Integrative Genomic Analyses of Sporadic Clear Cell Renal Cell Carcinoma Define Disease Subtypes and Potential New Therapeutic Targets

Vijay R. Dondeti1, Bradley Wubbenhorst3, Priti Lal4, John D. Gordan1, Kurt D’Andrea3, Edward F. Attiyeh5,7, M. Celeste Simon1,2,6, and Katherine L. Nathanson2,3

Abstract

Sporadic clear cell renal cell carcinoma (ccRCC), the most common type of adult kidney cancer, is often associated with genomic copy number aberrations on chromosomes 3p and 5q. Aberrations on chromosome 3p are associated with inactivation of the tumor suppressor gene von-Hippel Lindau (VHL), which activates the hypoxia-inducible factors HIF1α and HIF2α. In contrast, ccRCC genes on chromosome 5q remain to be defined. In this study, we conducted an integrated analysis of high-density copy number and gene expression data for 54 sporadic ccRCC tumors that identified the secreted glycoprotein STC2 (stanniocalcin 2) and the proteoglycan VCAN (versican) as potential 5q oncogenes in ccRCCs. In functional assays, STC2 and VCAN each promoted tumorigenesis by inhibiting cell death. Using the same approach, we also investigated the two VHL-deficient subtypes of ccRCC, which express both HIF1α and HIF2α (H1H2) or only HIF2α (H2). This analysis revealed a distinct pattern of genomic aberrations in each group, with the H1H2 group displaying, on average, a more aberrant genome than the H2 group. Together our findings provide a significant advance in understanding ccRCCs by offering a molecular definition of two subtypes with distinct characteristics as well as two potential chromosome 5q oncogenes, the overexpression of which is sufficient to promote tumorigenesis by limiting cell death. Cancer Res; 72(1); 112–21. ©2011 AACR.

Introduction

Approximately 210,000 individuals worldwide are diagnosed with renal cell carcinoma (RCC) each year, and RCC is responsible for more than 100,000 deaths annually (1). On the basis of histopathology, RCCs can be classified into several types, among which clear cell renal cell carcinoma (ccRCC) is the most common (2). Tumor stage and grade of ccRCCs are used to stratify patients and infer prognosis (3). However, because of tumor heterogeneity within ccRCCs, providing patients with reliable information about anticipated treatment response is challenging.

Some progress has been made in identifying underlying biologic determinants of ccRCCs. Importantly, the tumor suppressor von-Hippel Lindau (VHL) is inactivated in nearly 90% of sporadic ccRCC tumors (4, 5); pVHL, the protein encoded by VHL, is involved in multiple cellular pathways, but its best characterized function is the regulation of hypoxia-inducible factors HIF1α and HIF2α, the key mediators of the hypoxic response (6); pVHL is the recognition component of a multiprotein complex responsible for HIF1α and HIF2α degradation under oxygen-replete conditions and thus plays a key role in O2 homeostasis. VHL inactivation leads to constitutive HIF activity and promotes tumor growth by enhancing angiogenesis and cell proliferation (7, 8).

While both HIF1α and HIF2α likely play significant roles in ccRCC pathogenesis, they have been shown to have some differing properties (8, 9). Both HIF1α and HIF2α promote angiogenesis; however, HIF2α has been shown to be more important for tumor growth in RCC xenograft experiments (9, 10). Differences in HIF2α expression have been used to classify VHL-deficient ccRCC tumors into 2 subtypes, with one subtype expressing both HIF1α and HIF2α (H1H2) and another expressing only HIF2α (H2; ref. 11). Analysis of the 2 subtypes revealed that the H1H2 group shows increased mitogen-activated protein kinase and mTOR signaling, whereas the H2 group displays enhanced c-Myc activity. This subclassification, in part, explains the heterogeneity of ccRCCs (2), suggesting the...
possibility that the subtypes may have different clinical outcomes and need to be treated using distinct targeted therapies.

