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## RENAL CANCER BIOLOGY AND NOVEL TARGETS:

### The von Hippel-Lindau Tumor Suppressor Protein and Kidney Cancer Biology

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#### Introduction

Kidney cancer is one of the 10 most common forms of cancer and is responsible for over 10,000 deaths in the United States each year. The most common form of kidney cancer is clear cell renal carcinoma. Kidney cancer can be cured by nephrectomy if detected at an early stage. Treatment of recurrent or metastatic disease, however, is largely palliative. A minority of patients with advanced disease achievable durable remissions with high-dose interleukin 2. Unfortunately, this therapy is very toxic, must be administered in specialized care centers, and one cannot yet reliably predict which patients will benefit from this therapy. In the past decade, however, new therapies that modulate molecular pathways that are deregulated in clear cell carcinoma by virtue of mutations affecting the von Hippel-Lindau tumor suppressor gene (*VHL*) have been shown to significantly delay disease progression and/or to improve survival in patients with metastatic kidney cancer. This review will provide a brief update on the functions of the *VHL* encoded protein, pVHL, as they relate to kidney cancer therapeutics.

#### VHL tumor suppressor gene

Individuals who inherit a defective copy of the *VHL* tumor suppressor gene are predisposed to a variety of tumors including vascular tumors of the central nervous system and retina called hemangio-blastomas, adrenal gland tumors (pheochromocytomas), and clear cell renal carcinomas. Tumor development in this setting is linked to inactivation of the remaining wild-type *VHL* allele, thus depriving the cell of the wild-type pVHL. Biallelic *VHL* inactivation, either due to mutation or hypermethylation, is also very common (>50%) in sporadic (non-hereditary) clear cell renal carcinomas, especially if one eliminates tumors having variable or mixed histologies.

It is clear that *VHL* inactivation, although a common event in clear cell renal carcinoma, is not sufficient to cause this disease. Indeed, a number of non-random genomic alterations, including amplification of a region of chromosome 5q and loss of most or all of chromosome 14q, are frequently observed in clear cell renal carcinomas and are presumed to conspire with *VHL* loss to cause this disease.<sup>1-5</sup> Exon resequencing efforts recently identified mutations affecting the chromatin modifying enzymes in kidney cancer as additional culprits in this disease.<sup>6</sup>

pVHL has multiple functions but the most thoroughly studied, and the one that appears most tightly related to the suppression of kidney cancer, relates to its ability to inhibit a heterodimeric transcription factor called HIF (hypoxia-inducible factor), consisting of a labile alpha subunit and a stable beta subunit.<sup>7</sup> When oxygen is present pVHL binds directly to HIF $\alpha$  and targets it for polyubiquitination and proteasomal degradation. Under low oxygen conditions (hypoxia) HIF $\alpha$  is not recognized by pVHL and so is free to dimerize with its partner protein, HIF $\beta$  (also called aryl hydrocarbon receptor nuclear translocator or ARNT). The HIF heterodimer translocates to the nucleus, binds to specific DNA sequences

(hypoxia response elements) and increases the rate of transcription of ~100-200 genes, many of which promote survival under hypoxic conditions. Included amongst these genes are genes that promote the shift from oxidative metabolism to glycolysis (that is, can promote the Warburg effect),<sup>8</sup> autophagy, erythropoiesis, and angiogenesis. The latter two classes of genes can account for two clinical features of kidney cancer, namely, its ability to produce paraneoplastic erythrocytosis and its propensity to induce angiogenesis.

pVHL has a number of other functions that, although incompletely understood at the biochemical level, appear to be at least partly HIF-independent.<sup>7</sup> For example, loss of pVHL leads to the loss of a specialized epithelial structure called the primary cilium as well as altered microtubule dynamics.<sup>7</sup> Notably, the development of visceral cysts, including renal cysts, is a feature that is shared between a number of other so-called ciliopathies and von Hippel-Lindau disease.<sup>9,10</sup> pVHL also modulates apoptosis in response to nerve growth factor withdrawal, which might account for its role in pheochromocytoma development,<sup>11,12</sup> and also appears to suppress senescence in some contexts.<sup>13,14</sup> Nonetheless, deregulation of HIF appears to be a driving force in the development of pVHL-defective kidney cancers for the reasons outlined below.

