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RESEARCH BRIEF

L-2-Hydroxyglutarate: An Epigenetic Modifier and Putative Oncometabolite in Renal Cancer

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ABSTRACT

Through unbiased metabolomics, we identified elevations of the metabolite 2-hydroxyglutarate (2HG) in renal cell carcinoma (RCC). 2HG can inhibit 2-oxoglutaratre (2-OG)–dependent dioxygenases that mediate epigenetic events, including DNA and histone demethylation. 2HG accumulation, specifically the D enantiomer, can result from gain-of-function mutations of isocitrate dehydrogenase (*IDH1*, *IDH2*) found in several different tumors. In contrast, kidney tumors demonstrate elevations of the L enantiomer of 2HG (L-2HG). High-2HG tumors demonstrate reduced DNA levels of 5-hydroxymethylcytosine (5hmC), consistent with 2HG-mediated inhibition of ten-eleven translocation (TET) enzymes, which convert 5-methylcytosine (5mC) to 5hmC. L-2HG elevation is mediated in part by reduced expression of L-2HG dehydrogenase (L2HGDH). L2HGDH reconstitution in RCC cells lowers L-2HG and promotes 5hmC accumulation. In addition, L2HGDH expression in RCC cells reduces histone methylation and suppresses *in vitro* tumor phenotypes. Our report identifies L-2HG as an epigenetic modifier and putative oncometabolite in kidney cancer.

SIGNIFICANCE: Here, we report elevations of the putative oncometabolite L-2HG in the most common subtype of kidney cancer and describe a novel mechanism for the regulation of DNA 5hmC levels. Our findings provide new insight into the metabolic basis for the epigenetic landscape of renal cancer. *Cancer Discov; 4(11); 1290–8. ©2014 AACR.*

INTRODUCTION

One of the clearest examples of the role of metabolism in cancer is the recent identification of oncometabolites, small molecules with putative oncogenic properties. Mutations of fumarate hydratase (*FH*), succinate dehydrogenase (*SDH*), and isocitrate dehydrogenase 1 and 2 (*IDH1/2*) in tumors lead to elevated levels of fumarate, succinate, and 2-hydroxyglutarate (2HG), respectively (1, 2). In cells with *FH* and *SDH* mutations, precursor metabolites (fumarate and succinate) accumulate

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due to loss of FH and SDH enzymatic activity. In the case of *IDH1* and *IDH2*, highly conserved mutation "hot spots" in DNA of tumors result in the formation of a "neoenzyme" that leads to elevated 2HG (specifically the D enantiomer) in gliomas and acute myeloid leukemias (AML), as well as other malignancies (2, 3). A unifying theme linking these three oncometabolites is their ability to inhibit a class of enzymes referred to as 2-oxoglutarate (2-OG)–dependent dioxygenases (2-OGD). Members of this enzyme family include prolyl hydroxylases (PHD), which are involved in the regulation of the transcription factor hypoxia-inducible factor-1α (HIF1α), histone demethylases, and DNA hydroxylases (4). In the case of succinate accumulation, feedback inhibition is a proposed mechanism, as succinate is a product of 2-OGD–catalyzed reactions. In the case of fumarate and 2HG, competitive inhibition is the proposed mechanism due to structural similarity with the cofactor 2-OG. Collectively, these data suggest that metabolic perturbations in cancer can inhibit 2-OGD activities with potential effects on tumorigenesis.

Recent studies have focused on the ability of oncometabolites to inhibit DNA hydroxylation by the ten-eleven translocation (TET) enzymes (TET 1–3), which convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). The oxidation of 5mc to 5hmC has been proposed to promote the demethylation of DNA, via either active or passive means (5). Alternatively, 5hmC has been proposed to be its own epigenetic mark with distinct effects on DNA architecture and gene expression (6). Despite these conflicting views, emerging data demonstrate reduced 5hmC levels in human cancer, as well as animal tumor models, indicating a role for 5hmC loss in carcinogenesis (7, 8). Further evidence for the role of 5hmC loss in malignancy is provided by the fact that *TET2* is commonly mutated in human myeloid malignancies, including AML, as well as other myeloid disorders (9, 10). 2HG can inhibit TET enzymatic activity and promote loss of 5hmC (11). These studies have primarily examined the role of the D enantiomer of 2HG (D-2HG), which is markedly elevated in the setting of *IDH* mutations. Notably, both cell-free and *in vitro* studies demonstrate that the L enantiomer (L-2HG) is more potent at inhibiting 2OGDs, including the TET enzymes (11, 12).

In this report, we demonstrate elevation of 2HG in clear-cell renal cell carcinoma (ccRCC), the most common histologic subtype of kidney cancer. In contrast with *IDH*-mutant tumors, ccRCCs demonstrate elevations of L-2HG. In concordance with the ability of 2HG to inhibit TET enzymatic activity, tumors with elevation of 2HG had reduced levels of 5hmC in genomic DNA. We provide evidence that reduced mRNA and protein expression of L-2HG dehydrogenase (L2HGDH) in ccRCC promotes 2HG accumulation and 5hmC loss. Bioinformatic analysis demonstrates that copy-number loss is associated with reduced *L2HGDH* expression in ccRCC. L2HGDH reconstitution in RCC cells lowers L-2HG, promotes 5hmC accumulation, and suppresses *in vitro* tumor phenotypes. Collectively, our data demonstrate a putative oncometabolite elevated in ccRCC with effects on the kidney cancer epigenome.