Cyto genetic analysis also has contributed to our understanding of ccRCC and revealed that 3p losses (60%) and 5q gains (33%) are the most prevalent genetic abnormalities in sporadic ccRCCs (12, 13). Frequent VHL inactivation is, in part, explained by loss of 3p. However, specific targets on 5q have not yet been elucidated. Systematic sequencing of ccRCCs revealed mutations in the histone-modifying genes SETD2 (SET domain containing 2), KDM5C [lysine (K)-specific demethylase 5C], and KDM6A [lysine (K)-specific demethylase 6A] and the tumor suppressor NF2 (neurofibromin 2; ref. 14). Varela and colleagues identified truncating mutations in PBRM1 (polybromo 1), a SWI/SNF complex gene located on 3p (15). Of the targets identified as mutated by sequencing, only PBRM1 is involved in a large proportion (41%) of ccRCC tumors, whereas the others are present in approximately 3% of samples. Beroukhim and colleagues identified the tumor suppressors VHL, RUNX3, and CDKN2A/B as deleted and the MYC oncogene as amplified in ccRCCs; however, MYC amplification appears to be more important in renal cancer cell lines than in tumors (16). Most of the genes identified thus far by sequencing and copy number (CN) analysis are inactivated in ccRCCs and do not affect a large percentage of cases.

Materials and Methods

Sample acquisition

Frozen tumor samples for primary analysis were obtained through the Collaborative Human Tissue Network and from the Hospital of the University of Pennsylvania, Philadelphia, PA. Samples were embedded in optimum cutting temperature matrix and cut for genomic analysis or冻存 for the Collaborative Human Tissue Network and from the Hospital of the University of Pennsylvania, Philadelphia, PA. Samples were embedded in optimum cutting temperature matrix and cut for genomic analysis or frozen for further studies.

Immunohistochemistry

Immunostaining for HIF1α and HIF2α proteins was done as previously described in the work of Gordan and colleagues (11). DNA extraction and copy number analysis

Total genomic DNA was extracted from tissue sections using the Promega Wizard Genomic DNA Purification Kit. Illumina HumanHap550-2v3_B (561,466 SNPs) and Human610-Quadv1.0 (620,901 SNPs) arrays were used in this study. Sample hybridization and data collection were conducted at the Penn Genomics Facility according to the manufacturer’s protocol. To check for potential batch effects, we examined the hybridization controls present on all Illumina SNP arrays and box plots of signal intensities from the arrays in both batches to confirm that the data from both batches were in the same range. This analysis revealed no batch effects. The raw data were then processed using BeadStudio (Illumina) to obtain single-nucleotide polymorphism (SNP)-level signal intensities, which were then analyzed with Partek Genomic Suite to calculate SNP-level copy number. GLAD was used to segment the SNP-level copy number data (17). A segment needed to contain a minimum of 8 SNPs to be called a gain or loss. GLAD analysis resulted in segments with an average length of 13.5 megabases. Details about the segmentation analysis can be found in the Supplementary Methods. The segmented data were then analyzed using GISTIC (17). A total of 54 samples were analyzed: 33 new samples (GSE27852) and 21 samples (GSE13282) from a study previously published by our group (11).

Gene expression analysis

The gene expression data (GSE11904) was previously described in a study from our group (11).

Broad data

The Broad Data set was described in (16) and downloaded from GEO (GSE14994). The segmented copy number data were obtained from Tumorscape (18). Only data from sporadic ccRCC tumors were used for this analysis.

H1H2 and H2 genomic aberration analysis

The copy number data for each sample was used to determine what percentage of its genome was lost (CN < 1.7), normal (1.7 < CN < 2.3), or gained (CN > 2.3). In each sample, for percentage calculations, total genome length was determined by adding up the lengths of all segments (in bp) provided by GLAD segmentation, and lengths of lost, normal, and gained regions were determined by adding up the lengths of all segments (in bp) called lost, normal, or gained, respectively. The distribution of percentage genome lost, normal, or gained in H1H2 samples was compared with the corresponding distribution in H2 samples using the 2-tailed t-test for statistical significance.

