RESEARCH ARTICLE

Genetic and Functional Studies Implicate HIF1α as a 14q Kidney Cancer Suppressor Gene

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ABSTRACT

Kidney cancers often delete chromosome 3p, spanning the VHL tumor suppressor gene, and chromosome 14q, which presumably harbors ≥1 tumor suppressor genes. pVHL inhibits the hypoxia-inducible transcription factor (HIF), and HIF2α is a kidney cancer oncoprotein. In this article, we identify focal, homozygous deletions of the HIF1α locus on 14q in clear cell renal carcinoma cell lines. Wild-type HIF1α suppresses renal carcinoma growth, but the products of these altered loci do not. Conversely, downregulation of HIF1α in HIF1α-proficient lines promotes tumor growth. HIF1α activity is diminished in 14q-deleted kidney cancers, and all somatic HIF1α mutations identified in kidney cancers tested to date are loss of function. Therefore, HIF1α has the credentials of a kidney cancer suppressor gene.

SIGNIFICANCE: Deletion of 14q is a frequent event in clear cell renal carcinoma and portends a poor prognosis. In this study, we provide genetic and functional evidence that HIF1α is a target of 14q loss in kidney cancer. Cancer Discovery; 1(3); OF1-OF14. © 2011 AACR.

INTRODUCTION

Kidney cancer causes >10,000 deaths each year in the United States (1). Although surgery is potentially curative for kidney cancers that are detected at an early stage, recurrences after surgery remain common, and late-stage, inoperable kidney cancer is usually fatal.

Clear cell renal carcinoma is the most common form of kidney cancer and is usually linked to biallelic inactivation of the von Hippel–Lindau VHL tumor suppressor gene, which is located on chromosome 3p25. Individuals who carry a mutant VHL allele in the germline (von Hippel–Lindau disease) are at increased risk of clear cell renal carcinoma, in addition to pheochromocytomas and central nervous system hemangioblastomas. Somatic mutation, or hypermethylation, of the VHL locus is also common in sporadic clear cell renal carcinomas (2).

The VHL gene product, pVHL, has multiple functions, including serving as the substrate recognition subunit of an ubiquitin ligase complex that targets the alpha subunits of hypoxia-inducible factor (HIF), a heterodimeric transcription factor, for polyubiquitination and proteasomal degradation when oxygen is present (3). Accordingly, deregulation of HIF target genes, such as VEGF, is a signature abnormality in pVHL-defective neoplasms, and the degree of HIF deregulation correlates well with renal carcinoma risk linked to different VHL alleles (4–6). Notably, a number of drugs that inhibit VEGF, or its receptor KDR, have demonstrated significant activity in the treatment of metastatic kidney cancer (7).

Multiple lines of evidence suggest that HIF2α, and not its more intensively studied paralog HIF1α, acts as a driver in pVHL-defective renal carcinomas. For example, pVHL-defective renal carcinoma cell lines and tumors produce both HIF1α and HIF2α or HIF2α alone (6, 8), and the appearance of HIF2α in preneoplastic lesions in the kidneys of patients with von Hippel–Lindau disease correlates with increased histologic evidence of impending malignancy (9). Moreover, HIF2α, but not HIF1α, can override the tumor suppressor activity of pVHL (10–12), whereas eliminating HIF2α is sufficient to suppress tumor formation by pVHL-defective renal carcinoma cells in preclinical models (13, 14). A recent genome-wide association study linked the risk of renal carcinoma to HIF2α polymorphisms (15). Finally, HIF2α, rather than HIF1α, appears to be responsible for much of the disease that develops following pVHL inactivation in the mouse (16, 17).

Although HIF1α and HIF2α are similar, they can clearly antagonize one another in certain settings. For example, in some models HIF1α antagonizes, whereas HIF2α potentiates, c-Myc activity (8, 18, 19). In addition, HIF1α and HIF2α reciprocally regulate each other’s protein levels in some contexts, so that, for example, loss of HIF1α leads to induction of HIF2α and vice versa (10). In keeping with these observations, overproduction of wild-type HIF1α in pVHL-defective renal carcinoma cells suppresses tumor formation (10), whereas overproduction of HIF2α promotes tumor growth (10, 11). In contrast, HIF1α is believed to promote, rather than inhibit, many other tumor types of nonrenal origin (20).

A number of chromosomal abnormalities, in addition to chromosome 3p loss, have been described in clear cell renal carcinoma, including, most commonly, amplification of 5q and loss of chromosome 14q. In numerous studies, loss of 14q has been associated with poorer outcomes in renal carcinoma (21–24). The knowledge that HIF1α is located at chromosome 14q, together with the considerations outlined above, led us to explore further whether HIF1α might be a clear cell carcinoma tumor suppressor gene.

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org).