#### VHL link to kidney cancer

Genotype-phenotype correlations in *VHL* families suggest that the risk of developing kidney cancer is linearly related to the degree to which different *VHL* alleles deregulate HIF. In short, the *VHL* alleles linked to the highest risk of kidney cancer are also those that result in the highest levels of HIF.<sup>15</sup> This is in contrast to, for example, the risk of developing pheo-chromocytoma.<sup>16,17</sup> In preclinical models forced activation of HIF target genes is sufficient to override pVHL's tumor suppressor activity,<sup>18</sup> whereas suppression of HIF target genes in pVHL-defective renal carcinoma cells is sufficient to prevent tumor formation.<sup>19,20</sup>

#### Role of HIF

There are 3 HIF $\alpha$  genes in the human genome and hence "HIF" is actually a generic term. HIF1 $\alpha$  is the ubiquitously expressed, canonical, member of the family whereas the expression of HIF2 $\alpha$  is more restricted and HIF2 $\alpha$  has been less intensively studied. Both HIF1 $\alpha$  and HIF2 $\alpha$  are capable of activating transcription, while at least some HIF3 $\alpha$  isoforms appear to block HIF-dependent transcription.

There is solid evidence that HIF2 $\alpha$  acts as an oncoprotein in pVHL-defective kidney cancers and growing evidence that HIF1 $\alpha$  may, in fact, serve as tumor suppressor. For example, pVHL-defective tumors produce either both HIF1 $\alpha$  and HIF2 $\alpha$  together or exclusively HIF2 $\alpha$ .<sup>21,22</sup> Moreover, the appearance of HIF2 $\alpha$  in early renal lesions in the kidneys of *VHL* patients heralds malignant transformation.<sup>23</sup> Interestingly, HIF1 $\alpha$  resides on chromosome 14q, which is frequently deleted in kidney cancers. While HIF2 $\alpha$  can override pVHL's tumor suppressor activity in vivo, HIF1 $\alpha$  cannot.<sup>24</sup> Indeed, HIF1 $\alpha$  appears to suppress kidney cancer proliferation in vitro and in vivo.<sup>21,25</sup>

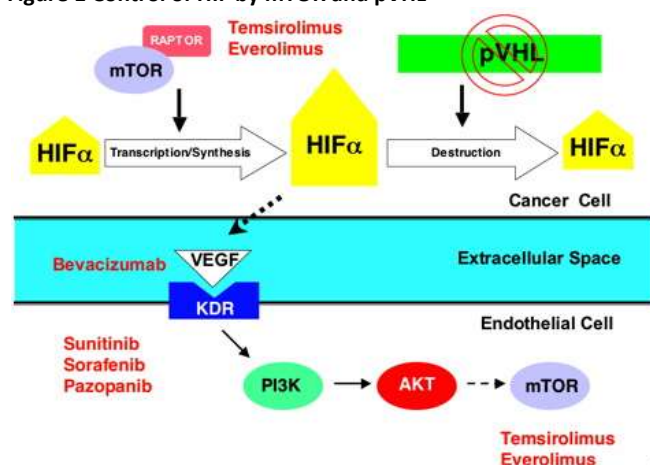
Why HIF1 $\alpha$  and HIF2 $\alpha$  would have opposite effects with respect to kidney carcinogenesis is not clear. It is clear, however, that the genes that are regulated by these two proteins are overlapping but not entirely congruent. For example, many glycolytic genes, as well