Cell culture, siRNAs, and transfections

The human ccRCC cell lines 786-O (obtained from American Type Culture Collection) and RCC10 (kind gift from W.G. Kaelin) were used for cell culture experiments. The cell lines were verified for VHL and HIFα expression status using quantitative PCR and Western blot analysis within the last 6 months. All siRNAs were purchased from Ambion, except for siEZH2-1, which was purchased from Qiagen. The following siRNAs were used in this study: siNeg (silencer select negative control#2), siEZH2-1 (s16387), siEZH2-2 (s16388), siSTC2-1 (s16389), siSTC2-2 (s16388), siVCAN-1 (s229334), and siVCAN-2 (s229335). HiPerFect (Qiagen) was used to transfect cells. For all experiments, 50,000 cells were plated in 6-well plates. After 24 hours, the cells were transfected with siRNAs and then grown for 3 days. All experiments were carried out in standard media containing 10% serum and grown under standard conditions.

Cell count, cell-cycle, and cell death analyses

Cells were counted three days after transfection using the Countess (Invitrogen). For cell-cycle analysis, cells were pulsed with 10 μmol/L bromodeoxyuridine (BrdUrd) for 15 minutes, stained with Alexa Fluor 488–conjugated anti-BrdU (Invitrogen), and then analyzed using standard protocols. For cell death analysis, cells were stained with FITC (fluorescein...
isothiocyanate)-Annexin V and propidium iodide (PI; BioVision) and analyzed using standard protocols. For cell-cycle analysis and cell death analysis, measurements were taken 3 days after transfection. All experiments were done in triplicate.

Results

Copy number profiling of all ccRCC

To identify aberrant genomic regions of ccRCCs, we conducted a genome-wide copy number analysis on 54 sporadic ccRCC tumors. Stages I, II, III, and IV comprise 46%, 15%, 30%, and 2% of the tumors in the study, respectively (Supplementary Table S1). Additional clinical information for the tumors is provided in Supplementary Table S1. The copy number data were segmented using GLAD (17). Segments with CN > 2.3 were considered “gains” and those with CN < 1.7 were considered “losses.” Thresholds for gains and losses were determined by analyzing copy number data of peripheral blood mononuclear cells (PBMC). Using these thresholds, 99.5% of PBMCs’ genomes were considered “normal” (Supplementary Fig. S1). Together, the gains and losses will be referred to as “aberrations.” Regions of gain on chromosomes 1 (5%), 5 (30%), 7 (17%), 11 (6%), 12 (13%), 16 (7%), 18 (6%), 19 (7%), 20 (7%), 21 (7%), and 22 (6%) and loss on chromosomes 1 (10%), 3 (80%), 6 (17%), 8 (25%), 9 (25%), 10 (11%), 13 (11%), 14 (31%), 15 (7%), and 18 (9%) were observed (numbers in parentheses indicate the percentage of samples in which the aberration was present; Fig. 1). These findings are consistent with previous reports (14, 16, 19). Although 3p losses and 5q gains were the most common alterations, considerable variation was observed (Fig. 1A). To determine the prevalence of different aberrations, a frequency histogram was plotted (Fig. 1C). Next, statistically significant regions of aberration were identified using GISTIC analysis (Fig. 1B; ref. 17). From Fig. 1, it is evident that aberrations are prevalent throughout the entire genome in ccRCCs.

Integrated copy number and gene expression analysis

The many putative target genes identified by GISTIC were too numerous to validate. Therefore, we integrated the copy number data with gene expression data to narrow the candidate gene set. Figure 2A provides an overview of the approach to derive integrated genomic data. We applied the integrated analysis to all 54 ccRCC tumors. We further applied the method to the 2 subclasses—H1H2 and H2—with the goal of identifying targets that are specific and unique to each subgroup. As the goal of the individual subtype analysis was to identify targets that are unique to each subtype, any targets common to both...
subtypes were removed from the final gene list for each subtype.

**Integrated genomic analysis of all ccRCCs**

The copy number data were segmented to delineate aberrant regions. Genomic segments aberrant in at least 10% (5 of 54) of tumor samples were selected for further analysis. Our data set includes gene expression data for 18 tumors that underwent copy number profiling (11). The expression patterns of genes in gained segments were inspected to identify those overexpressed at the mRNA level relative to normal kidney tissue. Genes statistically significantly overexpressed by at least 2-fold were taken into further analysis. This procedure led to the identification of 350 concordantly gained and overexpressed genes (Supplementary Table S2). A similar comparison of lost segments with underexpressed genes resulted in the identification of 523 lost and underexpressed genes (Supplementary Table S3). This set of 350 gained-overexpressed genes and 523 lost-underexpressed genes will hereafter be referred to as the "Penn Data" (Fig. 2A).