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doi: 10.1158/2159-8290.CD-11-0098
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RESULTS

Loss of Chromosome 14q Spanning the HIF1α Locus Is a Common Feature of Human Kidney Cancer

Kidney cancers frequently undergo deletions affecting chromosome 14q. To ascertain whether this abnormality occurs more often in kidney cancers than in other forms of cancer, we examined a recently published collection of copy number data generated with high-density single-nucleotide polymorphism (SNP) arrays from 3,131 cancers representing 26 different tumor types (25). The frequency of large deletions affecting most of chromosome 14q was highest in kidney cancer, followed by melanoma, gastrointestinal stromal tumor, and esophageal cancer (Fig. 1A). As expected, loss of chromosome 3p, which harbors the VHL tumor suppressor gene and other tumor suppressor genes such as PBRM1 (26), as well as amplification of 5q, was also extremely common in kidney cancer relative to other tumor types (Fig. 1B and C). These data do not, however, reflect a general proclivity for copy number alterations in kidney cancer because other copy number changes, such as loss of chromosomes 17p and 13q, which harbor p53 and RB1, respectively, did not occur more frequently in kidney cancer than in other cancers (data not shown).

HIF1α expression is lost in many VHL−/− renal carcinoma lines, can suppress tumor formation by VHL−/− renal carcinoma cells when overexpressed (10), and maps to 14q23. In contrast, previous studies, including our own, pinpointed 14q31-ter as the most likely region to harbor a kidney cancer tumor suppressor gene (23, 27, 28). Nonetheless, the 14q deletions in kidney cancer are typically very large, with the localization to 14q31-ter based on relatively rare kidney cancers with smaller deletions. For example, in our recent analysis of 90 clear cell renal carcinomas, 39 tumors (43%) had sustained 14q deletions (27). Of these, 36 (93%) were large deletions that also encompassed the HIF1α locus (27). This observation suggests the existence of multiple tumor suppressor genes on 14q, including, perhaps, HIF1α. Consistent with this hypothesis, deletions affecting HIF1α are more common in kidney cancer than in the other 16 tumor types for which we have ≥40 samples (Fig. 1D). This strong bias toward kidney cancer is not apparent, however, if one includes deletions elsewhere on 14q (Supplementary Fig. S1).

Figure 1. Frequencies of chromosomal abnormalities across different cancers. A, large deletions affecting most of 14q arm. B, large deletions affecting most of 3p arm. C, amplification of any region of 5q. D, deletions affecting HIF1α locus. ALL, acute lymphoblastic leukemia; GIST, gastrointestinal stromal tumor; MPD, myeloproliferative disorder; NSC, non-small cell; SC, small cell. See also Supplementary Fig. S1.
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Frequent Homozygous Deletions of the HIF1α Locus in Kidney Cancer Cell Lines

We next surveyed a panel of 16 clear cell renal carcinoma lines, most of which had undergone biallelic VHL inactivation, for HIF1α protein and mRNA production, along with HK-2 immortalized, diploid, human renal epithelial cells. In keeping with earlier reports (6, 8, 29), we found that many VHL−/− renal carcinoma lines produce no detectable wild-type HIF1α mRNA or protein, whereas all VHL−/− lines produce HIF2α (Fig. 2; Supplementary Fig. S2; data not shown). Of interest, some lines, such as RCC4, SKRC-20, A498, and 786-O cells, generate mRNAs with increased electrophoretic mobility (Fig. 2B). The truncated HIF1α mRNA in 786-O has also been noted by other investigators (30). SKRC-20, A498, and 786-O also produce abnormally migrating HIF1α proteins (Fig. 2A). These findings raised the possibility that the HIF1α locus, in addition to undergoing copy number loss, is rearranged in a subset of renal tumors.

To explore this concept further, we isolated genomic DNA from the 16 renal carcinoma lines and performed multiplex ligation-dependent probe amplification (MLPA) analysis to look for copy number changes affecting specific HIF1α exons (31). As controls, we also interrogated randomly chosen exons on chromosomes 1, 10, and 17. Two cell lines (SLR20 and SLR21) appeared to be diploid across the HIF1α locus (Fig. 2C; Supplementary Fig. S3).
these cell lines, which are phenotypically VHL+/−, were tested by us previously, using high-density SNP arrays, and did not exhibit 14q loss (27). Three cell lines (RCC4, Caki-2, and A704) appeared to have lost 1 HIF1α allele in its entirety and to have retained the other (Fig. 2C; Supplementary Fig. S3). Of interest, 7 of 16 cell lines (SKRC-20, A498, 769-P, 786-O, UOK101, SLR24, and SLR26) had clearly sustained homozygous deletions, which in some cases were very focal and involved only a subset of contiguous HIF1α exons (Fig. 2C; Supplementary Fig. S3). The remaining 4 cell lines (UMRC2, UMRC6, SLR23, SLR25) displayed more complex MLPA patterns that were intermediate between haploid and diploid across the HIF1α locus, with preferential loss of particular exons (Supplementary Fig. S3).

Several other putative tumor suppressor genes reside on chromosome 14, including the Hippo pathway genes SAV1 (14q22) and FRMD6 (14q22; ref. 32). In contrast to HIF1α, we did not detect altered transcripts for these genes (Supplementary Fig. S4B and C). We also did not find homozygous deletions of SAV1 by MLPA, with the exception of the previously reported deletion in 786-O cells (ref. 33; Supplementary Fig. S4A). The discovery of focal, homozygous HIF1α deletions provides genetic evidence that HIF1α has a tumor suppressor role in clear cell renal carcinoma.