as the proapoptotic/proautophagy gene BNIP3,<sup>26</sup> are primarily controlled by HIF1 $\alpha$  while the stem cell factor Oct4 is primarily under the control of HIF2 $\alpha$ .<sup>27</sup> It is also clear that HIF1 $\alpha$  and HIF2 $\alpha$  can have opposing effects on the c-Myc oncoprotein, with the former antagonizing c-Myc function and the latter cooperating with c-Myc in certain settings.<sup>28</sup> In addition to such qualitative differences, there are likely to be quantitative differences as well. Specifically, pVHL leads to the accumulation of both HIF1 $\alpha$  and HIF2 $\alpha$ , for the reasons outlined above. Once stabilized, however, HIF1 $\alpha$  remains enfeebled as a transcriptional activator by virtue of the FIH-1 asparaginyl hydroxylase, which hydroxylates a key asparaginyl residue within one of HIF1 $\alpha$ 's two transactivation domains.<sup>29,30</sup> HIF2 $\alpha$  largely escapes this modification.<sup>31,32</sup> As a result, occupancy of a HRE by HIF1 $\alpha$  would, at least for certain HIF targets, lead to diminished transcriptional activation relative to occupancy by HIF2 $\alpha$ . In other words, HIF1 $\alpha$  could act to blunt transcriptional activation by HIF2 $\alpha$  in such a scenario.

### Treating pVHL-defective kidney cancers

The above considerations provide a conceptual framework for treating pVHL-defective kidney cancers with drugs that inhibit HIF (especially HIF2 $\alpha$ ) or HIF-target genes linked to tumorigenesis. With respect to the latter, kidney cancers have the highest levels of the angiogenic growth factor VEGF, which is a HIF-responsive gene product, of any solid tumor examined. Four drugs that inhibit VEGF (bevacizumab) or its receptor KDR (sorafenib, sunitinib, pazopanib) have now been approved for the treatment of metastatic kidney cancer based on positive clinical trial data. Although the objective response rates (measured by RECIST criteria) differ amongst these agents the percentage of patients experiencing any tumor shrinkage/disease stabilization (as measured in "waterfall plots") is remarkably similar at about 75%. Indeed, kidney cancer is arguably the most sensitive solid tumor with respect to monotherapy with VEGF inhibitors. This presumably reflects the frequent inactivation of pVHL in this setting as well as the intimate relationship between pVHL and the control of HIF-dependent genes, including VEGF (Figure 1).

**Figure 1 Control of HIF by mTOR and pVHL**



The steady state levels of HIF $\alpha$ , particularly HIF1 $\alpha$ , are positively regulated by a complex containing mTOR and Raptor (TORC1 complex), which can be inhibited with rapamycin-like drugs. pVHL targets HIF $\alpha$  for proteasomal degradation and its loss, as a consequence, leads to the HIF $\alpha$  accumulation and activation of HIF target genes such as VEGF. VEGF is a secreted angiogenic polypeptide that engages the KDR receptor on endothelial cells and

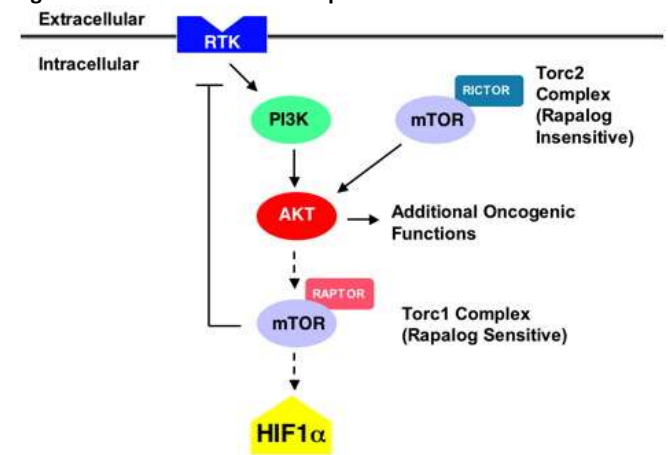
thereby promotes endothelial cell proliferation and survival. KDR signaling leads to mTOR activation.