To narrow this gene list further, the Penn Data were compared with that published by Beroukhim and colleagues to identify targets common to both datasets (16). Beroukhim and colleagues investigated both sporadic and VHL disease-associated cases of ccRCCs. Data from the sporadic ccRCC tumors were exclusively used for this study, as the Penn Data set only contains sporadic ccRCC tumors. The Beroukhim and colleagues data set contains copy number and gene expression data for 54 and 27 sporadic ccRCC tumors, respectively. Copy number and expression data were analyzed as described above for the Penn Data, leading to the identification of 453 gained-overexpressed genes (Supplementary Table S4) and 586 lost-underexpressed genes (Supplementary Table S5). This data set will henceforth be referred to as the "Broad Data" (Fig. 2A).

Comparison of the Penn and Broad Data sets revealed 72 gained and overexpressed target genes (Supplementary Table S6) and 187 lost and underexpressed target genes (Supplementary Table S7) present in both data sets (Fig. 2B). Table 1 lists the top 10 gained-overexpressed genes and top 10 lost-underexpressed genes when ordered by fold change in expression. From Table 1, it is evident that all the targets are aberrant in more than 10% of ccRCCs.

**Copy number analysis of ccRCCs based on HIF expression**

We have previously shown that VHL-deficient ccRCC tumors can be grouped into 2 subtypes—H1H2 and H2—on the basis of HIFα expression (11). Immunostaining was used to classify the 54 ccRCC tumors in this study into 29 H1H2, 19 H2, and 6 VHL wild-type tumors (Supplementary Fig. S2). The VHL wild-type tumors were not analyzed further because of their low number. Using the available copy number data, we profiled the gains and losses of the 2 subtypes. To determine the overall prevalence of aberrations, a frequency histogram was plotted for each subtype (Fig. 3A). Differences between H1H2 and H2 tumors were observed. Gains are found over the entirety of chromosome 3 in H1H2 tumors, whereas in H2 tumors, they localized to 5q. Only H1H2 tumors appear to have gains on chromosomes 16 (9%), 19 (7%), 20 (5%), and 22 (3%). The differences between the 2 subtypes are even more marked when losses are cataloged. Losses on chromosome 6q and 8p are more
that there are factors that influence tumorigenesis unique and specific to each subtype. Hence, any targets common to both subgroups were removed. Removing targets common to both subgroups helps us define targets that may specifically play a role in one subtype but not the other. This analysis uncovered 48 gained-overexpressed genes (Supplementary Table S8) and 106 lost-underexpressed genes (Supplementary Table S9) that are restricted to the H1H2 subtype and 121 gained-overexpressed genes (Supplementary Table S10) and 79 lost-underexpressed genes (Supplementary Table S11) that are restricted to the H2 subtype. Table 2 lists the top 5 gained-overexpressed genes and the top 5 lost-underexpressed genes from each subtype when sorted by fold change in expression. This analysis of H1H2 and H2 tumors suggests that there are factors that influence tumorigenesis unique and specific to each subtype.

