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VHL Gene Mutations in Renal Cell Carcinoma: Role as a Biomarker of Disease Outcome and Drug Efficacy

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Abstract

The therapeutic landscape for renal cell carcinoma has changed drastically over the last several years with the emergence of molecularly-targeted therapies. With previous prognostic and predictive tools based on studies of patients treated with cytokine therapies, confirmation of these prior methods and discovery of new markers in this new era of targeted therapy is of great importance. Alteration of the *VHL* gene by mutation, loss of heterozygosity, and promoter methylation has been found to be important to renal cell cancer pathogenesis. In this review, we will discuss the potential role of *VHL* mutation as a prognostic and predictive marker for renal cell cancer.

Introduction

Renal cell carcinoma (RCC) is an important disease with an expected 54,390 new cases predicted in 2008 with over 13,010 deaths.[1] There are several histologic subtypes of RCC including clear cell, papillary, chromophobe, and oncocytoma, with the clear cell variant being the most common, and making up nearly 70% of cases of RCC. Unfortunately, cure for RCC is only available to those with limited stage disease which can be surgically resected as to date systemic therapies cannot eradicate the cancer if it has spread distantly. In advanced stages, systemic therapies are given with the intent to provide debulking and disease stabilization and improvement in symptom control and survival. No conventional cytotoxic chemotherapy agents have demonstrated significant activity in this disease as monotherapy or combinations. [2] Previously systemic therapies consisted only of immune based therapies, such as the cytokines interferon and interleukin-2. However, the utility of cytokine therapy was limited by the small subset of patients with RCC who would derive clinical benefit and the limitation of widespread severe toxicities of these treatments which restricted their use[3]. These issues led to the advent of prognostic and predictive scoring systems to help with improved patient selection for immune-based therapy. One well known system is the Motzer or MSKCC criteria which implements clinical features such as functional status, prior nephrectomy, and blood levels of hemoglobin, calcium, and LDH at the time of presentation with metastatic disease [4]. These criteria and others like them were validated during this era of cytokine therapy and are still widely used today.

However, within the last several years, new molecularly targeted agents for the treatment of RCC have emerged from an improved understanding of the molecular biology of this disease. [5] One crucial finding has been the discovery of the *VHL* gene and its importance in regulation of the hypoxia pathway via the hypoxia inducible factors (HIFs).[•6] These recently introduced targeted agents which modulate this VHL-HIF pathway include the FDA approved multi-

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targeted tyrosine kinase inhibitors, sunitinib and sorafenib as well as the mTOR inhibitor, temsirolimus. These agents have shown superiority to previous cytokine therapies and are now part of the arsenal used in the standard treatment of RCC[••7–9]. Additional targeted agents likely to be considered for FDA approval on the immediate horizon include the mTOR inhibitor everolimus and the anti-angiogenic agent, bevacizumab. Many others are in active development. Since there has been a molecularly targeted shift in the RCC treatment paradigm and with multiple treatment choices available, further understanding and discovery of potential prognostic and predictive tools is paramount. Although validation of previous criteria, such as the previously described Motzer criteria is important, a further implementation of RCC molecular biology knowledge is ideal for new prognostic and predictive marker discovery and development in this new age of targeted therapies.

