Predictive Biomarkers and Personalized Medicine

The Role of Aberrant VHL/HIF Pathway Elements in Predicting Clinical Outcome to Pazopanib Therapy in Patients with Metastatic Clear-Cell Renal Cell Carcinoma

Toni K. Choueiri1,2,3, André P. Fay1, Robert Gagnon5, Ying Lin4, Brittany Bahamon2, Victoria Brown2, Jonathan E. Rosenberg5, Thomas E. Hutson6, Katherine L. Baker-Neblett4, Christopher Carpenter4, Yuan Liu4, Lini Pandite4, and Sabina Signoretti1,2,3

Abstract

**Purpose:** Inactivation of von Hippel-Lindau (VHL) gene in clear-cell renal cell carcinoma (RCC) leads to increased levels of hypoxia-inducible factors (HIF) and overexpression of HIF target genes, such as VEGF and others. VEGF-targeted agents are standard in advanced clear-cell RCC but biomarkers of activity are lacking.

**Experimental Design:** We analyzed tumor tissue samples from metastatic clear-cell RCC patients who received pazopanib as part of clinical trial VEG102616. We evaluated several components of the VHL/HIF pathway: VHL gene inactivation (mutation and/or methylation), HIF-1α and HIF-2α immunohistochemistry staining, and HIF-1α transcriptional signature. We evaluated the association of these biomarkers with best overall response rate (ORR) and progression-free survival (PFS) to pazopanib, a standard first-line VEGF-targeted agent.

**Results:** The VEG102616 trial enrolled 225 patients, from whom 78 samples were available for tumor DNA extraction. Of these, 70 patients had VHL mutation or methylation. VHL gene status did not correlate with ORR or PFS. Similarly, HIF-1α (65 samples) and HIF-2α (66 samples) protein levels (high vs. low) did not correlate with ORR or PFS to pazopanib. The HIF-1α transcriptional signature (46 samples) was enriched in tumors expressing high HIF-1α levels. However, the HIF-1α gene expression signature was not associated with clinical outcome to pazopanib.

**Conclusions:** In patients with advanced clear-cell RCC, several potential biomarkers along the VHL/HIF-1α/HIF-2α axis were not found to be predictive for pazopanib activity. Additional efforts must continue to identify biomarkers associated with clinical outcome to VEGF-targeted agents in metastatic RCC. Clin Cancer Res; 19(18); 5218–26. ©2013 AACR.

Introduction

Approximately 64,000 new cases of kidney cancer are diagnosed each year in the United States and 25% to 30% of these result in death (1). RCC is the most common subtype of kidney cancer and accounts for approximately 80% to 90% of cancers that arise in the renal parenchyma (2).

Distant recurrence after surgery is common, and 20% to 30% of patients present with de novo metastatic disease.

Germline mutations in the von Hippel-Lindau (VHL) gene are associated with a predisposition to develop hemangioblastomas of the retina, cerebellum, or spinal cord, and visceral cysts of the kidney and pancreas, as well as a variety of solid tumors, including clear-cell RCC (ccRCC; refs. 3, 4). Interestingly, somatic VHL mutations and chromosome 3p loss (VHL gene site locus) are found in the majority of sporadic ccRCC (5).

The VHL protein (pVHL) is a component of the ubiquitin-mediated proteolysis pathway that is important for degradation of many cellular proteins, including hypoxia-inducible factors (HIF; ref. 6). HIFs consist of 2 subunits (HIF-1α and HIF-2α) and induce the transcription of several genes that regulate angiogenesis, such as VEGF and platelet-derived growth factor (PDGF; refs. 7–9). Consistent with these findings, clinical studies of agents that target the VEGF ligand or the VEGF tyrosine kinase receptors in advanced RCC have yielded positive results leading to the approval of several agents in this disease (10), based on improved progression-free survival (PFS) rather than on overall...
Translational Relevance

Elucidation of the function of the VHL tumor suppressor gene and other regulators of angiogenesis, such as hypoxia-inducible factors (HIF) and its downstream target, VEGF, has led to important advances in the treatment of metastatic renal cell carcinoma (RCC). In the past few years, VEGF-targeted therapies became the standard in metastatic RCC treatment, showing significant improvements in clinical outcomes. An important challenge facing targeted therapies includes the selection of patients who are most likely to have clinical benefit. As a consequence, the establishment of biomarkers may help in understanding the biology underlying RCC and predicting response to therapies that target angiogenesis. In this article, we evaluated several potential biomarkers along the VHL/HIF-1α/HIF-2α axis to predict response and clinical outcomes to pazopanib, an oral, multi-kinase inhibitor of VEGF receptors.