### Comparison of level of genomic aberration in H1H2 and H2 tumors

We previously noted that a key difference between the 2 subtypes was the overexpression of DNA damage response genes, particularly those involved in double-strand break repair, in H2 samples relative to H1H2 samples (11). This finding led us to hypothesize that H2 tumors would exhibit fewer genomic aberrations than H1H2 tumors, which was supported preliminarily by copy number profiling of 10 H1H2 and 11 H2 tumor samples (11). Herein, we use a larger sample set and more detailed copy number distribution analysis to confirm our initial findings. Using the segmented copy number data of the H1H2 and H2 tumors, the average copy number distribution for each subtype was plotted (Fig. 4). The copy number distribution depicts the average percentage of the genome present in a given copy number bin (of 0.1 from 0 to 4) in each subtype. From Fig. 4, it is apparent that in H2 tumors, a greater percentage of the genome can be described as normal than H1H2 (1.7 \( \times \) \( \text{CN} \) < 2.3, \( P = 0.005 \)). Conversely, in H1H2 tumors, a greater percentage of the genome can be regarded as lost (CN < 1.7, \( P = 0.005 \)). Also, on average, a greater percentage of the genome of H1H2 tumors was gained (CN > 2.3) than in H2 tumors, and this finding showed a trend toward significance (\( P = 0.064 \)). This detailed analysis further establishes that H1H2 and H2 tumors are indeed 2 distinct subgroups of ccRCCs. Thus, the 2 subtypes have different gene expression profiles, copy number profiles, and genomic aberration patterns.

### Validation of integrated genomic analysis

The results above indicate that the integrated genomic approach can be used to identify targets that are important for ccRCCs as a whole and for H1H2 and H2 subtypes individually. Next, we wanted to validate the integrated genomic approach by functionally validating selected putative targets in cell culture and focused on those genes identified through our initial analysis of the whole set of ccRCCs. Following a review of the gained-overexpressed targets, EZH2 (7q36.1), STC2 (5q35.2), and VCAN (5q14.3) were chosen for further investigation. EZH2 was selected because it has been shown to be an unfavorable prognostic marker in ccRCCs (20) and inhibits apoptosis in ccRCC cells (21). STC2 was chosen as it has been found to be a negative prognostic marker in ccRCCs (22), but its functional role in ccRCCs has not yet been defined. Finally, VCAN was

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cytoband</th>
<th>Frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPCAT1</td>
<td>5p15.33</td>
<td>24</td>
</tr>
<tr>
<td>VCAN</td>
<td>5q14.3</td>
<td>19</td>
</tr>
<tr>
<td>HAPLN1</td>
<td>5q14.3</td>
<td>19</td>
</tr>
<tr>
<td>LOX</td>
<td>5q23.1</td>
<td>31</td>
</tr>
<tr>
<td>TGFBI</td>
<td>5q31.1</td>
<td>28</td>
</tr>
<tr>
<td>FABP6</td>
<td>5q33.3</td>
<td>30</td>
</tr>
<tr>
<td>PTTG1</td>
<td>5q33.3</td>
<td>30</td>
</tr>
<tr>
<td>STC2</td>
<td>5q35.2</td>
<td>31</td>
</tr>
<tr>
<td>NPTX2</td>
<td>7q22.1</td>
<td>17</td>
</tr>
<tr>
<td>EZH2</td>
<td>7q36.1</td>
<td>13</td>
</tr>
</tbody>
</table>

### Lost and underexpressed

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cytoband</th>
<th>Frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH1R</td>
<td>3p21.31</td>
<td>76</td>
</tr>
<tr>
<td>LPA</td>
<td>6q25.3</td>
<td>17</td>
</tr>
<tr>
<td>PLG</td>
<td>6q26</td>
<td>17</td>
</tr>
<tr>
<td>LRRC19</td>
<td>9p21.2</td>
<td>20</td>
</tr>
<tr>
<td>TYRP1</td>
<td>9p23</td>
<td>26</td>
</tr>
<tr>
<td>TRPM3</td>
<td>9q21.11</td>
<td>24</td>
</tr>
<tr>
<td>FBP1</td>
<td>9q22.32</td>
<td>20</td>
</tr>
<tr>
<td>ALDOB</td>
<td>9q31.1</td>
<td>22</td>
</tr>
<tr>
<td>SLC7A8</td>
<td>14q11.2</td>
<td>24</td>
</tr>
<tr>
<td>SERPINA5</td>
<td>14q32.13</td>
<td>20</td>
</tr>
</tbody>
</table>

NOTE: Frequency indicates how often the gene is aberrant in the Penn Data set.
chosen because, although it has not yet been linked to ccRCCs, it has been shown to be upregulated in ovarian cancer (23) and promote cell proliferation and inhibit apoptosis in cell culture assays (24). STC2 is at the distal region of the frequently gained 5q region, whereas VCAN is at the proximal region. We chose STC2 and VCAN so that we could evaluate 2 different and distant regions of the 5q gain.