Rationale of *VHL* Mutational Status as a Biomarker

von Hippel-Lindau (VHL) disease is an autosomal dominant hereditary disorder characterized by retinal and CNS hemangioblastomas, pheochromocytoma, and clear cell renal cell carcinoma.[10] With the discovery of the underlying gene linked to this disorder, the *VHL* gene, a revolution in clear cell RCC cancer biology has followed. The *VHL* gene which is located on chromosome 3p25, encodes for a 213 amino acid tumor suppressor protein that was found to be a key player in the regulation of the hypoxia response pathway, which is vital to tumor survival in low oxygen conditions.[11,12] The *VHL* protein (pVHL) functions as part of an E3 ubiquitin ligase which ubiquitylates a family of proteins known as the hypoxia inducible factors or HIFs and targets them for degradation by the proteasome.[13,14] In the absence of functional pVHL protein, HIF is allowed to accumulate and translocate to the nucleus where it acts as a transcription factor. Transcriptional targets of HIF include a variety of pro-tumorogenic genes such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), multidrug resistance pump (MDR-1), and erythropoietin (EPO).[15] It is by expression of these and other HIF target genes that RCC receives its phenotype of chemotherapy resistance, invasiveness, and vascularity. Functional loss of pVHL has therefore been linked as a sentinel event in clear cell RCC development and progression, with supportive evidence shown by re-introduction of functional wild-type *VHL* into a renal cell cancer cell line lacking *VHL* resulting in an inability to form tumor xenografts[16] as well as renal cancer cell growth inhibition *in vitro*[17]. Given the tight link to RCC tumorigenesis, the presence or absence of a *VHL* mutation seems to be a natural object for the development of a predictive or prognostic marker for this disease. In fact, functional loss of pVHL exists in the majority of sporadic cases of clear cell RCC which is the predominant variant of this disease. This functional loss may occur by a variety of methods of *VHL* gene alterations including somatic mutation events, loss of heterozygosity (LOH) by allele deletion with concomitant alteration of the contralateral gene, and epigenetic interference such as promoter hypermethylation (Figure 1).

VHL Alterations in Hereditary and Sporadic RCC

Distinct mutation events in hereditary von Hippel-Lindau disease lead to four separate phenotypes which are classified as types 1, 2A, 2B, and 2C. In this classification system type 1 is distinguished from type 2 due to their lack of pheochromocytoma development. Additionally, type 2A is at low risk of RCC development while type 2B is at increased risk. Type 2C is unique in that this variant has a tendency towards pheochromocytoma formation only. Several studies of families harboring these germline *VHL* mutations have resulted in the discovery that a predominance of large deletion events with premature protein truncation or complete protein loss occurs in type 1 patients and missense mutations commonly occurs in type 2 patients.[18,19] Analysis of missense mutations has been difficult due to presence of missense mutations signifying a significant risk of RCC in some cases and no significant risk

in others. Additionally, *in vitro* evidence suggests that missense mutations in *VHL* such as those associated with the Type 2 subtypes of VHL disease vary significantly with respect to the degree of HIF regulation (Rathmell 2004, Li 2007), with mutations less tightly linked with development of RCC displaying a milder deficiency in HIF regulation.

Concordant with the high risk of RCC associated with VHL disease, alteration in the *VHL* gene is a common finding in cases of sporadic clear cell RCC. Several studies have linked sporadic cases of clear cell RCC with both somatic *VHL* gene mutations as well as loss of heterozygosity.[20–23] The frequency of *VHL* mutation events in these studies ranges from 46 – 82% of sporadic cases, with mutational events including a heterogeneous assortment of frameshift deletions, frameshift insertions, missense, and nonsense mutations. Additionally, in each of these studies the vast majority (85–98%) of cases containing somatic mutations also exhibited contralateral allele loss or loss of heterozygosity, consistent with a two-hit hypothesis of tumorigenesis (Table 1). Other studies have evaluated *VHL* promoter methylation events in sporadic RCC with hypermethylation rates ranging from 11–19% of cases.[24–26] Together, these data suggest that disruption of the *VHL* tumor suppressive activity may account for the vast majority of cases of sporadic RCC in many series.

VHL Mutations as a Prognostic Biomarker

As a broad body of evidence points to *VHL* as a major tumor suppressor in this disease, it is logical to assume that *VHL* mutation might have important implications for disease prognosis. Somatic *VHL* mutation events and their impact on prognosis have been studied in a variety of case series. Findings from these studies have been mixed, demonstrating conflicting results when used to develop prognostic algorithms. The results of this efforts to link VHL mutation to disease prognosis are summarized in Table 1.

