Pseudohypoxic Pathways in Renal Cell Carcinoma

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Abstract

Mutations of the von Hippel-Lindau (VHL) or fumarate hydratase (FH) genes lead to morphologically different renal cell carcinomas with distinct clinical courses and outcomes. The VHL protein is a part of an ubiquitin ligase complex that targets proteins for proteosomal degradation. FH is one of the mitochondrial enzymes of the Kreb's cycle. Despite two different functionalities and cellular locations, loss of either VHL or FH products has been shown to alter expression levels of hypoxia-inducible factors (HIF-1α and HIF-2α) and their downstream targets. HIF proteins are key regulators of oxygen homeostasis. Tight regulation of HIF allows for cell survival and growth at the time of hypoxic stress. HIF acts via transcriptional regulation of vascular endothelial growth factor, platelet derived growth factor, endothelial growth factor receptor, glucose transporter protein 1, erythropoietin, and transforming growth factor-α. Loss of VHL or FH is thought to result in a pseudohypoxic state so that cellular response pathways mediated by HIF are activated despite normal oxygen conditions. Understanding of these pseudohypoxic pathways has provided a better appreciation of the molecular mechanisms of carcinogenesis in addition to providing a rationale for targeted therapeutic approaches.

Background

Von Hippel-Lindau (VHL) results from a germ-line mutation in the VHL gene, whereas hereditary leiomyomatosis (HL) and renal cell carcinoma (RCC) results from mutation in the fumarate hydratase (FH) gene. Despite arising in the same organ, RCC represents a heterogeneous disorder that differs morphologically, histologically, and genetically. Dissecting out the oncogenic pathways provides a rationale for development of novel therapeutic interventions.

VHL Gene and Its Role as a Tumor Suppressor

VHL disease is a hereditary disorder characterized by development of multiple visceral and central nervous system tumors. The most common VHL manifestations include RCCs or cysts, pancreatic neuroendocrine tumors or cysts, craniospinal and retinal hemangioblastomas, and endolymphatic sac tumors (1). As early as 1979, translocation of chromosome 3 was implicated in hereditary RCC (2). In 1987, loss of alleles of loci on the short arm of chromosome 3 was observed in patients with nonhereditary RCC, and in 1989, chromosome 3 to 6 translocation was reported in development of multiple bilateral RCC (2–4). Finally, in 1993, the VHL gene was identified and located on chromosome 3p25 bypositioning cloning in 221 VHL kindreds (5). The VHL gene was shown to behave as a tumor suppressor gene in accordance with Knudson's theory of carcinogenesis (6). Improved detection with quantitative Southern blotting, fluorescent in situ hybridization, and complete sequencing of the gene allowed identification of germ-line mutations in ~100% of affected individuals (7). Although in the majority of patients there is biallelic loss of the VHL gene via loss of heterozygosity, several VHL inactivations occur via hypermethylation of the CpG island in the promoter region of the VHL gene (5, 7–11). Variations of VHL germ-line mutations result in different phenotypes (12). Additionally, certain germ-line VHL mutations predispose to a higher frequency of RCC (13). Furthermore, VHL mutations have been identified in a high percentage of tumors from patients with sporadic, noninherited clear cell renal carcinoma, confirming importance of VHL loss in RCC tumorigenesis (8, 14).

The VHL gene has three exons that encode the 213-amino acid VHL protein (5). In 1995, elongin C and elongin B were identified as binding partners of the VHL protein (15, 16). Elongin is a heterodimer consisting of a transcriptionally active subunit (A) and two regulatory subunits (B and C; ref. 16). The VHL protein binds tightly and specifically to elongin C (15–17) by the presence of a stretch of 13 amino acids that are identical to elongin A (18). Subsequently, the VHL complex binds CUL2, a member of the Cdc53 family of proteins, which is known to form ubiquitin protein ligase complexes that target cell cycle proteins for proteosomal degradation (19). CUL2 specifically associates with trimeric pVHL-elongin B-elongin C complex in vivo and in vitro (Fig. 1A; ref. 19).

The ultimate role of the pVHL-elongin B-elongin C complex is tight regulation of hypoxia-inducible factor (HIF)-1α and HIF-2α levels via ubiquitin-mediated proteosomal degradation (21). Under normoxic conditions, the HIFs are hydroxylated by HIF prolyl hydroxylases (HPH), allowing direct binding...
of pVHL-elongin B-elongin C complex and subsequent degradation by the proteasome (22, 23). HPH activity requires molecular oxygen and 2-oxoglutarate as cosubstrates (24). Under hypoxic conditions, there is decreased HIF hydroxylation by oxygen-dependent prolyl hydroxylase (25–27). Unhydroxylated HIF does not bind VHL and therefore accumulates in the cell (27–29).