Validation of potential targets: EZH2, STC2, and VCAN

To evaluate the potential roles played by EZH2, STC2, and VCAN in ccRCCs, siRNA experiments were carried out using 786-O cells (expressing only HIF2α, “H2”) and RCC10 cells (expressing HIF1α and HIF2α, “H1H2”), human ccRCC cell lines. Two independent siRNAs each were used to silence EZH2, STC2, and VCAN. Real-time quantitative reverse transcription PCR (qRT-PCR) was used to monitor knockdown efficiency (Fig. 5A). Western blot analysis was used to confirm decrease in protein levels after silencing the targets (Supplementary Fig. S3C). First, we examined whether inhibiting these targets affects cell number. Suppressing EZH2, STC2, and VCAN significantly reduced cell numbers relative to the negative control siRNA in 786-O and RCC10 cells (Fig. 5B and Supplementary Fig. S3A). To determine whether the targets affected cell proliferation or cell death, cell-cycle studies and Annexin V staining analysis were undertaken (Fig. 5E and F and Supplementary Fig. S4). Interestingly, cell-cycle analysis failed to reveal any marked differences between cells treated with negative control and target siRNA (Fig. 5C). However, both 786-O and RCC10 cells treated with target siRNA showed a significant increase in cell death when compared with those exposed to negative control siRNA (Fig. 5D and Supplementary Fig. S3B). Silencing targets of interest increased cell death by 4% to 15%. These results indicate that EZH2, STC2, and VCAN primarily promote cell growth by inhibiting cell death. These experimental findings validate the integrated genomic approach used to identify the candidate genes.

We also tested whether simultaneous inhibition of STC2 and VCAN would have an additive effect on cell numbers. Upon simultaneously silencing STC2 and VCAN, we achieved more than 70% knockdown efficiency for both targets (Supplementary Fig. S5A) but did not observe any additive or synergistic effect on cell viability (Supplementary Fig. S5B and S5C). We also used terminal deoxynucleotidyl

Figure 3. Copy number profiling of H1H2 and H2 subtypes. A, frequency histograms showing the percentage of samples that are altered at each genomic segment. B, GISTIC analysis of the gained regions in H1H2 and H2 tumors. C, GISTIC analysis of the lost regions in H1H2 and H2 tumors. Gains are in red and losses are in blue.
transferase–mediated dUTP nick end labeling (TUNEL) staining to determine what effect gain of EZH2, STC2, or VCAN may have on apoptosis in tumors. We conducted TUNEL staining on 6 tumors without changes in the 3 targets and 12 tumors with a gain in one or more of the selected loci, comparing the percentage of nuclei that were TUNEL positive (Supplementary Fig. S6). We did not see a statistically significant difference in TUNEL positivity between tumors with a gain of at least one of the targets and those without. Tumors lacking gains in the EZH2, STC2, or VCAN loci may therefore possess other factors inhibiting apoptosis.

### Discussion

The goal of this study was to use an integrated genome-wide approach to identify genes which play an important role in sporadic ccRCCs and 2 individual ccRCC subtypes H1H2 and H2. Copy number analysis was integrated with gene expression data for 54 sporadic ccRCCs to find genes that were either concordantly gained and overexpressed or lost and underexpressed. The same analysis was also conducted on a publicly available data set (Broad Data; ref. 16), and the results compared with find overlapping targets. This process led to the identification of 72 gained-overexpressed and 187 lost-underexpressed genes.

We also used the integrated genomic approach to study the differences between the H1H2 and H2 subsets. Although H1H2 and H2 tumors share some overlapping copy number and expression changes, the integrated genomic analysis revealed that there are genes involved in tumorigenesis that are unique and specific to each subtype. Using copy number analysis, we found that the genome of the H1H2 group is on average more aberrant than the H2 group. This difference may be because H2 tumors express DNA damage response genes, particularly those involved in double-strand break repair, at a higher level than the H1H2 group (11). The genomic differences in H1H2 and H2 tumors may have clinical implications. GISTIC analysis revealed that copy number losses in 9p and 14q are more significant in the H2 group than in the H1H2 group. However, at the level of gene expression, we only see significant changes in the genes from 14q in the H2 subtype. Intriguingly, losses of 14q have been independently associated with a decrease in disease-specific survival in ccRCCs (12). The data presented herein support the postulate that ccRCCs can be subtyped on the basis of HIFα expression; the survival data from Klatte and colleagues indicate that the H2 subtype may be potentially