Brauch et al published a series of 227 sporadic clear cell RCC tumors that were analyzed for *VHL* altering events[27]. The combined rate of *VHL* mutation and promoter hypermethylation was 45% and the rate of LOH in this study was 93% of cases. This high rate of LOH suggests an even higher rate of *VHL* disruption, or the presence of an alternate tumor suppressor with tight genetic linkage to *VHL*. In this series, the presence of *VHL* mutation or hypermethylation events were significantly correlated with pT3 tumor grade (P=0.009). This was the first publication to demonstrate an association of *VHL* alteration events with a prognostic factor, in this case linking *VHL* alteration to a poor risk factor (pT3 tumor stage). In a separate study, 113 cases of sporadic clear cell RCC were evaluated for *VHL* mutational status as well as the proliferative marker, Ki-67, and microvessel density (via CD-34 staining).[28] Evaluating overall *VHL* mutations, there was no difference in outcomes for those with or without mutations. However, on multivariate analysis, mutations which were predicted to result in loss of pVHL protein expression or function resulted in poorer cancer specific survival (P=0.02). *VHL* mutational status was not associated with tumor grade, stage, microvessel density, or proliferative index (Ki-67). Loss of function mutations in this study were defined as events which altered *VHL* transcriptional read through such as nonsense and frameshift mutations predicted to interfere with protein stability and one missense mutation which altered the *VHL* start codon. Patients with other unpredictable mutation events that did not fall into these categories showed no significant difference in clinical outcome. These findings, while provocative, are difficult to interpret due to the small number of cases (12) that contained these loss of function mutations. Additionally, as mentioned above, emerging data suggests that subsets of *VHL* missense mutations will be predicted to have significant effects on HIF regulation, a subtlety which complicates assumptions about the impact of missense mutations on disease prognosis and has not been scrutinized to date in any series.

VHL alterations have also been linked to positive outcomes in several studies. Patard, et al performed *VHL* mutational analysis and carbonic anhydrase IX (CA-IX) staining on 100 clear cell RCC nephrectomy specimens.[29] Of these, fifty-eight were found to contain *VHL* mutations. In univariate analysis, patients who had *VHL* mutations in their tumors had improved 2 year overall survival rates compared to those who did not (76% versus 51%, p=0.037). Cancer specific survival showed a similar positive trend but did not reach significance (p=0.079). Additionally, those that showed high CA-IX staining, in addition to *VHL* mutation, had an even further improvement in 2 year survival rate (86%). In multivariate analysis, high levels of CA-IX staining continued to remain as a significant independent prognostic factor, while *VHL* mutation presence did not (HR 1.39 95% CI 0.59–3.31, p=0.455). CAIX, as a target of HIF transcriptional activation, may be an indicator of functional *VHL* loss, indicating *VHL* events which impart a significant failure of HIF suppression.

In a separate study, 187 Japanese patients with clear cell RCC were analyzed for *VHL* alteration events including mutation and promoter hypermethylation.[30] *VHL* mutation events were present in 98 patients (52%) and hypermethylation in 10 patients (5.3%). *VHL* alterations were associated with improved outcomes in those with Stage IIII disease, but no correlation was found in those with Stage IV disease, suggesting that VHL mutation may contribute most at the stages at which determinations of metastatic disease potential are made. Also importantly, these metastatic patients were treated with immunotherapy or cytotoxic chemotherapy and therefore it is unclear if the results would have been similar in the current landscape of treatment with targeted agents.