HIF1α Suppresses Kidney Cancer Proliferation
In Vitro and In Vivo

To address the suppression issue further, we made retroviral HIF1α expression vectors in which the amount of HIF1α produced can be regulated by the addition of doxycycline. VHL−/− renal carcinoma cells were then infected with the viruses and maintained in pools. Immunoblot analysis of these cells grown in the presence or absence of 1 μg/mL of doxycycline confirmed that HIF1α expression was induced by doxycycline and that the HIF1α levels achieved were similar to those observed after treating HK2 immortalized

Figure 3. Suppression of VHL−/− renal carcinoma proliferation by HIF1α. Immunoblot (A) and proliferation (B) assays of indicated cell lines infected with a doxycycline-inducible retrovirus encoding HIF1α and propagated in 5% serum in the presence or absence of doxycycline. HK-2 cells treated with vehicle, DMOG (1 mM), or MG132 (10 μM) were included as a control in A. See also Supplementary Fig. S5.
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renal epithelial cells with the prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG) or the proteasomal inhibitor MG132 (Fig. 3A; Supplementary Fig. S5). Re-expression of HIF1α in the VHL−/− renal carcinoma cell lines A498, 769-P, UOK101, and SLR24—all of which produce HIF2α, but not wild-type HIF1α—impaired proliferation in vitro (Fig. 3B). This effect was specific because induction of HIF1α did not diminish the proliferation of RCC4 and UMRC-2 VHL−/− renal carcinoma cells, which express both HIF1α and HIF2α (Fig. 3B; Supplementary Fig. S5). Therefore, HIF1α can suppress the proliferation of VHL−/− renal carcinoma cells when expressed at levels approximating those achieved after VHL inactivation.

In a reciprocal set of experiments, we downregulated HIF1α or HIF2α in 3 VHL−/− renal carcinoma cell lines that express both HIF1α and HIF2α (Caki-2, RCC4, and SLR25; Fig. 4A). In contrast to a recent report, we did not observe an increase in HIF2α in the cells treated with HIF1α short hairpin RNA (shRNA; ref. 10; Fig. 4A). The significance of this discrepancy is unclear. In all 3 cases, downregulation of HIF1α with 2 independent shRNAs enhanced proliferation in vitro, compared with cells expressing a scrambled control shRNA or HIF2α shRNA (Fig. 4B; Supplementary Fig. S6), in keeping with a recent study using RCC4 cells (8). The very modest inhibition of cell proliferation observed with HIF2α shRNA is consistent with earlier studies using cells grown under standard serum conditions.

In addition to affecting proliferation in vitro, downregulation of HIF1α promoted the growth of RCC4 renal carcinoma cells that had been implanted in the kidneys of nude mice (Fig. 5A–C). Similar results were obtained with Caki-2 cells grown s.c. in nude mice (Supplementary Fig. S6F and G), whereas SLR25 cells did not form tumors in nude mice, irrespective of HIF1α status (data not shown). Downregulation of HIF1α in UMRC2 renal carcinoma cells also dramatically enhanced tumor growth (Fig. 5D–G), despite having inconsistent effects in vitro (Supplementary Fig. S6D). Therefore, HIF1α suppresses tumor formation by VHL−/− renal carcinoma cells.

HIF1α Variants Resulting from HIF1α Genomic Deletions Are Defective as Tumor Suppressors

Next we attempted to recover the aberrant mRNAs that we had detected by Northern blot analysis in a subset of renal carcinoma lines. mRNA was harvested from these cell lines, converted to cDNA, and amplified by PCR. As predicted by MLPA analysis, the HIF1α transcript in SKRC-20 cells specifically lacked exons 3 and 4 (Δ3–4), the HIF1α transcript

Figure 4. Downregulation of HIF1α in VHL−/− renal carcinoma cells enhances cell proliferation. Immunoblot (A) and proliferation (B) assays of the indicated cell lines after infection with lentiviruses encoding HIF1α shRNA, HIF2α shRNA, or scrambled control shRNA and grown in the presence of 5% serum. See also Supplementary Fig. S6.
in A498 cells lacked exons 2 to 6 (Δ2–6), and the HIF1α transcript in SLR26 cells lacked exons 5 to 10 (Δ5–10; Fig. 6A). These transcripts presumably reflect the homozygous deletion detected by MLPA together with alternative splicing. In addition, PCR primers based on the 5′ and 3′ HIF1α untranslated regions detected a transcript lacking exons 2 to 12 in RCC4 and A498 cells, perhaps responsible for the faster migrating Northern blot band detected in these cells (Fig. 2B; data not shown). This variant (Δ2–12), in contrast to the other mRNA variants, was also detected in some normal kidney mRNA samples (Supplementary Table S2).

In 786-O cells, we recovered a transcript lacking exons 13 to 15, using 3′ rapid amplification of cDNA ends, in keeping with the MLPA data (Supplementary Fig. S3; data not shown) and with Western blot data indicating that these cells produce a HIF1α variant that reacts with a polyclonal antibody, but not a C-terminal monoclonal antibody (Fig. 2A). This transcript is predicted to encode a fusion protein, as it contains several in-frame exons from a neighboring gene (data not shown). For unclear reasons, however, this transcript greatly exceeds the apparent molecular weight of the mRNA detected in these cells by Northern blot analysis (Fig. 2B).