The biochemical and cellular events of HIF accumulation resulting from the presence of defective VHL protein are similar to hypoxic conditions, thus explaining the pseudohypoxic model for renal carcinogenesis. Similar to hypoxia, VHL mutations result in stabilization of HIF (21). This results from the inability of pVHL-elongin B-elongin C/CUL2 complex to bind HIF. Importantly, reexpression of wild-type VHL abrogates abnormal accumulation of HIF (21, 25, 30). Additionally, restoration of wild-type VHL abrogated tumor growth in a xenograft model (31, 32). However, in case of aberrant pVHL and its inability to bind and direct HIF for degradation, HIF levels accumulate in the cell and transcriptionally regulate multiple downstream targets, such as vascular endothelial growth

Fig. 1. A, HIF degradation is dependent on hydroxylation of conserved proline residues via enzymes referred to as HPHs. Molecular oxygen (O2) is required as a cosubstrate for HPH activity. Once hydroxylated, HIF is recognized by the VHL complex, which consists of the VHL protein, elongin B, elongin C, and CUL2. Once ubiquinated, HIF undergoes oxygen-dependent degradation (ODD). B, in the context of VHL mutations, HIF accumulation occurs with transcriptional up-regulation of genes promoting carcinogenesis, including VEGF, glucose transporter protein 1 (Glut-1), platelet derived growth factor (PDGF), and transforming growth factor-β (TGF-β). C, in the context of FH mutations, fumarate accumulation is thought to inhibit HPH activity. This also results in HIF accumulation with concomitant up-regulation of the aforementioned hypoxia-responsive genes. Modified from Linehan et al. (20).
factor (VEGF), platelet derived growth factor β, erythropoietin, endothelial growth factor receptor, glucose transporter protein 1, and transforming growth factor-α (Fig. 1B; refs. 30, 33–37). Over-expression of these gene products is associated with improved vascularity, autocrine stimulation, uncontrolled growth, and survival of the cell (Fig. 1B).

**FH Gene and Its Role as a Tumor Suppressor**

As noted earlier, kidney cancer represents a heterogeneous group of neoplasms linked by a common site of origin. In HLRCC, the most recently identified hereditary kidney cancer syndrome, affected individuals are at risk for the development of leiomyomas of the skin and uterus in addition to an aggressive form of kidney cancer (38). The initial study reporting this familial syndrome identified a link between cutaneous and uterine leiomyomas, which were transmitted in an autosomal dominant fashion (39). Several years later, Launonen et al. (38) identified the association of cutaneous/uterine leiomyomas with kidney cancer. Leiomyomatous manifestations are highly penetrant in HLRCC affected individuals (40). In contrast, kidney cancer has been detected in only one third of families evaluated at the National Cancer Institute (41). However, these tumors tend to be biologically aggressive as evidenced by the finding that, in the North American cohort of HLRCC families, ~70% of HLRCC affected individuals with kidney cancer succumbed to metastatic disease within 5 years (40).

Genetic linkage analysis led to the identification of the locus associated with the development of HLRCC (38, 42, 43). The responsible locus is on the long arm of chromosome 1 (1q) and was mapped to the gene encoding the enzyme FH (44). FH, also known as fumarase, catalyzes the hydration of fumarate to form malate. Similar to VHL, FH seems to act as a tumor suppressor gene (44). Kidney cancer formation is thought to result from somatic alteration of the wild-type copy of the FH allele (44).

The exact link between Kreb’s cycle dysfunction and tumor formation remains to be determined. Given the reliance of a cell on the Kreb’s cycle for generation of energy equivalents via oxidative phosphorylation, it seems inconsistent that FH alterations would lead to tumorigenesis. Despite this apparent contradiction, alterations of succinate dehydrogenase, another Kreb’s cycle enzyme, have also been linked to a hereditary cancer syndrome. Mutations of the genes encoding three of the four subunits that comprise succinate dehydrogenase have been identified in families predisposed to the development of pheochromocytomas (45–47). This familial cancer syndrome is referred to as hereditary paraganglioma and pheochromocytoma. Succinate dehydrogenase catalyzes the Kreb’s cycle reaction immediately preceding the reaction catalyzed by FH. The identification of these two cancer syndromes has provided a unique opportunity to identify the link between mitochondrial dysfunction and tumor formation. Although the exact mechanism of tumor formation in these cancer syndromes remains to be elucidated, proposed models include diminished apoptosis, oxidative stress via generation of reactive oxygen species, and pseudohypoxic drive.

The preponderance of reports supports pseudohypoxic drive as the link between mitochondrial dysfunction and tumor formation. As noted earlier, pseudohypoxic drive refers to the activation of hypoxia-responsive pathways under normal oxygen conditions. There is evidence of pseudohypoxic drive at multiple levels in kidney tumors associated with HLRCC. Multiple investigations have examined the expression of the HIF proteins, which are key regulators of oxygen homeostasis (29, 34, 48). Pollard et al. (49) identified increased HIF-1α expression by immunohistochemistry in HLRCC kidney tumors compared with normal stroma. This finding was validated by immunoblotting of tissue lysates. HIF-1α was easily detected in tumor cell lysates, whereas no detectable HIF-1α could be identified in normal kidney (49). Isaacs et al. (24) also identified significantly enhanced expression by immunohistochemistry of both HIF-1α and HIF-2α proteins in HLRCC kidney tumors compared with normal matched renal parenchyma. Interestingly, there was evidence of preferential expression of HIF-1α compared with HIF-2α in HLRCC renal tumors.