RESULTS

We analyzed 59 matched tumor/normal pairs using an unbiased metabolomics profile (S. Sudarshan, manuscript in preparation). This initial analysis identified statistically significant elevations of 2HG (greater than 5-fold) in ccRCC relative to normal renal parenchyma (Fig. 1A). However, multiple tumors demonstrated elevations of 2HG more than 10-fold higher than in normal tissue. Investigation for somatic mutations in RCC using both the cBioPortal for Cancer Genomics [to analyze The Cancer Genome Atlas (TCGA) datasets] and the Sanger Catalogue of Somatic Mutations in Cancer (COSMIC) database did not demonstrate any evidence for *IDH* mutations in ccRCC (data not shown). 2HG is known to occur in two enantiomers, D(*R*) and L(*S*). We therefore analyzed metabolite extracts from both high-2HG and low-2HG tumors via liquid chromatography/tandem mass spectrometry (LC-MS/MS) for levels of each 2HG enantiomer (Fig. 1B). Of notable significance is that the predominant enantiomer present in these cells is the L enantiomer (Fig. 1B). Analysis of high-2HG tumors demonstrated that the L enantiomer accounted for approximately 90% of the 2HG present in these tumors (Fig. 1C). We validated our findings in a separate cohort (Fig. 1D). Clinical information present on this cohort is provided in Supplementary Table S1. We therefore analyzed a panel of RCC lines for 2HG elevations, in addition to nontransformed renal epithelial cells HK-2 and HRE 152. Consistent with our tissue analysis, several RCC lines demonstrated increased 2HG, with a predominant contribution made by the L enantiomer (Fig. 1E). Notably, L-2HG elevation was found in cells both with and without alterations of *VHL*, the most commonly altered gene in ccRCC. Analysis of tumor samples confirmed L-2HG elevation in tumors both with and without *VHL* coding mutations (Supplementary Table S2). Collectively, these demonstrate elevations of L-2HG in ccRCC.

Elevated 2HG has been shown to inhibit TET enzymatic activity, thereby leading to reduced levels of 5hmC in the context of *IDH* mutation (11). We used an ELISA-based assay to quantitate absolute 5hmC levels. To validate the assay, we overexpressed the catalytic domains (CD) of TET1 and TET2 in HEK-293 cells, in addition to catalytically inactive mutants (CM) of TET1 and TET2. Consistent with previous data (11), cultured cells, including HEK-293 cells, express low levels of 5hmC (Fig. 2A). However, transient expression of the TET1 or TET2 CD was able to raise 5hmC levels, whereas CM forms of either TET1 or TET2 could not (Fig. 2A). We therefore examined levels of 5hmC in the context of 2HG elevation. Consistent with prior data, L-2HG octyl-ester treatment reduced DNA 5hmC levels in HK-2 renal epithelial cells (Fig. 2B). We confirmed increases in intracellular 2HG levels following ester treatment (data not shown). Tumors with elevated 2HG levels demonstrate significantly reduced levels of 5hmC relative to matched normal tissue on ELISA analysis (Fig. 2C, top). Dot-blot assay with an antibody specific to 5hmC in DNA identified that high-2HG tumors had reduced levels of 5hmC relative to normal kidney (Fig. 2C, bottom). In contrast, 5hmC levels were not reduced in tumors with low 2HG levels (Fig. 2D). Collectively, these data indicate that raised levels of 2HG in ccRCC are associated with 5hmC loss.

We next wanted to identify potential factors that promote L-2HG accumulation in ccRCC. L-2-hydroxyglutaric aciduria is an inborn error of metabolism linked to loss-of-function mutations of the gene *L2HGDH* (13). Notably, several RCC lines had reduced mRNA and protein

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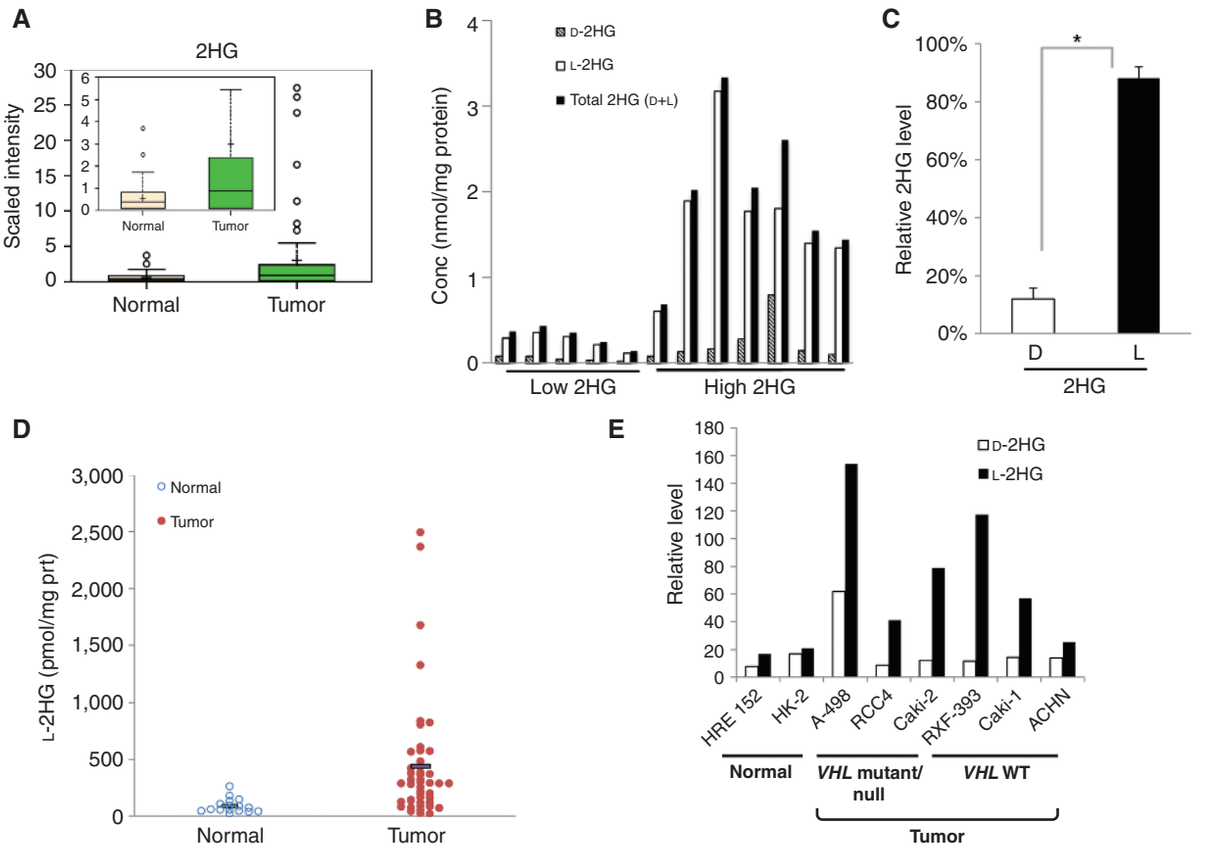