The Δ3–4, Δ2–6, and Δ5–10 HIF1α variants, as well as wild-type HIF1α, were introduced into 769-P VHL–/– renal carcinoma cells, using the doxycycline-inducible retroviral expression vector described above. Notably, each variant, except for the Δ5–10 one, preserves the proper reading frame. An N-terminal hemagglutinin (HA) epitope tag was introduced to facilitate the detection of wild-type and mutant HIF1α proteins after induction with doxycycline. Each variant migrated in accordance with its predicted molecular weight and, with the exception of the Δ2–6 variant, was produced at levels that were similar to those of wild-type HIF1α (Fig. 6B).

Notably, wild-type HIF1α suppressed the proliferation of 769-P cells to a greater extent than did any of the 3 variants tested (Fig. 6C). In the next set of experiments, the 769-P cells that had been engineered to inductively express wild-type or mutant HIF1α were pooled and propagated in vitro in the presence or absence of doxycycline (Fig. 6D). Growth in the presence of doxycycline led to progressive loss of cells expressing wild-type HIF1α relative to those expressing mutants, suggesting that cells expressing wild-type HIF1α are at a growth disadvantage in such competition assays (Fig. 6E). Similar results were obtained when the cells were injected into the kidneys of NOD/SCID mice and propagated in vivo in the presence or absence of doxycycline (Fig. 6F), indicating that the HIF1α variants tested in this study are enfeebled as tumor suppressors relative to wild-type HIF1α.

**HIF1α Activity Is Diminished in Human Kidney Cancers Harboring 14q Deletions**

To determine if our cell line data were relevant to human kidney cancers, we next asked whether HIF1α activity is diminished in human kidney cancers that have sustained 14q deletions encompassing the HIF1α locus. Toward this end, we performed gene expression profiling on the cell lines described above that either expressed both HIF1α and HIF2α (either naturally or by virtue of induced expression of HIF1α) or expressed HIF2α alone (either naturally or by virtue of a HIF1α shRNA), followed by supervised clustering to arrive at...
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Figure 6. Differential effects of cell line-derived HIF1α variants on proliferation and tumor cell fitness. A, HIF1α variants identified in renal carcinoma lines. B and C, immunoblot (B) and proliferation (C) assays of 769-P cells infected with doxycycline-inducible retroviruses encoding the indicated HIF1α variants and propagated in the presence of doxycycline. 769-P cells infected with an inducible virus encoding wild-type HIF1α but grown in the absence of doxycycline were included as a control in B, D, in vitro and in vivo competition assays. E and F, PCR-based analysis indicating relative abundance of cells, as in B and C, after growth in vitro for 9 days (E) or orthotopically in vivo for 6 weeks (F) in the presence or absence of doxycycline. Neg., PCR reaction with no input DNA. WT, wild-type. See also Supplementary Table S2.

a “HIF1α transcriptional signature,” which included known HIF1α-specific targets, such as BNIP3, PGK1, HK1, and TPI1 (Fig. 7A; refs. 10, 34). Gene set enrichment analysis (GSEA) using this HIF1α signature and gene expression data from 52 VHL−/− clear cell renal carcinomas, including 32 without a HIF1α deletion and 20 with a HIF1α deletion, confirmed that HIF1α activity is diminished in tumors that have sustained a 14q deletion spanning the HIF1α locus (P < 0.01; Fig. 7B).

Somatic HIF1α Mutations in Human Clear Cell Carcinomas Are Loss of Function

We have not yet been able to definitively identify focal, homozygous deletions in renal tumor DNA by MLPA (data not shown). This inability might be related to technical issues because MLPA is very sensitive to contamination by host DNA. In 3 of 23 primary clear cell renal carcinomas, however, we did detect presumptively pathogenic HIF1α mRNA...
**Figure 7.** HIF1α activity is impaired by copy number changes and mutations in pVHL-defective kidney cancers. **A**, heat map depicting genes that are differentially expressed between VHL−/− renal carcinoma cell lines that do (H1H2) or do not (H2) express high levels of HIF1α. Genes (right) are ordered from top to bottom according to P values showing the degree to which they are significantly altered in H1H2 cells compared with H2 cells. A t-test and hierarchical clustering were performed using software GENE-E at the Broad Institute (57). **B**, GSEA plot showing location of the enrichment score of 71 HIF1α upregulated genes in a set of ccRCC tumors with biallelic VHL inactivation. The HIF1α positively regulated genes are significantly enriched (P ≤ 0.01) in tumors that have not sustained 14q deletions encompassing HIF1α. **C**, HIF1α mutations (mu) identified in renal carcinoma patients. Immunoblot (D) and proliferation (E) assays of 769-P cells infected with doxycycline-inducible retroviruses encoding wild-type HIF1α or the indicated HIF1α variants and propagated in the presence of doxycycline. Cells grown in the absence of doxycycline were included as controls in D.
splice variants (absent in normal kidney RNA samples and absent in publicly available databases), analogous to those present in renal carcinoma cell lines (Supplementary Table S2).