Further evidence for the role of pseudohypoxic drive in HLRCC tumors is derived from studies examining downstream targets of the HIF proteins. Enhanced expression of glucose transporter protein 1 was identified in HLRCC kidney tumors compared with matched normal renal parenchyma (24). In addition, studies of HLRCC uterine fibroids revealed enhanced expression of VEGF (49). Consistent with this finding, HLRCC fibroids were also found to have down-regulation of the antiangiogenesis factor TSP1 (49). Up-regulation of the hypoxia-responsive gene BNIP3 in HLRCC fibroids provides further evidence of pseudohypoxic drive in HLRCC tumors (49). In a parallel fashion, multiple studies have implicated pseudohypoxic drive in pheochromocytoma formation in the context of hereditary paraganglioma and pheochromocytoma (49, 50).

Although several lines of evidence suggest pseudohypoxic drive in the genesis of tumors of HLRCC, the exact mechanism by which this occurs has not yet been defined. Unlike the VHL protein, which has a clear role in HIF degradation, there is no evidence to support a direct link between FH and HIF. The link between these two may be a consequence of the biochemical milieu resulting from loss of FH. As stated earlier, FH catalyzes the hydration of fumarate to form malate. Consequently, loss of FH may result in elevated intracellular levels of fumarate. Elevated levels of fumarate have been identified in HLRCC fibroids (49). Isaacs et al. (24) investigated the role of fumarate in regulating HIF stability. Because of the lack of a HLRCC cell line, the FH-deficient phenotype was simulated in AS49 lung carcinoma cells cotreated with exogenous fumarate as well as with a FH inhibitor. Combination treatment resulted in elevated nuclear HIF-1 levels. Notably, cotreatment with fumarate and a FH inhibitor resulted in diminished levels of the hydroxylated form of HIF. As stated previously, hydroxylation of HIF is required for VHL complex recognition and subsequent ubiquitin-mediated proteosomal degradation. Diminishment of hydroxylated HIF under these circumstances led these investigators to examine the enzymatic activity of HPH, the enzyme catalyzing the hydroxylation of HIF. Cell-free assays under various conditions showed that fumarate inhibits HPH catalytic activity. Furthermore, it was determined that fumarate acts as a competitive inhibitor of the HPH cosubstrate 2-oxoglutarate.

Taken together, these data suggest that loss of FH activity is thought to occur in HLRCC renal tumors, resulting in a...
biochemical environment that inhibits HPH activity (Fig. 1C). Under these conditions, inhibition of HPH activity may result in HIF accumulation with concomitant up-regulation of HIF downstream targets, such as glucose transporter protein 1 and VEGF. Hence, data derived from both clinical and basic studies provide a clear rationale for targeting hypoxia-responsive elements, such as angiogenic factors, in the treatment of patients with HLRCC kidney tumors.

**Clinical Translational Advances**

As the molecular pathway of the VHL gene has been dissected over the past decade, new molecular targets have been identified. This has led to progress in the development of therapeutic options for metastatic RCC. In a recent randomized placebo-controlled trial, Yang et al. (51) showed a prolonged progression-free survival in patients with metastatic RCC using the anti-VEGF antibody bevacizumab. Identification of small molecules that inhibit kinase domains of HIF downstream targets, such as sorafenib, showed significant disease-stabilizing activity for metastatic RCC with an acceptable toxicity profile (52–54). Additionally, in a recent placebo-controlled randomized trial, Motzer et al. (55) showed that multitargeted inhibition of VEGF receptor and platelet-derived growth factor receptor with SU11248 (sunitinib malate) is associated with significant response rates and reasonable tolerability. These agents were recently approved by the Food and Drug Administration for treatment of metastatic RCC. These were the first new agents approved for treatment of advanced renal cancer in more than 10 years. Current therapeutic approaches to the treatment of patients with advanced kidney cancer include interleukin-2, bevacizumab, sorafenib, and sunitinib. Potential treatment for HLRCC renal carcinomas could include agents targeting the FH/HIF pathway or agents targeting glucose uptake.

The identification of the genes involved in VHL and HLRCC expands our understanding of the molecular mechanisms of pathogenesis of kidney cancer. Although VHL and HLRCC are hereditary forms of kidney cancer, study of the mechanism of these cancer genes has provided the basis for a better understanding of the molecular pathogenesis of sporadic kidney tumors. It seems apparent that pseudohypoxic pathways are integral to both the inherited as well as the sporadic forms of kidney cancer. Further study of pseudohypoxic pathways will hopefully facilitate the identification of novel targets for molecular therapeutic strategies.

**References**

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