VHL mutational status does not appear to be a highly robust predictor of response to VEGF blockade although there is a trend toward better responses amongst patients with VHL mutations.<sup>33,34</sup> It is likely, however, that many VHL "wild-type" tumors are phenotypically pVHL-defective, either because VHL hypermethylation, alterations in other pathways that phenocopy pVHL loss, or false-negative mutational readouts.

The steady-state levels of HIF $\alpha$  are determined by its rate of synthesis and by its rate of destruction (Figure 1). HIF $\alpha$  has a very high metabolic turnover rate. Accordingly, HIF $\alpha$  species are amongst the first proteins to disappear when transcription or translation are impaired. This caveat should be borne in mind when analyzing many of the "HIF1 inhibitors" described in the literature. It is very clear, however, that the transcription and translation of HIF is extremely sensitive to changes in the activity of the mTOR kinase,<sup>36</sup> which participates in two complexes called TORC1 and TORC2<sup>36</sup> (Figure 2). The former is under the control of the PI3K, AKT, TSC pathway<sup>35</sup> (Figure 2). Mutations affecting this pathway have been linked to the development of hamartomas.<sup>35</sup> TORC1 can be inhibited with rapamycin-like drugs. Notably, mTOR also plays a role downstream of KDR in endothelial cells (Figure 1). In short, inhibition of mTOR might downregulate HIF within pVHL-defective tumor cells as well as blunt VEGF signaling (Figure 1). It has also been shown that pVHL-defective cells are highly sensitive to drugs, including rapamycin, that induce autophagy.<sup>37,38</sup>

Some or all of these considerations likely relate to the fact that two rapamycin-like drugs, temsirolimus and everolimus,<sup>39,40</sup> have proven to be beneficial in kidney cancer patients who have high risk-features or who have failed KDR inhibitors, respectively.

**Figure 2 TORC1 and TORC2 complexes**



mTOR exists in two complexes, called TORC1 and TORC2. The former contains the protein Raptor and can be inhibited with rapamycin-like drugs. The latter contains the protein Rictor and is relatively insensitive to rapamycin-like drugs. The Raptor complex feedback inhibits signaling by particular receptor tyrosine kinases (RTK). Accordingly, rapamycin-like drugs can lead to paradoxical increases in RTK signaling, including signals flowing through the AKT kinase.

Two factors might conspire to limit the overall effectiveness of rapamycin-like inhibitors for the treatment of kidney cancer. First, in other settings blockade of TORC1 has caused a paradoxical increase in upstream receptor tyrosine kinase signaling due to a loss of TORC1-dependent negative feedback loop (Figure 2).<sup>41,42</sup> Our preliminary evidence indicates that this might occur in kidney cancer cells as well (Sungwoo Lee and W.G.K-unpublished data). Second, TORC1 inhibition seems to preferentially downregulate HIF1 rather than HIF2 $\alpha$ .<sup>43</sup> Instead, HIF2 appears to be more sensitive to loss of TORC2, which is largely (but not completely), inured to rapamycin-like drugs.<sup>43</sup> A number of dual TORC1/2 inhibitors are now being developed and preliminary data in preclinical kidney cancer are encouraging.<sup>44</sup> As an alternative, Iliopoulos and colleagues have identified small molecules that suppress HIF2 $\alpha$  translation in an mTOR-independent manner.<sup>45,46</sup> Whether these compounds can be converted into therapeutics remains to be determined.

### Conclusion

It is assumed that more complete inhibition of VEGF signaling will translate into enhanced clinical activity in patients with kidney cancer. In this regard, a number of more potent and more selective VEGF inhibitors are in clinical development and might eventually replace the existing VEGF inhibitors by virtue of superior VEGF blockade, decreased toxicity and/or enhanced ability to be combined with other agents. A caveat, however, is that on-target toxicities, such as microangiopathy<sup>47</sup> and cardiomyopathy,<sup>48,49</sup> will likely limit the degree to which VEGF signaling can be safely blocked in patients. Indeed, a recent trial of combined bevacizumab and sunitinib was halted because of such microangiopathic changes (<http://www.cancernetwork.com/rcc/content/article/10165/1265295>).