Figure 1. L-2HG is elevated in RCC tumors and cell lines. Human kidney samples were obtained by surgical resection and metabolites were extracted for metabolite profiling analysis by GC/MS analysis. **A,** 2HG was significantly increased in primary tumor (tumor) compared with adjacent benign kidney tissue (normal). Inset graph is presented with smaller scale. **B,** high-2HG and low-2HG tumors were analyzed by tandem MS to resolve the enantiomeric distribution of 2HG within these tumors. **C,** relative ratio of D-2HG and L-2HG against total 2HG in high-2HG RCC samples. Error bars, SEM. *, $P < 0.05$. **D,** L-2HG levels were measured in another cohort of samples from a separate biorepository. **E,** enantiomeric resolution of 2HG in a panel of nontransformed and transformed lines of renal origin. WT, wild-type.

expression of *L2HGDH* relative to nontransformed renal epithelial cells (Fig. 3A and B). We found that A498 and RXF-393 demonstrate elevated L-2HG levels with concomitant reduced L2HGDH mRNA/protein expression (Figs. 1E and 3A and B). These data prompted us to examine the relationship between L-2HG levels and L2HGDH expression in patient samples. We analyzed *L2HGDH* mRNA expression in normal kidney, low–L-2HG tumors, and high–L-2HG tumors. Elevated L-2HG tumors had a higher *L2HGDH* ΔC_t (C_t *L2HGDH* − C_t RPLP0), indicating lower *L2HGDH* expression when compared with low–L-2HG tumors and normal kidney (Fig. 3C and D). Immunoblot analysis confirmed the mRNA findings, as primary tumors with elevated 2HG had reduced protein levels of L2HGDH relative to normal tissue (Fig. 3E). Immunohistochemical analysis also demonstrated reduced L2HGDH expression in high–L-2HG tumors relative to normal kidney. Notably, proximal tubule cells, the likely cell of origin for ccRCCs, have prominent L2HGDH expression relative to distal tubule cells (Fig. 3F). We next examined whether reexpression of L2HGDH in RCC cells could lower L-2HG levels. Using lentivirus, we stably expressed *L2HGDH* cDNA in RCC cells and confirmed expression by immunoblotting (Supplementary Fig. S1). L2HGDH expression significantly reduced intracellular L-2HG levels in A498 cells (Fig. 3G). The *L2HGDH* locus is located on chromosome 14q, a region commonly lost in ccRCC (14). We therefore examined the relationship between copy-number loss and gene expression alterations with Level 3 RNA sequencing data from The Cancer Genome Atlas. ccRCC tumors with loss of a single copy (i.e., loss of heterozygosity) were associated with significantly reduced mRNA expression of L2HGDH compared with diploid tumors (Fig. 3H). Collectively, these data demonstrate that reduced expression of L2HGDH promotes L-2HG accumulation in ccRCC.

We next determined whether modulation of *L2HGDH* expression could affect the levels of 2HG, as well as 5hmC levels. We therefore cotransfected HEK-293 cells with the TET1 CD (to raise 5hmC levels to a detectable range) and either *L2HGDH* siRNA or control siRNA. Immunoblotting confirmed TET1 CD expression and knockdown of L2HGDH (Supplementary Fig. S2). Consistent with its role in L2HG metabolism, L2HGDH knockdown led to raised levels of