Survival (OS). With multiple available approved agents that have not been compared head-to-head, coupled with the fact that no biomarkers have been developed to guide treatment decisions, the choice of a particular anti-VEGF therapy depends on the clinical setting (previously treated or untreated), the prognostic stratification (good/intermediate vs. poor), and the histology (clear-cell vs. non-clear-cell; refs. 10, 11).

The biology underlying RCC with the central role of the VHL/HIF/VEGF axis sheds new light on potential molecular biomarkers to predict response to therapies that target angiogenesis and potentially guide treatment decisions. For example, Choueiri and colleagues showed that certain mutations in the VHL gene may predict response (but not PFS or OS) to VEGF-targeted agents (12). Gordon and colleagues identified 2 subgroups of VHL-deficient tumors distinguished by HIF expression: one that expresses both HIF-1α and HIF-2α (H1H2) and another that expresses only HIF-2α (H2; ref. 7). In keeping with these findings, the authors speculated that H1H2 tumors may respond better to VEGF inhibitors than H2 tumors (7, 13).

Recently, Shen and colleagues developed an HIF-1α transcriptional signature based on transcriptional profiling of ccRCC cell lines expressing both HIF-1α and HIF-2α or HIF-2α alone. However, whether this genomic profile correlates with the response to agents that target angiogenesis, such as VEGF-targeted therapy, remains unclear (14).

Pazopanib (Votrient; GlaxoSmithKline), an oral, multi-kinase inhibitor of VEGF receptors (VEGFR)-1, -2, and -3, PDGF receptors (PDGFR)-α/ß, and c-Kit, significantly prolonged PFS compared to placebo in patients with metastatic ccRCC who were treatment-naive or pretreated with cytokines (15). This agent was approved in the United States in 2009 and in Europe in 2010 and is considered category 1 by National Comprehensive Cancer Network for first-line therapy in metastatic RCC. Despite this important addition to the arsenal of RCC systemic therapeutics, the majority of patients fail to experience long-term durable tumor response to pazopanib, or to other currently approved anti-VEGF drugs (10). Using data from a randomized, prospective, clinical trial of pazopanib in advanced RCC, we thought to investigate whether several elements of the VHL/HIF pathway, including VHL gene status, HIF1/HIF2 protein expression, and the HIF1 gene signature described by Shen and colleagues (14), could be associated with outcome to a specific angiogenesis inhibitor, pazopanib.

Materials and Methods

Patients

All patients provided written informed consent for participation in the clinical trial (VEG102616; NCT00244764). This study was conducted in accordance with the Declaration of Helsinki; protocols and informed consent forms were reviewed and approved by institutional review boards/ independent ethics committees according to local guidelines. Patient characteristics have been described previously (9). This randomized, phase II discontinuation study enrolled patients with metastatic ccRCC to receive pazopanib (800 mg daily, orally); those who had stable disease at week 12 were randomly assigned to pazopanib or placebo. This study was subsequently amended to an open-label pazopanib trial on the recommendation of the data monitoring committee, based on the favorable response rate observed in the first 60 enrolled patients (9).

Tumor tissue samples

Formalin-fixed paraffin-embedded (FFPE) archival tumor tissue samples were collected from 131 consenting patients (Supplementary Fig. S1). Tumor samples (n = 80) from patients consenting for DNA/RNA analysis underwent histopathology review, microdissection, and nucleic acid extraction. Based on quantity and quality of DNA and RNA isolated from FFPE tumor tissue samples, 78 (of 80) DNA samples qualified for VHL mutation and methylation analysis, and 46 RNA samples qualified for gene expression analysis. Tissue slides from 101 and 99 patients were available for HIF-2α and HIF-1α immunohistochemistry (IHC), respectively. These included both paraffin-dipped and non-paraffin-dipped slides.