### Table 2. Genes determined to be either gained and overexpressed or lost and underexpressed uniquely in H1H2 or H2 subtypes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cytoband</th>
<th>Frequency, %</th>
<th>Gene</th>
<th>Cytoband</th>
<th>Frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H1H2</strong></td>
<td></td>
<td></td>
<td><strong>Lost and underexpressed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBE2I</td>
<td>16p13.3</td>
<td>45</td>
<td>LPA</td>
<td>6q25.3</td>
<td>28</td>
</tr>
<tr>
<td>C16orf42</td>
<td>16p13.3</td>
<td>45</td>
<td>PLG</td>
<td>6q26</td>
<td>28</td>
</tr>
<tr>
<td>TPM4</td>
<td>19p13.12</td>
<td>14</td>
<td>GLDC</td>
<td>9p24.1</td>
<td>21</td>
</tr>
<tr>
<td>AP3D1</td>
<td>19p13.3</td>
<td>38</td>
<td>ALDOB</td>
<td>9q31.1</td>
<td>21</td>
</tr>
<tr>
<td>GADD45B</td>
<td>19p13.3</td>
<td>38</td>
<td>ADD3</td>
<td>10q25.1</td>
<td>14</td>
</tr>
<tr>
<td><strong>H2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELL2</td>
<td>5q15</td>
<td>21</td>
<td>WBSCR16</td>
<td>7q11.23</td>
<td>21</td>
</tr>
<tr>
<td>C5orf13</td>
<td>5q22.1</td>
<td>26</td>
<td>ALDH6A1</td>
<td>14q24.3</td>
<td>26</td>
</tr>
<tr>
<td>EPB41L4A</td>
<td>5q22.2</td>
<td>26</td>
<td>FOS</td>
<td>14q24.3</td>
<td>26</td>
</tr>
<tr>
<td>RAPGEF6</td>
<td>5q31.1</td>
<td>26</td>
<td>ACOT1</td>
<td>14q24.3</td>
<td>26</td>
</tr>
<tr>
<td>HAVCR1</td>
<td>5q33.33</td>
<td>32</td>
<td>C14orf1</td>
<td>14q24.3</td>
<td>26</td>
</tr>
</tbody>
</table>

NOTE: Frequency indicates how often the gene is aberrant.

Figure 4. Copy number distribution in H1H2 and H2 subtypes. In H2 tumors, a greater percentage of the genome is normal (1.7 < CN < 2.3, P = 0.009) than in H1H2 tumors, and conversely, in H1H2 tumors, a greater percentage of the genome is gained (CN > 2.3, P = 0.064) or lost (CN < 1.7, P = 0.006) relative to H2 tumors.
linked to clinical outcome (12). Together, these findings potentially suggest that different therapeutic regimens may need to be used to treat patients with H1H2 and H2 tumors.

To validate the integrated genomic approach, we chose to study 3 of the identified targets using cell culture studies. Genome-wide studies of ccRCC to date have primarily revealed genes which are inactivated during tumorigenesis. Thus, we focused on the concordantly gained and overexpressed genes. Three gained-overexpressed genes, EZH2 (7q36.1), STC2 (5q35.2), and VCAN (5q14.3), were chosen for study in cell culture experiments. EZH2 is thought to promote cell proliferation and inhibit cell differentiation by silencing tumor suppressors (25, 26), and known to be activated in breast (27) and prostate cancers (28). It also has been implicated in ccRCCs (20, 21). It is interesting that while EZH2, a histone methyltransferase, is gained, KDM6A, a histone demethylase, is mutated in ccRCC (14). EZH2, a member of the PcG family, is believed to play a role in cancer by silencing tumor suppressors, such as ARF (26, 29). KDM6A has been shown to demethylate many retinoblastoma-binding proteins leading to their activation and subsequent cell-cycle arrest (30). Thus, both KDM6A mutation and EZH2 gain will lead to increased cell-cycle progression. STC2 has been shown to be overexpressed in prostate (31), breast (32), and colorectal cancers (33) and has been linked to ccRCCs (22), but its functional role has not yet been determined. STC2 is upregulated under hypoxia and thought to help cells adapt to the stress of the tumor microenvironment (33).