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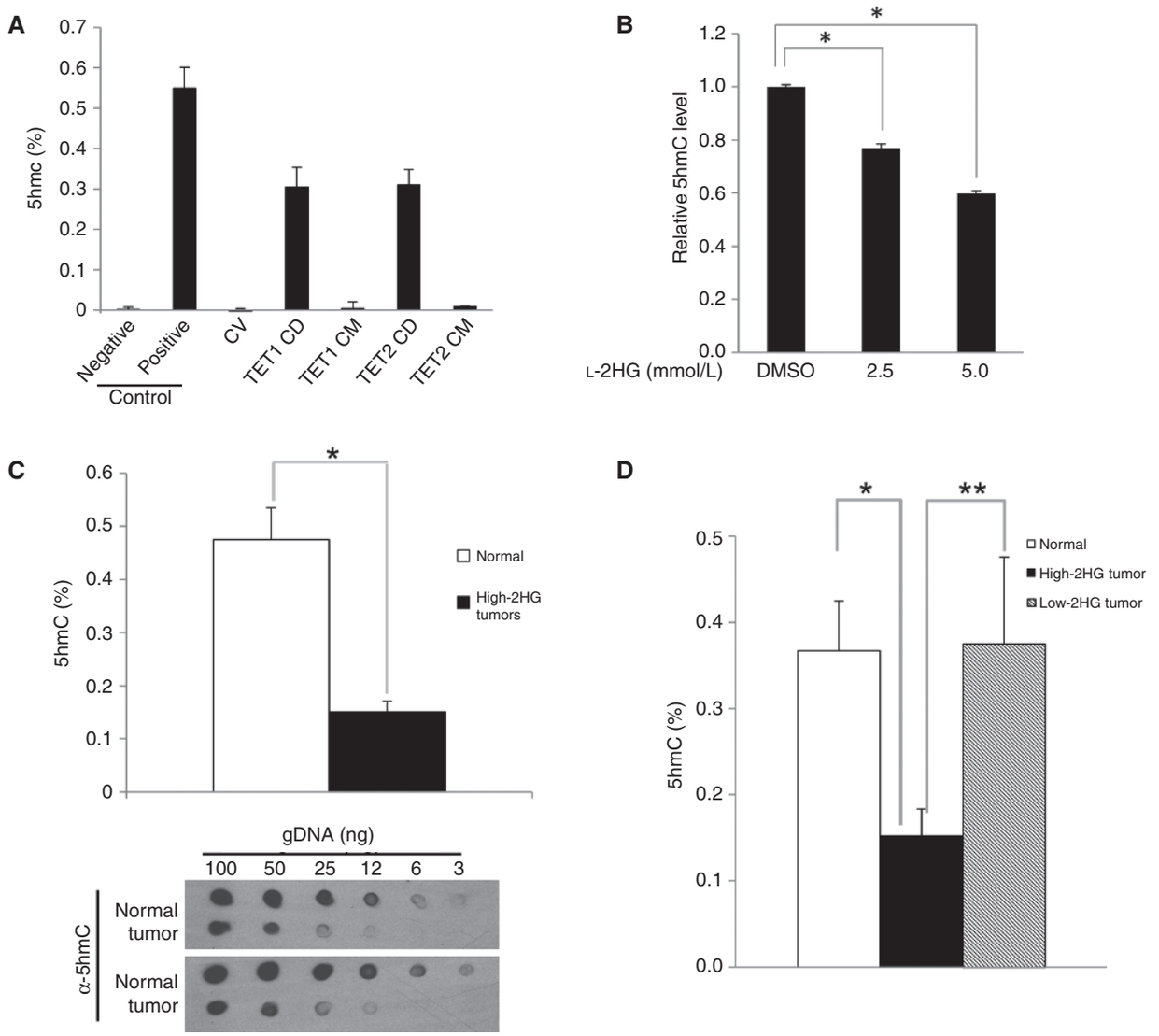

Figure 2. Increased L-2HG is associated with loss of 5hmC in RCC. **A,** validation of ELISA for 5hmC. HEK293 cells were transiently transfected with plasmids expressing control vector (CV), TET1 wild-type catalytic domain (CD) or mutant catalytic domain (CM), or TET2 CD or TET2 CM. Cells were harvested and genomic DNA was examined to determine the 5hmC level. **B,** HK-2 renal epithelial cells were treated with L-2HG octyl ester for 4 hours and assayed for 5hmC levels via ELISA. **C,** 5hmC levels were compared between normal and high-L-2HG RCC tumor samples by ELISA (top) and dot-blot assay (bottom). **D,** 5hmC levels in normal, low-2HG tumors, and high-2HG tumors were determined by ELISA. Error bars, SEM. *, $P < 0.005$; **, $P < 0.05$.

cellular 2HG (Fig. 4A, left). Concomitantly, we identified decreased levels of 5hmC with L2HGDH knockdown, consistent with the ability of L2HG to inhibit TET enzymatic activity (Fig. 4A, right). Similar results were obtained in HK-2 renal epithelial cells (Fig. 4B and Supplementary Fig. S2). As a complement to our knockdown approach, we overexpressed the TET1 CD and either control vector or *L2HGDH* cDNA in HEK-293 cells. Immunoblotting confirmed transgene expression (Supplementary Fig. S3). Under basal culture conditions, L2HGDH overexpression had little impact on cellular 2HG levels, consistent with the fact that HEK-293 cells express high levels of L2HGDH mRNA and protein levels (data not shown). We therefore challenged cells with esterified L-2HG (octyl-2HG) to raise intracellular levels of 2HG. Following octyl-2HG challenge, *L2HGDH* cDNA overexpression in HEK-293 cells led to reduced cellular 2HG levels relative to control cells, consistent with the role of L2HGDH in the metabolism of L-2HG (Fig. 4C, left). Consistent with reduced 2HG levels, L2HGDH-overexpressing cells demonstrate increased 5hmC levels compared with vector control–transfected cells (Fig. 4C, right). Consistent with these data, reexpression of L2HGDH in A498 cells led to an increase in 5hmC levels as compared with control vector (Fig. 4D). As noted, prior studies have demonstrated that L-2HG can inhibit histone demethylases and hence promote histone methylation. We therefore examined the impact of