Other clinical trials have shown no correlation between *VHL* mutational status and prognosis. Kondo et al reported 240 cases of sporadic RCC tumors that were analyzed for mutational status, methylation and LOH.[31] Rates for these events within this cohort were 51%, 5%, and 90% respectively. No correlation was identified between *VHL* altering events and prognostic features such as tumor size, grade, or rate of metastasis. In another series of 185 cases of clear cell RCC evaluated for *VHL* mutation and methylation events (57.3% with *VHL* alteration, 42.7% without), there was no significant difference noted in cancer specific survival.[32] Finally in a series of 96 sporadic RCC cases reported by Gimenez-Bachs, 21.9% of patients were positive for a *VHL* mutation and there was no correlation between the presence of mutation and tumor stage, size or histological grade.[33]

Ultimately, it is not clear that RCC associated with a *VHL* gene mutation has specific prognostic value. Studies to date have been limited by the available technology of the time for identifying mutation or methylation patterns in *VHL*, as well as by potential confounders such as the stage of the tumors analyzed and the potential influence of *VHL* missense mutations without variable effects on HIF regulation. What is clear is that *VHL* gene mutation is tightly linked with the clear cell histology subtype, and that not all tumors of this class will display *VHL* mutation. Correlating *VHL* mutation with clearly defined prognostic patterns requires highly defined samples within highly annotated data sets which unfortunately exist only rarely. Future studies will likely include *VHL* mutation as a prospectively defined stratifying factor, and necessary to eventually clarify this issue.

VHL Mutation as a Predictive Biomarker

There have been very few studies regarding *VHL* gene alteration as a potential predictive marker for response to therapy. Chiefly, this stems from the long dearth of effective therapy for the majority of patients with renal cell carcinoma, leaving the field now in a position of needing to catch up with recent advances in treatment. It was established that clear cell histology, which is most closely associated with *VHL* mutations, and in particular tumors with alveolar features and no evidence of papillary or granular features, were the most likely of all

histologic categories to respond to interleukin-2 (IL-2) therapy.[34] This trend translated a survival benefit as well, and led to the future exclusion of patients with non-clear cell histology from treatment with high dose IL-2. Although suggestive, this data does not directly implicate *VHL* mutation as a predictive marker of response to cytokine therapy.

Association of *VHL* mutation with the risk of recurrence following nephrectomy has been evaluated in a series of 56 patients with clear cell RCC who underwent nephrectomy.[35] Sixteen of these patients (29%) had findings of either *VHL* somatic mutation or methylation events, a surprisingly low percentage compared to other studies, which raises concerns that *VHL* gene alterations may have been unrecognized among the wild type group. In this investigation, there was no association of *VHL* mutation or methylation status with either progression-free survival or overall survival. However, further analysis of a subset of these sixteen *VHL* mutations that predicted for loss of function showed that patients with these more "severe" mutational types had a significantly decreased progression-free survival (P=0.016) and overall survival (p=0.046). Patients that were treated with immunotherapy showed no significant correlation between presence of any *VHL* mutation and overall survival. When specific types of mutations were evaluated, patients with missense mutations had a tendency toward better cytokine response. Unfortunately, this trial can only be considered hypothesis generating due to the very small number of *VHL* mutations analyzed.