Somatic HIF1α mutations have been described at low frequency in human kidney cancer (refs. 29, 35; H. Greulich, M. Meyerson, and W.G. Kaelin, unpublished observations). We therefore made doxycycline-inducible retroviral vectors corresponding to 3 of these mutations (c.2120delA, c.2180 C→A, and V116E), as well as a presumed benign SNP (A475S; Fig. 7C and D). All 3 mutations suspected of being pathogenic compromised the ability of HIF1α to suppress renal carcinoma growth, whereas the presumptive SNP did not (Fig. 7E). A 4th mutant recently reported (35), exon 4 -5 del (tacagTTTGAACTAACTGGA), was not tested because it was predicted to induce a frameshift after exon 3. Collectively, these results support that HIF1α activity is diminished in a subset of VHL−/− kidney cancers because of a reduction in gene dosage and, in some cases, as a result of intragenic, loss-of-function mutations.

DISCUSSION

Loss of the region of chromosome 3p spanning the VHL gene is the most frequent genomic change in clear cell renal carcinoma, the most common form of kidney cancer. The next 2 most common genomic abnormalities in renal cancer are chromosome 5q amplification and chromosome 14q loss. Moreover, in this article we show that kidney cancer has the highest rate of chromosome 3p loss, chromosome 5q amplification, and 14q loss among a wide variety of tumor types.

VHL loss leads to increased abundance of HIF1α and HIF2α, and deregulation of HIF-dependent transcription is a signature abnormality in kidney cancer. Mounting evidence suggests that HIF2α, rather than HIF1α, promotes pVHL-defective renal carcinogenesis. Indeed, many pVHL-defective renal carcinomas produce low, or undetectable, levels of HIF1α (6, 8, 36), and restoring HIF1α expression in a VHL−/− renal carcinoma line was previously shown to suppress tumorigenesis (10). We confirmed the latter finding and revealed that HIF1α, at levels comparable to those seen when pVHL function is impaired, suppresses renal carcinoma proliferation and tumor growth.

Prompted by this knowledge, we asked whether HIF1α, which resides on chromosome 14q23.2, might be one of the genes targeted by 14q deletions in kidney cancer. Indeed, we found that the vast majority of 14q deletions detected in renal carcinoma encompass HIF1α. Moreover, we documented that HIF1α, but not neighboring genes on chromosome 14q, is often subject to focal deletions in kidney cancer cell lines. Some of these deletions led to the production of aberrant mRNAs and proteins that compromised the ability of HIF1α to suppress VHL−/− renal carcinoma proliferation and tumorigenesis. We also discovered that downregulation of wild-type HIF1α promotes growth of renal carcinoma in vivo. Finally, we showed that somatic, presumably pathogenic, HIF1α mutations in human clear cell carcinomas eneefble HIF1α as a tumor suppressor in cell proliferation assays. Collectively, these genetic and functional data credential HIF1α as a clear cell renal carcinoma suppressor gene. Hence loss of pVHL simultaneously leads to activation of an oncoprotein (HIF2α) and a tumor suppressor protein (HIF1α). This finding would explain the frequent loss of chromosome 14q in kidney cancer and is consistent with the observation that loss of HIF1α protein in preneoplastic lesions in the kidneys of patients with von Hippel-Lindau disease heralds further malignant transformation (9).

In a recent study the percentage of clear cell renal carcinomas with low HIF1α expression approximated the frequency of 14q loss for this tumor type (8), and 14q loss was enriched among the HIF1α-negative tumors (K. Nathanson, personal communication). Moreover, we confirmed that HIF1α transcripational activity is indeed decreased in VHL−/− kidney tumors that have sustained 14q deletions encompassing HIF1α, compared with those that have not. Although homozygous HIF1α deletions appear to be common in clear cell renal carcinoma cell lines, we have not documented a similarly high frequency in primary renal tumors. This discrepancy might be due to technical factors, but it does raise the possibility that HIF1α haploinsufficiency is sufficient to promote primary tumor growth in vivo, and that reduction to nullizosity is selected for during tumor progression in vivo or the propagation of clear cell carcinoma lines in vitro.

HIF1α is usually thought to promote tumor growth, but precedent exists for its function as a tumor suppressor. For example, loss of HIF1α enhances tumor formation by embryonic stem cell–derived teratoma cells and by murine astrocytes (37–39). In the context of renal carcinoma, HIF1α might act as a tumor suppressor specifically by antagonizing HIF2α. For example, transactivation by 1 of the 2 HIFα transactivation domains [the C-terminal transactivation domain (CTAD)] is inhibited by the asparaginyl hydroxylase factor inhibiting HIF1 (FIH1). HIF2α is less sensitive than HIF1α to FIH1-mediated inhibition (40, 41). Competitive displacement of HIF2α by HIF1α from HIF-responsive promoters that depend upon the CTAD for full activation would therefore potentially decrease promoter activity. Moreover, in some contexts HIF1α can suppress HIF2α levels via mechanisms that are as yet unclear (10). In short, loss of HIF1α might, paradoxically, increase the activity of certain HIF-responsive promoters in pVHL-defective tumor cells. Experimental evidence exists to support this contention (ref. 10; C. Shen and W.G. Kaelin, unpublished observations and data not shown).