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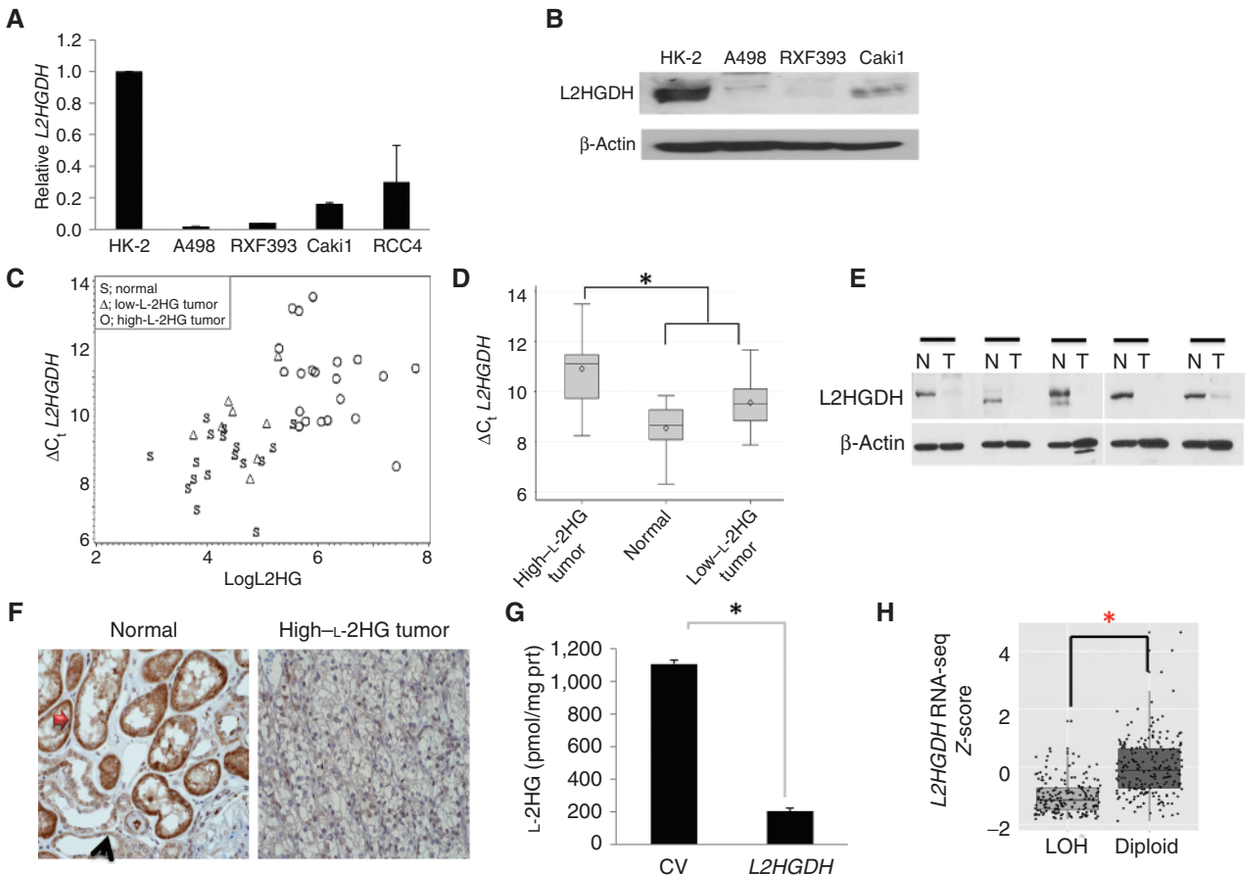

Figure 3. *L2HGDH* is reduced in RCC tumors and cell lines. **A,** the mRNA expression of *L2HGDH* in RCC lines relative to nontransformed renal epithelial cells (HK-2). **B,** the protein expression of L2HGDH in RCC lines relative to nontransformed renal epithelial cells. **C** and **D,** *L2HGDH* mRNA levels measured by real-time RT-PCR in normal, low-L-2HG tumor, and high-L-2HG tumor and plotted as a function of L-2HG levels and graphically displayed. *, $P < 0.001$. **E,** high-2HG tumors and matched normal tissue were analyzed for L2HGDH protein levels by immunoblotting (T, RCC tumor; N, benign tissue). **F,** immunohistochemistry for L2HGDH in normal kidney and high-L-2HG tumor. Red and black arrows, proximal and distal tubular epithelial cells, respectively. **G,** L-2HG levels were measured in A498 cells stably transduced with control vector and *L2HGDH* cDNA. **H,** analysis of TCGA data assessing the effects of copy loss on mRNA gene expression of *L2HGDH*. LOH, loss of heterozygosity; RNA-seq, RNA sequencing. *, $P < 0.0001$.

L2HGDH effects on histone methylation patterns. Stable expression of L2HGDH in both A498 and RXF393 cells led to reduced H3K27me3 and H3K9me3 levels, consistent with reduced L-2HG levels and ensuing activation of histone demethylase activity (Fig. 4E). Collectively, these demonstrate that modulation of L2HGDH expression can impart effects on both DNA and histone modifications. Given the emerging role of epigenetic alterations in RCC, we examined the effects of L2HGDH expression on *in vitro* phenotypes. Notably, L2HGDH expression reduced proliferation and colony formation in A498 and RXF393 RCC cells (Fig. 4F and G).