In a more recent study, Choueiri et al evaluated 123 patients with metastatic clear cell RCC who had undergone treatment with any VEGF targeted therapy (including the receptor tyrosine kinases: sorafenib, sunitinib, axitinib; or the monoclonal VEGF antibody: bevacizumab). [•36] DNA was extracted from archival tumor samples and analyzed for *VHL* mutations. If wild-type *VHL* was identified, then analysis of methylation status was performed. In this series, 60 patients (49%) had *VHL* mutations discovered, with 42% of these events in exon 1, 32% in exon 2, and 27% in exon 3. Additionally 12 patients (10%) had promoter methylation noted in the setting of wild type *VHL*. Overall the response rate for the entire series of patients was 37% with 2 patients obtaining a complete response and 43 patients with a partial response. In patients with evidence of either promoter methylation or *VHL* mutation, the response rate was 41% compared to 31% in the wild-type group (p=0.34). Upon multivariate analysis, patients with *VHL* mutational events which predicted for pVHL loss of function obtained a significant response rate of 52% compared to those with wild-type *VHL* who had a response rate of 31% (p=0.04). Specifically, the responses seen in patients treated with the very potent VEGF receptor inhibitors sunitinib or axitinib were independent of *VHL* mutational status. Conversely, no responses were noted in patients with wild-type *VHL* treated with sorafenib or bevacizumab. The authors hypothesized that these treatment specific findings may be due to off-target effects of sunitinib and axitinib or that they may be more potent VEGF receptor inhibitors, with effects that supercede the VEGF upregulation predicted to be associated with a loss of function *VHL* mutation. Despite these disease response correlations, presence of a *VHL* gene mutation was not correlated with either progression free or overall survival. This data set represents an expanded analysis initially reported by Rini, et al which in a small pilot study suggested a trend toward improved progression free survival upon treatment with VEGF targeted therapy in patients with loss of function *VHL* mutations.[37] Finally, in a much smaller cohort (n=13) of RCC patients treated with axitinib no correlation was seen between somatic *VHL* mutational status and response.[38] Although each of these investigations has limitations, in terms of VEGF targeted therapy, *VHL* mutation status as a predictive biomarker of response or survival remains to be established.

VHL gene mutation has also been evaluated as a potential predictive marker for mTOR directed therapy in a recent study. In this report by Cho et al, archival tumor specimens were evaluated from 20 patients who received treatment for advanced RCC with temsirolimus.[39] In this small series, no correlation was seen between *VHL* mutation and treatment response. However

protein expression of phospho-AKT and phospho-S6, two important proteins indicating activity of the mTOR pathway were positively associated with response to mTOR directed treatment.

Conclusions

The discovery of *VHL* mutation as an integral component of the development of the great majority of sporadic renal cell carcinomas has been a defining feature of the therapeutic revolution that has occurred for this disease. Drugs of multiple classes have been developed which expoit targets tightly linked with the deregulated HIF signaling pathway. With increasing treatment options becoming available for the treatment of RCC, there is now a great need for the development of biomarkers to help guide treatment decisions. Previous prognostic scoring systems have utilized clinical findings such as performance status and simple laboratory findings. Although these scoring systems may continue to be useful in this new era of targeted therapy, improvement upon prognostic and predictive tools is necessary. Molecular targets, such as mutational status, have proven prognostically useful in other disease states, most notably in the case of lung cancer where activating mutations in the EGF receptor have been tightly associated with the therapeutic efficacy of EGFR targeted therapy.[40] As clear cell renal cell cancer is frequently associated with genetic alteration of *VHL* via somatic mutation, promoter hypermethylation, and/or LOH, the presence or absence of *VHL* alteration would seem to be an ideal potential biomarker for therapeutic strategies which impinge upon this signaling pathway. Unfortunately, such a correlation has not been easy to discern. Studies to-date have shown mixed results in regards to *VHL* alterations having a correlation to prognosis, known prognostic factors, or treatment outcomes. Therefore, currently there seems to be no role for screening tumors for *VHL* mutation outside of the setting of a clinical trial.

Clear limitations have included limited sample size, a heterogeneous population mixed with multiple stages of disease and varied treatments. Additionally, methods of *VHL* mutation detection are not uniform across the published literature which may explain some discrepancy in *VHL* detection rates and may skew attempts to draw statistically significant correlations. As methods for detection of *VHL* gene alteration events have been improved, generally higher rates of events are being discovered. If over time it becomes clear that the vast majority if not all of patients with clear cell RCC have *VHL* alteration events, then this finding would cease to become a potentially useful predictive or prognostic marker as the finding would be unable to discriminate significantly sized subgroups of patients for treatment efficacy.