In addition to quantitative differences on shared HIF-responsive promoters, a number of qualitative differences between HIF1α and HIF2α might relate to HIF1α scoring as a tumor suppressor protein. For example, some genes regulated by HIF1α are not regulated by HIF2α and vice versa. Conceivably, some genes that are preferentially activated by HIF1α decrease renal carcinoma cell fitness. In this regard, 3 genes found in our HIF1α signature—TXNIP, KCTD11, and PLAGL1—have been implicated as tumor suppressors in other contexts (42–45). Further, HIF1α and HIF2α differ in terms of their ability to engage collateral signaling pathways, such as those involving c-Myc and Notch. For example, HIF1α, via
a variety of mechanisms, can inhibit c-Myc activity in certain settings, whereas HIF2α does not (8, 18, 19).

The 14q deletions in kidney cancer are typically very large and usually span HIF1α, as noted above. Nonetheless, rare tumors with small deletions had pinpointed 14q31-ter as the likely location for a kidney cancer tumor suppressor gene (23, 27, 28). The simplest reconciliation of these findings would be the existence of multiple kidney cancer tumor suppressor genes on 14q, in addition to HIF1α, with perhaps some acting through haploinsufficiency. Alternatively, these small deletions might have been passenger, rather than driver, mutations.

It will be of interest to determine whether pVHL-defective clear cell renal carcinomas that retain wild-type HIF1α expression use alternative mechanisms to circumvent the tumor suppressor activity of HIF1α. We note, for example, that PLAG1 maps to a region of 6q frequently deleted in VHL-associated neoplasms and sporadic clear cell renal carcinomas (27, 46, 47). Moreover, it will be important to determine whether retention of HIF1α expression alters the response of pVHL-defective tumors to targeted agents that directly or indirectly target HIF. In this regard, it is possible that the salutary effects of rapamycin-like mTOR inhibitors (rapalogs) in kidney cancer are partially mitigated by their ability to downregulate HIF1α (48), especially in light of a recent report predicting that HIF2αΔ3–4, cDNAs (HIF1αΔ3–4, HIF1αΔ2–6, and HIF1αΔ5–10) were PCR amplified from HK-2, SKRC-20, A498, and SLR26 cells, respectively, with a 5′ primer that introduced a BamHI site and an HA epitope and a 3′ primer that introduced a MluI site. The products were digested with BamHI and MluI and cloned into pRetroX-Tight-pur vector (Clontech) cut with these 2 enzymes.

The tumor-derived HIF1α mutations in Fig. 7 were generated by the site-directed mutagenesis of the pRetroX-Tight-HIF1α-WT vector by QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and confirmed by DNA sequencing. The forward primers for mutagenesis of HIF1α mu1-mu4 are as follows: 5′-CGTCTTCGGAGGAAGAATCCAAAGATACTAGCTTT GCAG-3′; 5′-GGACATGATGTTAACCTTTTCAAGGACAGTGG-3′; 5′-CATGATTACATTCTCTGTAATTGAAAGAACATTACATGGGA TTAC-3′; and 5′-CTGCAGCTCAAATGAAAGAACATTCAACAAATGTTCA TTTAAATT AGAACCGCGAACCT-3′, respectively.

Lentiviral shRNA vector pLKO.1, lentiviral HIF1α shRNA vector (TRCN0000003810, target sequence: 5′-GTGATGAAAGAATCCGGATTACG AAT-3′; TRCN0000003811, target sequence: 5′-CGGGCAAGTAAG AATCTGAA-3′; TRCN0000003809, target sequence: 5′-CCAGTTAT GATTTGAAAGATGTTA-3′), and lentiviral HIF2α (EPAS1) shRNA vector (TRCN0000003806, target sequence: 5′-CAGTACCCAGACGGTA TTCCCA-3′) were obtained from the Broad Institute TRC shRNA library.

**Methods**

Cell Lines

HK-2, 786-O, A704, 769-P, Caki-2, RCC4, and A498 cells were purchased from the American Type Culture Collection. UMRC-2 (50), UMRC-6 (50), and UOK101 (51) cells were provided by Drs. Bert Zbar and Martson Linehan (National Cancer Institute, Bethesda, Maryland). SLR20, SLR21, SLR23, SLR24, SLR25, and SLR26 cells were supplied by Drs. Mark A. Rubin and Kirsten Mertz (Weill Cornell Medical College, New York, New York; ref. 52). SKRC-20 cells (53) were provided by Drs. Gerd Ritter and Beatrice Yin (Memorial Sloan-Kettering Cancer Center, New York, New York). DNA from these cell lines was subjected to SNP analysis by us previously (27), and the cell lines were monitored regularly for mycoplasma contamination. No other validation was performed. Whenever possible, freshly thawed vials of cells were used at early passage. HK2 immortalized human renal epithelial cells were maintained in keratinocyte serum-free medium supplemented with 0.05 mg/mL of bovine pituitary extract and 5 ng/mL of human recombinant epidermal growth factor. Renal carcinoma cells 786-O, A498, RCC4, UMRC-2, UMRC-6, and UOK101 were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS; 769-P, A704, SKRC-20, SLR20, SLR21, SLR23, SLR24, SLR25, and SLR26, in RPMI-1640 containing 10% FBS; and Caki-2, in McCoy’s 5A containing 10% FBS. Following retroviral or lentiviral infection, cells were maintained in the presence of puromycin (2 μg/mL) or G418 (500 μg/mL) depending on the vector. All cells were kept at 37°C in 10% CO2.