DISCUSSION

Here, we observe elevation of L-2HG in ccRCC, the most common RCC histologic subtype. We also demonstrate a novel mechanism for L-2HG elevation via reduced mRNA and protein expression of L2HGDH in part due to loss of the *L2HGDH* gene. Previous studies demonstrate that elevated cellular levels of L-2HG are present in an inborn error of metabolism that results from L2HGDH deficiency (13). Although rare, a significant proportion of these patients develop tumors, suggesting a role for L-2HG in carcinogenesis. In particular, L2HGDH deficiency has been linked to brain tumors and Wilms' tumor (15, 16). To the best of our knowledge, this is the first report to demonstrate specific elevation of the L enantiomer in cancer. This is in contrast to elevation of D-2HG, which has been identified in a growing list of cancers with *IDH* mutations. Notably, a recent study reported increased 2HG in breast cancers in the absence of *IDH* mutations (17). However, the specific enantiomer was not reported.

Our data do not exclude alternative mechanisms for L-2HG accumulation in ccRCC. Prior studies have demonstrated that "off-target" activity of malate dehydrogenase (MDH1/MDH2) can convert 2-OG to L-2HG (18). Substrates for this reaction include glucose and glutamic acid (19). It is well established that glutamine can be converted to glutamate (via glutaminases), which can eventually be converted to 2-OG. This is particularly relevant given recent studies by Wise and colleagues (20) demonstrating that HIF1α can promote modest

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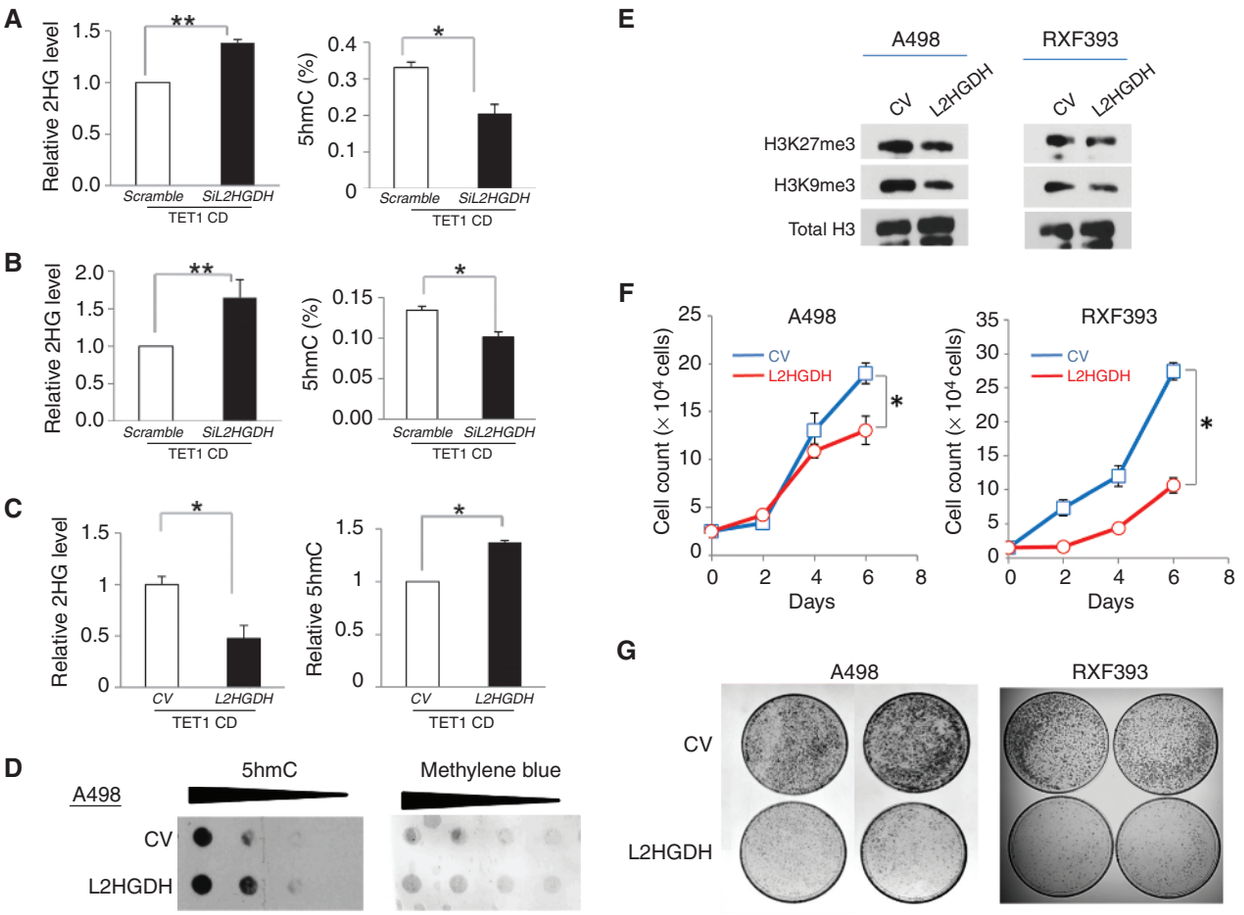