DNA extraction

DNA was extracted from ten 5-μm-thick sections of paraffin-embedded tissue. Samples were microdissected to achieve a minimum of 90% tumor tissue and DNA was extracted according to a proprietary procedure of Response Genetics, Inc. Extracted DNA was eluted in a 50 μl final volume.

VHL sequencing

Sequencing of VHL gene was conducted by Genzyme Analytical Services. VHL exons were amplified by PCR (see Supplementary Methods and Supplementary Table S1 for details).
VHL methylation

Methylation status of the VHL promoter was assessed by methylation-specific PCR (MS-PCR) following DNA bisulphite treatment using the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer’s instructions. The modified templates were amplified using methylated and unmethylated-specific primers (Supplementary Table S2) and results visualized on a 10% polyacrylamide gel.

HIF-1α and HIF-2α immunohistochemical analysis

For HIF-1α and HIF-2α IHC, the primary antibodies (anti-HIF-1α antibody from Neomarkers, Cat #MS-1164-PABX, anti-HIF-2α antibody, clone UP15 provided by Dr. William G. Kaelin at Dana-Farber Cancer Institute, Boston, MA) were applied to sections for 1 hour at a 1:10,000 dilution for HIF-1α and a 1:5,000 dilution for HIF-2α. For HIF-1α, detection was conducted using the CSA II kit (Dako) according to the manufacturer’s instructions. For HIF-2α, detection was conducted by incubation with the Dako EnVision+ System HRP labeled polymer. Slides were slightly counterstained with hematoxylin. FFPE cells with high (786-O-vector) or low (786-O-VHL) HIF-2α levels were used as positive and negative controls, respectively, to validate the specificity of the HIF-2α immunohistoassay.

Immunostained slides were scanned using the ScanScope System (Aperio Technologies Inc.). Digital slides were reviewed by a single expert kidney cancer pathologist who identified the tumor regions for analysis. Quantification was conducted using a modified Aperio IHC nuclear image system (Aperio Technologies Inc.). Digital slides were used as positive and negative controls, respectively, to validate the specificity of the HIF-2α immunohistoassay.

GSEA analysis was conducted using programs provided (16). A priori criteria. Baseline clinical characteristics (age, gender, risk category, and performance status) were compared among patients with available tissue for biomarker analysis versus those without available tissue, to ensure that patients with available biomarker data were representative of the rest of the trial population. Exact χ² tests were used to assess the relationship between ORR and VHL, HIF-1α, and HIF-2α expression.

Patterns of expression among the mapped HIF-1α transcriptional signature genes were assessed using multivariate analysis (hierarchical two-way clustering using complete linkage with correlation distance), and putative patterns were assessed for association with HIF-1α gene expression levels using the Kruskal–Wallis test. PFS was estimated using Kaplan–Meier analysis. Gene set enrichment analysis (GSEA) was applied to determine if mapped HIF-1α transcriptional signature genes were enriched for HIF-1α gene expression. GSEA ranks genes in the signature based on association with class distinction, in this case low or high HIF-1α relative to the median (16). GSEA calculates an enrichment score (ES) that reflects whether rankings are random or are overrepresented in patients expressing low or high HIF-1α levels. High ES is indicative of enrichment. Statistical analyses were conducted using SAS 9.2 (SAS Institute); GSEA analysis was conducted using programs provided (16). A P value < 0.05 was considered statistically significant. The analysis includes only the samples for which reliable results were obtained.

Results

VHL gene status and clinical outcome

DNA was extracted from 78 available tumor samples. Overall, 68 of 78 patients (87%) had at least one VHL mutation and 8 of 69 patients (12%) showed promoter hypermethylation; 6 patients had both VHL mutation and methylation; and 8 did not have either VHL mutation or methylation. All mutations were heterozygous. VHL mutation types included frameshifts (n = 33, 48%), nonsense (n = 6, 9%), deletion/insertion (n = 3, 4%), splice junction (n = 6, 9%), and missense (n = 20, 29%). When examined in relation to clinical outcome, VHL mutation and/or methylation status did not correlate with either ORR (ORR 37.5% vs. 41.4% in patients with VHL [n = 70] vs. without VHL gene inactivation [n = 8], respectively; Table 1) or PFS (median PFS was 13.8 months vs. 17.4 months in patients with vs. without VHL gene inactivation, respectively; Fig. 1).