An additional confounding factor to the utilization of *VHL* mutation as a predicitive biomarker of response to therapy may be the targeted nature of drugs currently used to treat kidney cancer. As we have seen for mTOR directed therapy, definitive evidence of pathway activation conveys a greater potential for response to appropriately targeted therapy. Therapy targeting VEGF or the VEGF receptor lies several steps removed from a *VHL* mutation, and in fact, tumor-specific VEGF expression can be induced by a wide variety of mechanisms in addition to *VHL* mutation, such as NO levels, p53 activation, and growth factor signaling.[•41] Therefore, such correlations may be limited by alternate genetic events that promote a tumor cell environment that favors treatment with VEGF targeted therapy. This is apparent by the observations that drugs targeting VEGF signaling have been found to be effective in a wide array of tumor types which have never been associated with *VHL* mutation.[42–44] Conversely, the effects of the tyrosine kinase inhibitors on many alternate targets in addition to the VEGF receptor may negate statistically significant correlations. Relevant to VEGF-targeted therapy are tumor or serum assessments of the activity of that ligand/receptor pair, as are currently ongoing. It may not be until therapeutic strategies specifically achieve successful replacement of pVHL cellular activities that tight correlations with *VHL* mutation may be detected. At this point *VHL* mutation remains the hallmark of clear cell RCC, and will continue to be interesting as a prognostic tool.

Further evaluations of the role of *VHL* alterations as a predictive biomarker may be challenging and need to be performed within well designed large clinical trials utilizing state of the art detection methods and modern therapies.

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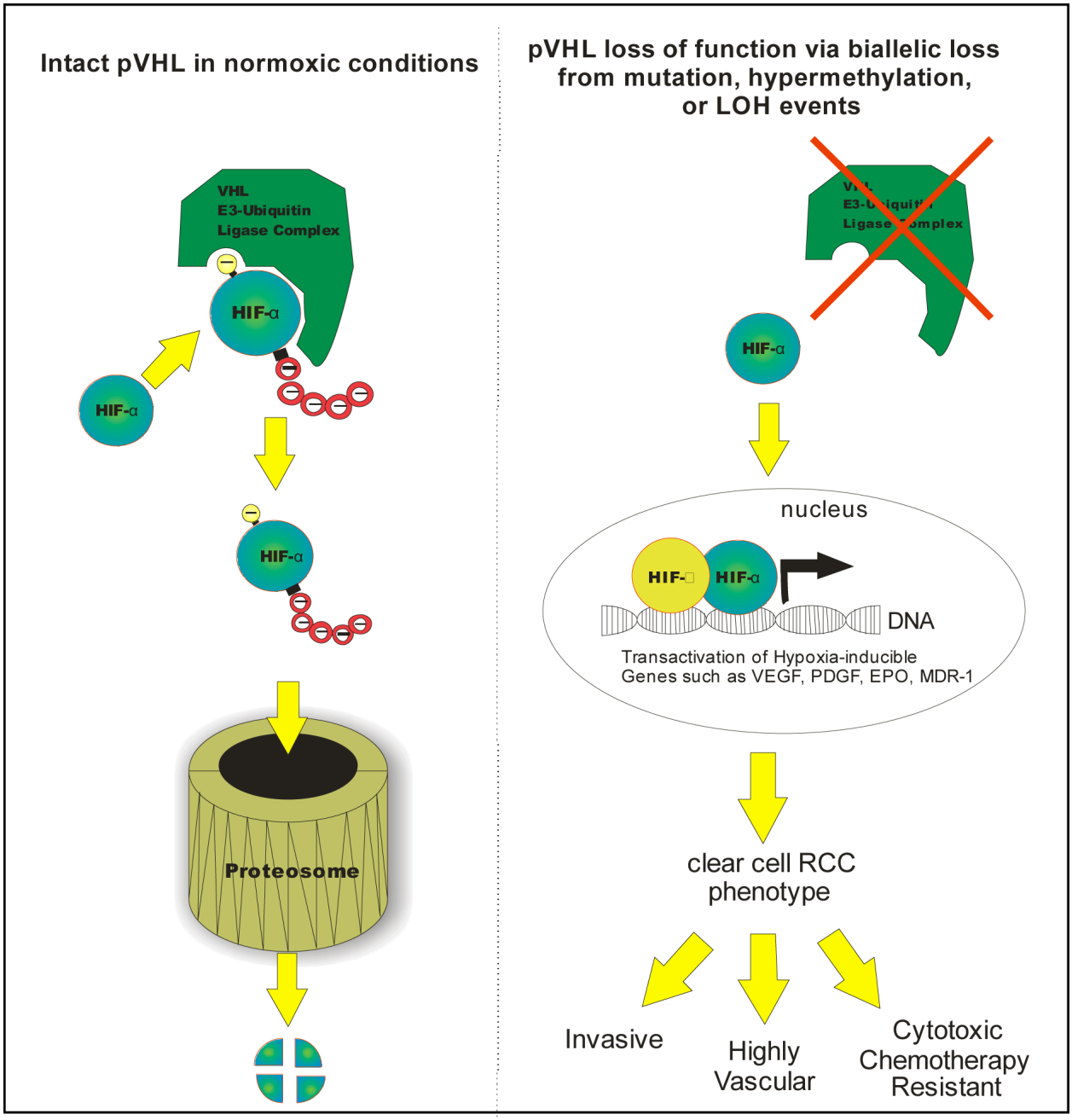