Figure 4. Knockdown or ectopic expression of *L2HGDH* is associated with changes of intracellular L-2HG concentration and DNA 5hmC level. siRNA to L2HGDH was cotransfected with the TET1 catalytic domain (CD) in HK-2 cells (**A**) and HEK293 cells (**B**). A noncoding scramble was used as a control siRNA (siControl). Mass spectrometry confirmed raised 2HG with L2HGDH knockdown (left). Genomic DNA was also isolated to determine 5hmC level by ELISA (right). **C,** HEK293 cells were transiently cotransfected with the TET1 CD and either *L2HGDH* cDNA or control vector (CV). Cells were subsequently challenged for 4 hours with 1 mmol/L L-2HG octyl ester. Metabolites were extracted for measurement of intracellular total 2HG level and analyzed by LC/MS (left). Genomic DNA was also isolated to determine 5hmC level by ELISA (right). Error bars, SD from at least two independent experiments. **, $P < 0.005$; *, $P < 0.05$. **D,** 5hmC dot-blot assay in A498 cells ± L2HGDH cDNA. Methylene blue blot is included for loading control. **E,** histone immunoblotting in A498 and RXF393 cells ± *L2HGDH* cDNA. **F** and **G,** proliferation and colony formation assays in RCC cells ± *L2HGDH* cDNA.

increases in 2HG synthesis via IDH-mediated reductive carboxylation of glutamine-derived 2-OG. However, enantiomeric resolution of 2HG was not performed in this study. Nevertheless, given recent studies on reductive glutamine metabolism in VHL-deficient RCC cells (in which HIF is stabilized; refs. 21, 22), these alternative mechanisms warrant further investigation.

2HG is part of a growing list of small molecules referred to as oncometabolites, or small molecules with putative transforming properties. Recent studies have linked D-2HG to the promotion of leukemogenesis (23). In particular, D-2HG promotes cytokine independence and blocks differentiation in hematopoietic cells. Mutations in tumors of *FH* and *SDH* lead to elevated levels of fumarate and succinate, respectively, due to loss of enzymatic activity. Germline mutations of *FH* predispose individuals to the development of renal cancer as well as cutaneous and uterine leiomyomas (24). Germline *SDHB* mutations have also been linked to renal cancer as well as pheochromocytomas and paragangliomas (25, 26). Hence, a notable finding is that all three metabolites are linked to RCC.

A unifying theme among these three oncometabolites is their ability to inhibit 2OGDs. Members include PHDs, 5mC DNA hydroxylases (TET 1–3), and histone demethylases. Multiple studies have shown that fumarate, succinate, and 2HG can inhibit TET enzymatic activity (11, 27, 28). Consistent with these data, we identified reduced 5hmC levels in tumors with elevated 2HG. The fold changes of 2HG we have identified in RCC are more modest relative to the 2HG elevations noted in the context of *IDH* mutations. A prior study by Choi and colleagues (29) analyzed 2HG (both D and L) in *IDH*-mutant tumors by the same methodology as our study. The reported range of D-2HG in these tumors ranged from approximately 20 to 200 nmol/mg protein.

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We identified multiple tumors with L-2HG levels within 10-fold of *IDH*-mutant tumors. Notably, prior studies have demonstrated that the L enantiomer of 2HG is a far more potent inhibitor of 2OGDs (including TETs) than the D enantiomer (11, 12, 27).

Given the multiple 2OGDs, it may be difficult to discern which of these are significant from a tumorigenic standpoint. Several lines of evidence suggest the importance of 2-OGDs relating to histone and DNA biology in kidney cancer. Recurrent mutations of *UTX*, which encodes an H3K27 demethylase that requires 2-OG, have been found in RCC (30). In addition, recent studies demonstrate increased expression of the catalytic subunit of the polycomb repressive complex 2 (PRC2), enhancer of zeste homologue 2 (EZH2), in ccRCC (31, 32). The PRC2 promotes H3K27 methylation. Our findings demonstrate that reexpression of L2HGDH in RCC cells reduces H3K27 methylation. Collectively, these data strongly suggest the importance of this histone mark in renal carcinogenesis. A notable finding from two independent studies in AML is that *TET2* mutations are mutually exclusive from *IDH* mutations (10, 33). The common link between these two seemingly unrelated mutations is their effect on TET enzymatic activity—through either loss-of-function mutations (*TET2* mutations) or, in the case of *IDH* mutation, 2HG-mediated inhibition of TET enzymatic activity through competition with the cofactor 2-OG. As noted, mutations of *TET2* have been identified in myeloid disorders such as AML and glioma. Notably, recent deep-sequencing efforts on RCC have identified *TET2* mutations in RCC (34). However, whether these mutants result in loss of function with effects on 5hmC levels has not been determined. In addition, reduced expression of TET enzymes has been demonstrated in a variety of tumors (7, 8). Correspondingly, reduced levels of 5hmC have been identified in cancer as well (7, 8). Given these data, L-2HG likely has multiple biologically relevant 2-OGD targets.

As noted previously, the TETs promote the conversion of 5mC to 5hmC, which is currently thought to promote DNA demethylation. Hence, it might be expected that loss of TET activity, due to either mutation or inhibition, would result in DNA hypermethylation. However, the reported effects on global DNA methylation in the context of *TET2* mutations in myeloid disorders have been conflicting (10, 35). Alternatively, 5hmC may represent its own epigenetic mark with specific effects on gene expression, perhaps through the recruitment of specific transcriptional regulators. These data point to the complexity of 5hmC biology and the need for further studies clarifying the mechanisms by which this DNA modification affects gene methylation, regulation, and expression in the setting of elevated L-2HG.

