not cause tumors.

Dr. Kaelin: In studies of multiple cancer genes, there are many examples where the same mutation in mouse and humans gives you a different phenotype, meaning you might see different organs affected. Also, maybe HIF is not sufficient by itself.

Dr. Iliopoulos: One possibility is that we are talking about the relative importance of HIF in different genetic backgrounds.

Dr. Michael Atkins: Is there any uniformity in the way the hemangiomas and renal tumor in VHL patients respond to therapy?

Dr. Daniel George: VHL syndrome is a disease with multiple sites of tumors and/or vascular proliferations. We have evidence of some responses in these tumors; however, we are seeing a variable response in different disease sites to fairly specific agents. I think there are probably multiple factors in play.

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Clin Cancer Res 2004;10:6290S-6295S.

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