**Plasmids**

The wild-type and variant HIF1α cDNAs (HIF1αΔ3–4, HIF1αΔ2–6, and HIF1αΔ5–10) were PCR amplified from HK-2, SKRC-20, A498, and SLR26 cells, respectively, with a 5′ primer that introduced a BamHI site and an HA epitope and a 3′ primer that introduced a MluI site. The products were digested with BamHI and MluI and cloned into pRetroX-Tight-pur vector (Clontech) cut with these 2 enzymes.

The tumor-derived HIF1α mutations in Fig. 7 were generated by the site-directed mutagenesis of the pRetroX-Tight-HIF1α-WT vector by QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and confirmed by DNA sequencing. The forward primers for mutagenesis of HIF1α mu1-mu4 are as follows: 5′-CGTCTTCGGAGGAAGAATCCAAAGATACTAGCTTT GCAG-3′; 5′-GGACATGATGTTAACCTTTTCAAGGACAGTGG-3′; 5′-CATGATTACATTCTCTGTAATTGAAAGAACATTACATGGGA TTAC-3′; and 5′-CTGCAGCTCAAATGAAAGAACATTCAACAAATGTTCA TTTAAATT AGAACCGCGAACCT-3′, respectively.

Lentiviral shRNA vector pLKO.1, lentiviral HIF1α shRNA vector (TRCN0000003810, target sequence: 5′-GTGATGAAAGAATCCGGATTACG AAT-3′; TRCN0000003811, target sequence: 5′-CGGGCAAGTAAG AATCTGAA-3′; TRCN0000003809, target sequence: 5′-CCAGTTAT GATTTGAAAGATGTTA-3′), and lentiviral HIF2α (EPAS1) shRNA vector (TRCN0000003806, target sequence: 5′-CAGTACCCAGACGGTA TTCCCA-3′) were obtained from the Broad Institute TRC shRNA library.

**Immunoblot Analysis**

Cells were lysed with 1 × EBC buffer [50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40] supplemented with a protease inhibitor cocktail (Complete; Roche Applied Science, Indianapolis, Indiana), resolved by SDS-PAGE (30 μg/lane), and transferred to nitrocellulose membranes (Bio-Rad). Primary antibodies were rabbit polyclonal anti-HIF1α (NB100-479; Novus, Littleton, Colorado), mouse monoclonal anti-HIF1α (BD Transduction Laboratories), mouse monoclonal anti-HA (HA-11; Covance Research Product), and mouse monoclonal antitubulin (B-512; Sigma-Aldrich). Bound antibody was detected with horseradish peroxidase (HRP)-conjugated secondary antibodies (Pierce, Rockford, IL) and Immobilon Western chemiluminescent HRP substrate (Millipore).

**Northern Blot Analysis**

Total RNA was isolated using TRIzol reagent (Invitrogen), reversed by avian-foiraldehyde gel electrophoresis (10 pg RNA per lane), transferred to nitrocellulose membranes, and probed with a HIF1α Agel-Pstl fragment labeled with [32P]dCTP using a Prime-It II Random Primer Labeling Kit (Stratagene). Hybridizations were performed in QuikHyb (Stratagene) according to the manufacturer’s instructions. Signals were detected by X-ray film.

**Multiplex Ligation-Dependent Probe Analysis**

Multiplex ligation-dependent probe amplification was performed as described in ref. 31, using the probe sets listed in Supplementary Table S1 and reagents provided by MRC-Holland (54).

The capillary electrophoresis and peak height determination of amplification products were performed by Mei Lin at the DNA Sequencing Facility, Brigham and Women’s Hospital, Boston, Massachusetts. Briefly, amplification products were 10× diluted in Hi-Di Formamide (ABI, Foster City, California) containing 1/16 volume of ROX500 size standard (ABI) and then were separated by size on an ABI 3100 Genetic Analyzer (ABI). Electropherograms were analyzed by GeneMapper v3.5 (ABI), and peak height data were exported to an Excel table.

**Cell Proliferation Assays**

Renal carcinoma cells that had been infected to inducibly express HIF1α were plated in 96-well plates (~500 cells per well; 6 wells per condition and time point) in RPMI-1640 media supplemented.
with 5% FBS in the presence or absence of the indicated amount of doxycycline, with a media change every 2 days. The number of viable cells per well at each time point was measured using an XTT assay (Cell Proliferation Kit II; Roche, cat. no. 11465015001) according to the manufacturer’s instructions. Spectrophotometric absorbance at 450 nm was measured 6 hours after the addition of the XTT labeling reagent/electron coupling reagent, using a microtiter plate reader (Perkin Elmer Life and Analytical Science).

Renal carcinoma cells stably infected with lentiviral shRNAs were plated, in triplicate, in 6 well plates (10^4 cells/well) in RPMI (RC4C, SLR25 and UMRC-2) or McCoy’s 5A (Caki-2) supplemented with 5% FBS. Cells were trypsinized and collected at the indicated time points. The number of viable cells, as determined by Trypan blue staining, was determined with a hemocytometer.