In summary, our data demonstrate elevated levels of 2HG, specifically the L enantiomer, in RCC. We show that 2HG elevation is associated with loss of 5hmC levels in RCC samples and that these changes are mediated by the reduced expression of L2HGDH. Specifically, we identify the first putative oncometabolite in the most common form of kidney cancer and its effects on the epigenetic landscape. Our data add to the growing body of evidence demonstrating the interplay between intermediary metabolism and nucleic acid biology in cancer.

METHODS

Plasmids

Expression plasmids of FLAG-tagged TET1 CD, TET1 CM, TET2 CD, and TET2 CM were kindly provided by Dr. Yi Zhang through Addgene. The *L2HGDH* expression plasmid pcDNA3.1-L2HGDH was constructed by cloning the full-length human *L2HGDH* cDNA amplified from human kidney epithelial HK-2 cells into pcDNA3.1 using primers 5′-TTTGAATTCATGGTGCCAGCGCTGCGTTAT-3′ and 5′-TTTGG TACCTTATAATTCAAATCTTTGTTGTACTTCATCTGCAATC-3′.

Cell Culture and Transfection

All lines were acquired from the ATCC, except RXF-393 (NCI), HRE152 (J.A. Copland, Mayo Clinic, Jacksonville, FL), and RCC4 (P. Ratcliffe, University of Oxford, Oxford, UK). Cell lines were periodically tested for *Mycoplasma*. No other authentication was performed. For transient transfection in HEK293, cells were transfected using Lipofectamine 2000 (Invitrogen) and calcium phosphate methods for RNA interference and cDNA expression, respectively. For *L2HGDH* knockdown in HK-2 cells, cells were transfected with pooled siRNA reagent (Thermo Fisher) using the Amaxa 4D Nucleofector 4D nucleofector (Lonza) system according to the manufacturer's protocol. A nontargeting scramble siRNA pool was used as a negative control (Thermo Fisher).

RNA Extraction and Quantitative RT-PCR

Total RNA from RCC patient samples and RCC cell lines was extracted with TRizol (Invitrogen). cDNA was generated using the VILO RT Kit (Invitrogen) and then used as a template for the *L2HGDH* Taqman expression assay probe (Applied Biosystems). Ribosomal protein (*RPLP0*) was used to normalize data in tissue samples. For cell line analysis, *L2HGDH* mRNA expression level was quantified by using $2^{-\Delta\Delta CT}$ method normalized to *GAPDH*. For tissues, the ΔC_t *L2HGDH* (C_t *L2HGDH* − C_t *RPLP0*) was measured for normal tissue, low–L-2HG, and high–L-2HG tumor. Multivariate analyses of variance (MANOVA) followed by ANOVA were conducted in SAS Version 9.3 to compare the expression level (ΔC_t *L2HGDH*) between the three groups. We used SAS GLM contrast to compare the High group with the others (Normal and Low). L-2HG values were log-transformed to meet the assumptions for parametric tests.

Quantitative 5-hydroxymethylation (5-hmC) Analysis

For DNA dot blotting, genomic DNA was denatured, serially diluted in NaOH/EDTA solution, and spotted on positively charged Nylon membranes (Roche Applied Science). The membrane was cross-linked (UVP) and then blocked with 5% milk in TBST for 30 minutes, followed by incubation with the anti–5-hmC antibody (Active Motif) overnight at 4°C and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody for 1 hour at room temperature. After washing three times with TBST, the membrane was treated with enhanced chemiluminescence reagent and scanned. For quantitation of 5hmC, Quest 5-hmC DNA ELISA kits (Zymo Research) were used following the manufacturer's protocol. Briefly, the bottom of the well was coated with anti–5-hydroxymethylcytosine polyclonal antibody, and the denatured 100 ng genomic DNA was added. To detect DNA bound to the anti–5-hmC pAb, anti-DNA HRP antibody and HRP developer were applied. Greenish-blue color was analyzed in the wells by a plate reader at 405- to 450-nm detection.

Metabolite Extraction and Chromatography/Mass Spectrometry of 2HG

For GC/MS and LC/MS analysis of tissues, human kidney samples, including normal and RCC, were extracted and prepared for

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analysis as described in the Supplementary Methods and as previously described (36).

For 2HG enantiomer analysis (i.e., D-2HG and L-2HG quantification), samples were analyzed as previously described (37). Briefly, extracts were derivatized with DATAN (diacetyl-L-tartaric acid), which permits enantiomeric analysis, followed by LC-MS/MS analysis and normalized to protein levels.

For total 2HG (D-2HG + L-2HG) measurement of samples from *in vitro* studies, cell pellets were washed in 1× PBS three times and extracted with 10% cold trichloroacetic acid (TCA), and precipitate was removed by centrifugation. TCA in the supernatant was removed by vortexing with four volumes of 1,1,2-trichlorotrifluoroethane (FREON)-trioctylamine (Sigma) mixture, and the upper aqueous layer was collected for analysis after centrifugation. Samples were analyzed by ion chromatography coupled with negative electrospray mass spectrometry (RFIC-MS; Dionex), and 2HG was determined by selected ion monitoring (SIM 147.1). 2HG in cell extracts was quantified by using a calibration curve of 2HG and normalized to protein content. Unless otherwise noted, 2HG refers to total levels (D + L).

Statistical Analysis

Unless otherwise noted, statistical analyses were carried out using the program "R" and Microsoft Excel software. Comparisons between groups for statistical significance were performed with a two-tailed paired *t* test and ANOVA. A P value of <0.05 was considered statistically significant in all cases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

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