Seminars in Cancer Biology 23 (2013) 18–25

Contents lists available at SciVerse ScienceDirect

Seminars in Cancer Biology

journal homepage: www.elsevier.com/locate/semcancer

Review

The VHL/HIF axis in clear cell renal carcinoma

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ARTICLE INFO

Keywords:
Kidney cancer
von Hippel-Lindau
Hypoxia
Angiogenesis
VEGF

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 HIF2α appears to be a driving force in pVHL-defective clear cell renal carcinomas. In contrast, genetic and functional studies suggest that HIF1α serves as a tumor suppressor and is a likely target of the 14q deletions that are characteristic of this tumor type. Drugs that inhibit HIF2α, 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.

1. 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 is 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].

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

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http://dx.doi.org/10.1016/j.semcancer.2012.06.001

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) HIFα escapes recognition by pVHL, dimerizes with HIFβ, 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 HIFα [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.

2. The HIF transcription factor

There are three HIFα family members (HIF1α, HIF2α, HIF3α) and two HIFβ family members (HIF1β and HIF2β) [22]. HIFβ is often referred to as ARNT (aryl hydrocarbon receptor nuclear translocator). HIF1α is ubiquitiously expressed and is the canonical HIFα family member. The expression of HIF2α 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 HIF1α and HIF2α 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]. HIF3α undergoes extensive mRNA splicing and many of the resulting splice variants lack a transactivation domain and can competitively inhibit transcriptional activation by transactivation-competent HIFα protein isoforms [23–26].

Recognition of HIFα by pVHL requires that HIFα 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 HIFα ubiquitination by pVHL (and hence stability) to oxygen availability. It appears that HIF1α, but not HIF2α, can also be recognized by at least one hydroxylation-insensitive ubiquitin ligase complex that does not contain pVHL [33–36].

HIFα 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 HIFα and thereby tunes the hypoxic response [41,42]. Of note, the HIF2α CTAD is relatively resistant to FIH1 relative to the HIF1α 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 HIF1α vs HIF2α (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 HIF1α and HIF2α 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 HIF1α [46] while HIF2α 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 HIF2α in pVHL-defective renal carcinoma cells but by HIF1α in breast cancer cells [45]. Differential control of genes by HIF1α and HIF2α presumably reflects a variety of factors including differential engagement with cis-acting non-HIF transcription factors.

3. HIF2α is a renal oncoprotein

Multiple lines of evidence underscore the importance of HIF, and particularly HIF2α, in pVHL-defective clear cell renal carcinoma. For example, all *VHL*−/− clear cell renal carcinoma lines examined to date express HIF2α whereas many do not express HIF1α [52–54] (see also below). Elimination of HIF2α in *VHL*−/− clear cell lines can, like restoration of pVHL function itself, suppress their ability to form tumors in nude mice [55,56]. Conversely, overproduction of HIF2α, but not HIF1α, can override pVHL's tumor suppressor activity in such xenograft assays [48,57,58]. HIF2α 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 HIF2α in preneoplastic renal lesions in VHL patients heralds incipient transformation [5] and HIF2α single nucleotide polymorphisms (SNPs) have been linked to the risk of developing kidney cancer in the general population [63].

The importance of HIF2α 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 HIF1α activity in cells lacking pVHL. In addition, it is possible that some genes that are preferentially activated by HIF2α relative to HIF1α are particularly oncogenic.

4. HIF1α is a renal tumor suppressor

HIF1α 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, *HIF1α* deletions leading to absent protein production [52]. In other cases alternative mRNA splicing around deleted *HIF1α* exonic sequences leads to the production of aberrant HIF1α isoforms [52]. Reintroduction of wild-type HIF1α, but not these aberrant HIF1α species, in clear cell renal carcinoma lines that lack endogenous, wild-type, HIF1α, suppresses their proliferation in vitro and in vivo [48,52]. Conversely, shRNA-mediated downregulation of HIF1α in clear cell renal carcinoma lines that retain endogenous, wild-type, HIF1α, 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 HIF1α and 14q deleted tumors display a transcriptional signature indicative of decreased HIF1α activity [52–54,65]. Clear cell renal carcinoma tumors, in contrast to lines, however, frequently appear to retain a wild-type *HIF1α* allele [52]. Although the failure to document biallelic *HIF1α* 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 *HIF1α* 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