HIF-2α expression and clinical outcome

HIF-2α staining was conducted in available tumor samples (101 of 225). The analysis included 66 samples (65%) for which reliable IHC data were obtained. The reasons for excluding 35 samples were: low immunoreactivity (n = 9), uneven staining (n = 8), overstained tissue (n = 5), low-quality section (n = 10), or other (n = 3). Among the 66
sample patients, 25 were classified as responders and 41 as nonresponders. Intensity scores were 1+ for 27 patients, 2+ for 38 patients, and 3+ for 1 patient. The mean percentage (standard deviation) of positively stained nuclei was 30.8% (12.2) for score 1+, 59.8% (18.7) for score 2+, and 79.1% (not available; n = 1) for score 3+. Notably, higher overall intensity score correlated with higher positivity of stained nuclei cell (P < 0.001).

The correlation between intensity score and ORR is described in Table 2. ORR among patients with HIF-2α staining 1+ versus those staining 2+ and 3+ was not statistically different (P = 0.36). Similarly, the mean percentage of positively stained nuclei was 45.5% for responders and 45.6% for nonresponders, with no statistical difference (P = 0.29).

When we examined PFS among both groups with low and high HIF-2α staining, there was no statistically significant difference between the 2 groups (P = 0.76; Fig. 2A and B). The percentage of positively stained nuclei was also not correlated with PFS (P = 0.75, not shown).

HIF-1α expression and clinical outcome

HIF-1α staining was conducted in available tumor samples (99 of 225). Of these, 65 (65.6%) were included in this analysis. Reasons for excluding 34 specimens were: very low immunoreactivity (n = 11), uneven staining (n = 6), over-stained tissue (n = 4), low-quality section (n = 1), or other (n = 12). Of these, 23 patients were classified as responders and 42 as nonresponders. Intensity scores were 1+ for 17 patients and 2+ or 3+ for 48 patients. The mean percentage (standard deviation) of positively stained nuclei was 36.5% (16.099) for score 1+ and 57.6% (16.3) for both 2+ and 3+. Similar to HIF-2α, the correlation between intensity and percent stain was statistically significant (P < 0.001).

The correlation between intensity score and ORR is described in Table 3. There was no statistically significant difference between the 2 groups (P = 0.13). Similarly, no statistically significant difference was found in terms of PFS between the higher intensity and the lower intensity scores (P = 0.25; Fig. 2C and D). The percentage of positively stained nuclei was also not correlated with PFS (P = 0.6915, not shown).

HIF-1α gene signature and clinical outcome

Recently, an HIF-1α transcriptional signature based on gene expression profiles from 10 VHL-inactivated RCC cell lines that either express or do not express HIF-1α was reported (14). Of note, this signature was enriched in tumors that had not sustained 14q deletions encompassing HIF1 locus. To evaluate whether this genetic profile could be related to clinical outcome in metastatic ccRCC patients receiving pazopanib, we examined the 69 genes described in the HIF-1α transcriptional signature by Shen and colleagues (14) in patients enrolled in the VEG102616 trial.

RNA was available for 46 of 225 patients. Study results and baseline characteristics for this subset of patients were similar to the overall study. Forty-one patients had VHL heterozygous mutations and 5 patients were VHL wild type. Sixty-four of 69 genes were mapped to the Affymetrix platform and HIF-1α gene expression was detected in all 46 patients (Affymetrix statistical test [P < 0.05]: median intensity = 5,330, minimum = 1,389, maximum = 14,067).

We conducted a multivariate analysis that resulted in 4 possible gene expression clusters (Supplementary Fig. S2). Each cluster was associated with a different level of HIF-1α gene expression (P = 0.0136, Kruskal–Wallis test, box
plots; Fig. 3A). GSEA analysis (Fig. 3B; ref. 14) provided additional evidence that patients expressing high HIF-1α tend to be enriched for genes in the HIF-1α transcriptional signature \((P = 0.064)\). However, clusters were not associated with ORR \((P = 0.7455)\) or PFS \((P = 0.6058)\) in patients receiving pazopanib.