VCAN is involved in cell adhesion, proliferation, migration, and angiogenesis and thought to promote cell proliferation by increasing the propagation of signals from mitogens such as platelet-derived growth factor (PDGF) and TGF-β1 (34). VCAN has been linked to prostate, breast, and ovarian cancers (23, 34), but not yet to ccRCCs. Using siRNA experiments, we showed that EZH2, STC2, and VCAN promote tumor growth by inhibiting cell death in ccRCC cells. These results strongly suggest that EZH2, STC2, and VCAN play roles in ccRCCs and validate the integrated approach used to identify them.

While the experiments described here examine EZH2, STC2, and VCAN individually, it is evident from the copy number data
that the genes are gained simultaneously. In tumors that show a gain of STC2, there is a 29% and 48% chance that EZH2 or VCAN will also be gained, respectively. In approximately 4% of the tumors, all 3 targets are gained. To test whether STC2 and VCAN have an additive effect on cell numbers and cell death, we simultaneously silenced them. We did not detect any additive or synergistic effects. More studies will be required to better understand the detailed functions of EZH2, STC2, and VCAN and whether they interact in vivo.

EZH2, STC2, and VCAN are commonly gained in both the H1H2 and H2 subtypes of ccRCCs. EZH2, STC2, and VCAN are gained in 7%, 31%, and 24% of H1H2 samples respectively, and in 11%, 32%, and 11% of H2 samples, respectively. In addition to these common targets, the individual subtype analysis has revealed that there are targets that are unique to each subtype. EZH2, STC2, and VCAN may be responsible for the earlier steps in tumorigenesis in both subtypes, whereas the targets that are specific to each subtype may be more important in the later steps of tumorigenesis. More work is needed to understand the different roles played by the common targets and roles played by the targets specific to each subgroup. It is also important to note that most (46%) of the tumors in this study are stage I tumors and that it is possible that alternative targets may be important during different tumor stages.

In summary, we are the first to identify and functionally validate 2 potentially important targets on 5q (STC2 and VCAN), a region gained in more than 30% of ccRCC samples. We also have further established that ccRCCs can be classified into subtypes on the basis of HIFα expression with each group having its own specific pattern of copy number alterations.

Disclosure of Potential Conflicts of Interest

M.C. Simon is an Investigator of the Howard Hughes Medical Institute. No potential conflicts of interest were disclosed by other authors.

Acknowledgments

The authors thank John Maris, Brad Johnson, and Frank Lee for useful discussions; Penn Genomics Facility for sample hybridization and data collection; and Hakon Hakonarson for providing U7 data for PBMCs. Tissue samples were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute. Other investigators may have received samples from these same tissues.

Grant Support

This work was supported by NIH Program Project grant CA104838 (to M.C. Simon), the Howard Hughes Medical Institute (to M.C. Simon), NIH R01 grant CA135509 (to K.L. Nathanson), and the Abramson Cancer Center. This project is funded, in part, under a grant with the Pennsylvania Department of Health (to K.L. Nathanson). The Department specifically disclaims responsibility for any analyses, interpretations, or conclusions. Services provided in the Penn Genomics Facility are supported by the Abramson Cancer Center core grant 5P30CA016660.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 23, 2011; revised November 9, 2011; accepted November 11, 2011; published OnlineFirst November 17, 2011.

References

Integrative Genomic Analyses of Sporadic Clear Cell Renal Cell Carcinoma Define Disease Subtypes and Potential New Therapeutic Targets


Cancer Res 2012;72:112-121. Published OnlineFirst November 17, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-1698

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/11/17/0008-5472.CAN-11-1698.DC1

Cited articles
This article cites 34 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/72/1/112.full.html#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
/content/72/1/112.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.