Renal carcinoma 769-P cells that had been induced to inductible express tumor-derived HIF1α mutants were plated, in triplicate, in 6 well plates (5,000 cells/well) in RPMI with 5% FBS. Cells were trypsinized and collected at the indicated time points. The number of viable cells was determined by using an automated cell counter (Countess, Invitrogen).

**Xenograft Assays and Bioluminescence**

Orthotopic and s.c. growth of tumor cells was as described in ref. 55. For RCC4 cells, 1 × 10^6 viable RCC4 HIF1α shRNA cells (right kidney) and 1 × 10^5 viable RCC4 scrambled shRNA cells (left kidney) were injected orthotopically into Swiss nude mice (Taconic, Hudson, New York). Bioluminescence detection and quantification of tumor burden were performed as described in ref. 55. For each mouse, total photons from the right kidney were divided by total photons from the left kidney and normalized to the ratio for that mouse at the first week after tumor cell injection. For UMRC-2 cells, 2 × 10^6 viable UMRC-2 HIF1α shRNA cells (right side) and 2 × 10^6 viable UMRC-2 scrambled shRNA cells (left side) were injected s.c. into Swiss nude mice. The mice were sacrificed 4 weeks after injection, and tumors were excised and weighed. For Caki-2 cells, 1 × 10^5 viable Caki-2 HIF1α shRNA cells (right side) and 1 × 10^5 viable Caki-2 scrambled shRNA cells (left side) were injected s.c. into Swiss nude mice. The mice were sacrificed 16 to 20 weeks after injection, and tumors were excised and weighed.

The 5-week-old female NOD scid gamma mice (The Jackson Laboratory, Bar Harbor, Maine) were orthotopically injected with 1 × 10^6 viable 769-P cells (1:1:1 mixture of 769-P cells stably infected with retroviruses encoding wild-type HIF1α or 3 different HIF1α variants). The mice were randomized to Chow that did or did not contain 6 g doxycycline/kg chow (Bioserv) 1 week after tumor cell implantation and were sacrificed 6 to 8 weeks later. Then 300 ng of genomic DNA, extracted from the injected kidney with the DNeasy Blood & Tissue Kit (Qiagen), was PCR amplified using a forward primer (5′-ATGTACCCATACGATGTTCCAGATTACGC-3′) based on the sequence encoding the shared HA epitope tag and a reverse primer (5′-TGTTGCTTGGAGGATCTTGCGCTTTC-3′) based on the HIF1α C-terminus.

**Microarray and GSEA**

Gene expression profiling was performed at the Microarray Core at Dana-Farber Cancer Institute, using Gene 1.0ST Arrays (Affymetrix), and the data were deposited in the Gene Expression Omnibus database (GSE27415). In brief, total RNA was extracted using the RNeasy Mini Kit with on-column DNase digestion (Qiagen). Biotin-labeled cRNA was prepared from 1 μg of total RNA, fragmented, and hybridized to a Human Gene 1.0 ST Array. The arrays were scanned and raw expression data normalized using DNA-Chip Analyzer (dChip) custom software (56). Genes were assessed for significantly altered expression between H1H2 and H2 subgroups by t-test analysis using the software GENE-E (57). The 71 genes with P-values ≤ 0.05 and an average signal ratio ≥ 1.3 between 2 subgroups were selected as positively regulated by HIF1α. This gene set was evaluated by GSEA (58) across a panel of 52 ccRCC tumors with biallelic inactivation of VHL (with 32 non-HIF1α deletion tumors and 20 HIF1α deletion tumors) for which expression data were previously obtained (GSE14994; ref. 27). We used the GSEA parameters: weighted scoring, signal-to-noise metric, and gene set permutations.

**Disclosure of Potential Conflicts of Interest**

W.G. Kaelin owns equity in, and consults for, Fibrogen, Inc., which is developing drugs that modulate HIF activity. No other potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Drs. Mark A. Rubin and Kirsten Mertz at Weill Cornell Medical College for the SLR20, SLR21, SLR23, SLR24, SLR25, and SLR26 cell lines; Drs. Gerd Ritter and Beatrice Yin at Memorial Sloan-Kettering Cancer Center for the SKRC-20 cell line; Dr. Marston Linehan at NCI for ccRCC tumor samples; and Dr. David Kwiatkowski for advice on the MLPA assays.

**Grant Support**

This work was supported by NIH (W.G. Kaelin), Howard Hughes Medical Institute (W.G. Kaelin), Doris Duke Charitable Foundation (W.G. Kaelin), Dana-Farber/Harvard Cancer Center Kidney Cancer Career Development Award (C. Shen; funded by Genentech). W.G. Kaelin is a Doris Duke Distinguished Clinical Investigator and Howard Hughes Medical Institute Investigator.

Received May 2, 2011; revised May 25, 2011; accepted June 2, 2011; published OnlineFirst June 7, 2011.

**REFERENCES**

HIF1α is a 14q Kidney Cancer Suppressor Gene

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\textit{Cancer Discovery} Published OnlineFirst June 7, 2011.

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