Virtually all kidney cancer patients eventually develop resistance to VEGF inhibitors, although the underlying resistance mechanism(s) remain poorly understood. Fortunately, some forms of resistance to VEGF blockade can be circumvented by simply changing the choice of inhibitor. Clearly, however, a more detailed understanding of the molecular circuits that allow kidney cancers to escape VEGF inhibition is needed. In one recent study, which awaits confirmation, enhanced secretion of interleukin-8 was implicated as a potential resistance mechanism.<sup>50</sup> Interestingly, interleukin-8 has been shown to conspire with VEGF before to enhance angiogenesis.<sup>51</sup> Clearly additional targets, and the agents with which to attack them, are needed in kidney cancer so as to build more effective combinations moving forward. It is anticipated that such targets will emerge from a variety of sources including cancer genome resequencing projects, unbiased chemical and genetic screens aimed at identifying vulnerabilities created upon VHL loss, and identification of the genetic alterations, including copy number changes that, together with VHL loss, are responsible for this disease.

### Discussion

**Dr. Atkins:** In many tumors HIF-1 alpha is believed to be associated with poor prognosis, it is hypoxia driven. This situation seems to be different in kidney cancer. To what extent are we looking at HIF-1 alpha as a tumor suppressor in a context-dependent fashion where it is a tumor suppressor if Hif-2 alpha is up or if VHL is lost and the resultant downstream target genes are up? To what extent is that also related to other potential genetic changes in RCC?

**Dr. Kaelin:** This brings up the point of correlation versus causation. When people in Biotech ask where they should test a HIF-1 alpha inhibitor, I say I do not know because almost all of the data is guilt

by association. You may have an aggressive tumor that is growing rapidly, outgrows its blood supply, gets hypoxic, up-regulates Hif-1 alpha and ergo Hif-1 alpha is associated with a bad prognosis. But of course that does not mean that Hif-1 alpha is causing the bad behavior; it could be the result of the bad behavior. I do not know of a solid tumor today where I can say definitively Hif-1 alpha is a driver. Now, there are some animal models where you can make a case that Hif-1 alpha is acting as a driver in a particular cell line growing subcutaneously in a nude mouse, but I think the data are pretty soft at the moment in terms of Hif-1 alpha.

**Dr. Atkins:** Might there be effects of Hif-1 alpha upregulation that interact with the stroma such as increases in LDH, or decreases in immune function, so that it may be associated with poor prognosis by creating an environment that allows the tumor to grow? You might not see it when you are only testing at the tumor cell level.

**Dr. Kaelin:** Well, yes. I think Hif-1 alpha has plausibility on its side and correlations on its side, but I do not know that in all cases we can say definitively that it is the driver. And this is not the first time Hif-1 alpha has paradoxically scored as a tumor suppressor. There are other models now where knocking out Hif-1 alpha promotes tumor growth.

**Dr. Rathmell:** There was a paper in the past year suggesting that histone demethylases were mutated in many RCCs. Can you comment on their potential role as therapeutic targets in this disease?

**Dr. Kaelin:** The nice thing about working with histone-methylation is that both the methyltransferases and the demethylases are potentially drug-able, in contrast to, for example, the situation with kinases and phosphatases where if you lose the kinase you cannot develop a drug that targets the phosphatase. We have done an experiment recently where in tumors that lack the MEN1 methyltransferase complex when we block the corresponding demethylase, tumor growth is diminished. So first of all I think we have to figure out whether all of these mutations that were just reported were all loss of function or whether some are gain of function. But then I think to your point I think potentially we can play games on both sides of the equation. So if it is a gain of function methyltransferase mutation then we can target the methyltransferase with a drug. If it is a loss of function methyltransferase mutation then you want to drug the demethylase that acts as the counterbalance to that.

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