Figure 1.

Under normal oxygen conditions (left side), the VHL protein forms a complex that exhibits E3 ubiqtuitin ligase complex activity, recognizing prolyl hydroxylated HIF-α and marking it with a polyubiquitin tail. The cellular proteasome recognizes and degrades polyubiquinated HIF-α. In the absence of functional VHL (right side), HIF-α accumulates and migrates to the nucleus where it joins with HIF-β and CBP to initiate transactivation of hypoxia inducible genes, leading to the characteristic phenotype of clear cell renal cell cancer (RCC).

Table 1

Summary of key clinical trials evaluating VHL gene alterations such as mutation, hypermethylation, or loss of heterozygosity(LOH) and correlation with prognostic or predictive findings. ccRCC=clear cell renal cell carcinoma VEGF=vascular endothelial growth factor

Study	Number of cases/Histologic Subtype included	Rate of VHL Mutation	Rate of Hypermethylation	Rate of LOH	Prognostic or Predictive Findings
Brauch, et al	151/ccRCC	45%	17%	93%	*VHL* mutation or hypermethylation was significantly associated with pT3 tumor stage (poor risk factor)
Yao, et al	187/ccRCC	52%	5.3%	Not reported	*VHL* alterations correlated with improved clinical outcomes for Stage I–III but not Stage IV in patients treated with immunotherapy
Schraml, et al	113/ccRCC	34%	Not reported	Not reported	Loss of function mutations associated with worse clinical outcomes
Patard, et al	100/ccRCC	58%	Not reported	Not reported	Patients with a *VHL* mutation had a significantly improved 2 year survival
Kondo, et al	240/sporadic RCC cases	51%	5%	90%	No correlations found between mutational status and prognostic factors
Gimenez-Bachs, et al	96/sporadic RCC cases	21.9%	Not reported	Not reported	No correlations found between mutational status and prognostic factors
Smits, et al	185/ccRCC	52.4% (with loss of function mutations)	10.9%	Not reported	No correlations found between mutational status and prognostic factors
Kim, et al	56/ccRCC	19.6%	14.2%	Not reported	Loss of function mutations associated with poorer PFS and OS. No correlation between *VHL* alteration and immunotherapy response
Choueiri, et al	123/ccRCC	49%	10%	Not reported	Loss of function mutations were associated with improved response to VEGF targeted therapy. No association with PFS and OS.
Gad, et al	12/ccRCC	16.6%	Not reported	Not reported	No correlation between *VHL* mutation and axitinib response