**Discussion**

The establishment of biomarkers may help in understanding the biology underlying RCC and the efficacy of therapies that target the VEGF axis. Significant efforts have been undertaken thus far to discover useful and clinically relevant biomarkers to customize therapy in metastatic RCC patients, but at this time no biomarkers are ready for routine clinical use.

In this study, we explored several elements of the VHL/HIF/VEGF axis along the same molecular pathways that could be associated with pazopanib activity. Unfortunately, no correlation between several tested biomarkers, including VHL mutation and methylation status, and defined clinical endpoints, ORR or PFS, were observed. This is in contrast to data from a large retrospective study in 123 patients treated with various VEGF-targeted agents, which showed that ORR was significantly higher (52% vs. 31%) in patients with "severe/loss of function" mutations (e.g., frameshift, stop, deletions); however, this study also showed that PFS and OS did not differ based on VHL gene status (12). In this study, the presence of the wild-type allele did not preclude response to pazopanib. It is interesting to note the high prevalence of VHL mutations in the 78 patients tested. Several studies, including a recent study (17), suggest a prevalence of VHL mutations varying from 27% to 55%. Nevertheless, few studies showed >80% VHL mutations in tested RCC specimens. This difference may be thought largely due to differences in sequencing technologies, as highlighted in the paper by Nickerson and colleagues (18).

A high proportion of RCC is characterized by deregulation of the VHL/HIF pathway and overexpression of HIF-1α, HIF-2α, or both. These genes have some overlapping effects on angiogenesis, but they also exhibit opposing properties. VHL-defective tumors can be divided into 2 subgroups regarding HIF expression, with one subtype expressing both HIF-1α and HIF-2α (H1H2) and another only HIF-2α (H2; refs. 7, 19). HIF-1α antagonizes c-myc activity and suppresses tumor formation in renal cells. However, overproduction of HIF-2α promotes cell proliferation by activation of c-myc (7). Another difference is that HIF-1α, but not HIF-
2α, remains susceptible to ubiquitination, suggesting an alternative pathway not dependent of pVHL (13). More evidence is accumulating to suggest that HIF-2α, rather than HIF-1α, promotes RCC carcinogenesis (20, 21). In contrast, overproduction of HIF-1α is believed to suppress tumor growth (3, 14, 22). In addition, HIF-1α and HIF-2α regulate
each other. Overexpression of HIF-1α strikingly downregulates HIF-2α and vice versa (23). These findings suggest that the subtypes may have different clinical outcomes and possibly different responses to VEGF-targeted agents. To that end, HIF-1α has been correlated with prognosis in other cancer subtypes (24). Birner and colleagues reported HIF-1α as a marker for tumor progression in cervical cancer (25). Relevant to RCC, Patel and colleagues (26) evaluated 43 metastatic RCC patients who received sunitinib. Pre-treatment HIF levels by Western blot analysis were associated with response to sunitinib in this small subset of patients. The final report from this small study is eagerly awaited. During the last 2012 American Society of Clinical Oncology (ASCO) meeting, Hudes and colleagues presented an evaluation of 75 tumor samples from pazopanib-treated patients. Loss of chromosome 14 or 14q- (HIF-1α locus) was not correlated with PFS or clinical response to pazopanib. This was an important hypothesis-driven study, as prior work showed that HIF-1α activity is diminished in 14q-deleted kidney cancers (27). At the same meeting, a preliminary study by Saez and colleagues showed that HIF-1α and HIF-2α staining intensity >10% represented an independent predictive factor of outcome (PFS and OS) for VEGFR tyrosine kinase inhibitor therapy in 71 patients with metastatic RCC. That study used different antibodies against HIF1 and HIF2 than our study. In the Saez study, VHL mutation was not correlated with outcome, and HIF-1α expression was associated with a response (28).

In our study, 73.8% of the samples expressed HIF-1α intensity score 2+ or 3+. In the same way, 59% of tumors expressed HIF-2α in higher levels. Interestingly, we identified a higher expression of HIF-1α than HIF-2α. Nevertheless, the level of expression of HIF-1α and HIF-2α was not associated with clinical response to pazopanib. The genomic profile observed among 46 tumor samples available for DNA analysis from the prospective RCC study of pazopanib was consistent with the cell-line-derived HIF-1α transcriptional signature proposed by Shen and colleagues (14). A multivariate analysis identified 4 different patterns of HIF-1α gene expression. Although we externally validated the Shen data, neither patterns nor HIF-1α expressions were associated with clinical outcome in this small subset of patients. Despite these genes having been considered key players in RCC pathogenesis, they were not able to select patients who will or will not benefit from pazopanib. There are several alternative factors that should be explored as potential biomarkers of response to VEGF inhibitors, and potentially, pazopanib. We are now aware of other recurrent gene mutations in ccRCC on chromosome 3p, part of the large group of histone-modifying enzymes. Patients with ccRCC who have PBRM1 (40%) and BAP1 (15%) mutations may display different adverse pathologic features and survival patterns, and it is not inconceivable that these can be predictive of response to systemic therapies such as VEGF inhibitors (29, 30).

Our study has several limitations. Although the patients were part of a well-conducted clinical trial with a single agent, in contrast to other studies (12), the sample size for each tested biomarker was small. We had 78 patients tested for VHL gene status, ~100 patients for HIF1 or HIF2, and 46 patients for HIF1 gene signature. The study population was somewhat selected, with potentially unmeasured clinical differences, despite the fact that baseline characteristics such as age, gender, and Memorial Sloan-Kettering Cancer Center risk groups were similar and balanced (Supplementary Table S3).

Another limitation is the variable quality of the tissue sections used for IHC analysis. It is well recognized that tissue specimen collection, processing, and storage procedures (i.e., preanalytical variables) can profoundly impact the result of biomarker analyses in FFPE tissues (31–33). For instance, we have previously shown that HIF-2α immunoreactivity is significantly affected by the long-term storage of tissue slides (34). The tissue slides used in this study were preserved under 2 different conditions (i.e., paraffin-dipped and non-paraffin-dipped) and this might have influenced immunoreactivity. Indeed, the proportion of HIF-2α-positive cells in the paraffin-dipped tissue was higher compared with non-paraffin-dipped sample cohorts (Supplementary Fig. S3).

Although standardization of procedures for tissue sample acquisition, storage, and analysis is a challenge, especially in the context of multi-institutional clinical trials, many efforts should be directed toward this goal in the future. Pathologist involvement in clinical trial design will be essential in this endeavor.

In summary, targeted therapies are the standard of care for patients with advanced RCC. Several elements of the VHL/HIF pathway have been extensively correlated with RCC tumorigenesis. In this study, the expression levels of HIF-1α and HIF-2α were not predictive for a single anti-VEGF therapy response and the role of these proteins as biomarkers to clinical outcomes remain unclear. Thus, efforts to identify predictive biomarkers and develop new drugs are critical for long-term survival of patients with RCC.

Disclosure of Potential Conflicts of Interest
T.K. Choueiri is a consultant/advisory board member in Pfizer, GlaxoSmithKline, Aveo, and Novartis. R. Gagnon is employed as a statistician in GlaxoSmithKline. Jonathan Rosenberg is a consultant/advisory board member in GlaxoSmithKline. K. Baker-Neblett is employed as a clinical research manager in GlaxoSmithKline. C. Carpenter is employed as a Director, Clinical Research and has ownership interest (including patents) in GlaxoSmithKline. L. Pandite is employed as a Vice President and has ownership interest (including patents) in GlaxoSmithKline. S. Signoretti has a commercial research grant from GlaxoSmithKline and also is a consultant/advisory board member in GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.K. Choueiri, Y. Lin, B. Bahamon, T.E. Hutson, K. Baker-Neblett, Y. Liu, S. Signoretti

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.K. Choueiri, R. Gagnon, Y. Lin, V.E. Gagnon


Writing, review, and/or revision of the manuscript: T.K. Choueiri, A. Fay, R. Gagnon, Y. Lin, J. Rosenberg, T.E. Hutson, K. Baker-Neblett, C. Carpenter, Y. Liu, L. Pandite

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.K. Choueiri, B. Bahamou, V.E. Brown, T.E. Hutson, Y. Liu

Study supervision: T.K. Choueiri, K. Baker-Neblett, Y. Liu, S. Signoretti

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