Combined Gene Expression Profiling and RNAi Screening in Clear Cell Renal Cell Carcinoma Identify PLK1 and Other Therapeutic Kinase Targets

Yan Ding1, Dan Huang1, Zhongfa Zhang1, Josh Smith1, David Petillo1, Brendan D. Looyenga2, Kristin Feenstra3, Jeffrey P. MacKeigan2, Kyle A. Furge1,4, and Bin T. Teh1,5

Abstract

In recent years, several molecularly targeted therapies have been approved for clear cell renal cell carcinoma (ccRCC), a highly aggressive cancer. Although these therapies significantly extend overall survival, nearly all patients with advanced ccRCC eventually succumb to the disease. To identify other molecular targets, we profiled gene expression in 90 ccRCC patient specimens for which tumor grade information was available. Gene set enrichment analysis indicated that cell-cycle–related genes, in particular, Polo-like kinase 1 (PLK1), were associated with disease aggressiveness. We also carried out RNAi screening to identify kinases and phosphatases that when inhibited could prevent cell proliferation. As expected, RNAi-mediated knockdown of PLK1 and other cell-cycle kinases was sufficient to suppress ccRCC cell proliferation. The association of PLK1 in both disease aggression and in vitro growth prompted us to examine the effects of a small-molecule inhibitor of PLK1, BI 2536, in ccRCC cell lines. BI 2536 inhibited the proliferation of ccRCC cell lines at concentrations required to inhibit PLK1 kinase activity, and sustained inhibition of PLK1 by BI 2536 led to dramatic regression of ccRCC xenograft tumors in vivo. Taken together, these findings highlight PLK1 as a rational therapeutic target for ccRCC. Cancer Res; 71(15); 5225–34. ©2011 AACR.

Introduction

In 2010, in the United States, an estimated 58,000 people will be diagnosed with, and 35,000 deaths will be attributed to, cancers of the kidney and renal pelvis (1). Kidney cancer can be divided into several histologic subtypes, but the majority of the cases (about 75%) are of the clear cell renal cell carcinoma (ccRCC) subtype (2). Surgery offers the best opportunity to cure localized ccRCC. However, most patients who experience recurrence after surgery or who have metastatic disease at the time of diagnosis will ultimately succumb to the disease. Immunotherapy with agents such as interleukin-2 (IL-2) has been the only choice for metastatic renal cell carcinoma (RCC) patients, with 7-8% of individuals showing complete remission following treatment (3, 4), but IL-2 is beneficial to only a small number of patients. New agents such as sunitinib, sorafenib, and pazopanib, which target the receptor tyrosine kinases of vascular endothelial growth factor and platelet-derived growth factor, have recently been approved by the Food and Drug Administration for ccRCC therapy, but the responses are usually partial and most patients eventually experience progression (5, 6). Therapies directed against the mTOR have also been used to treat advanced disease, but again most patients have disease progression (7). Thus, there remains a critical need for effective and specifically targeted therapies for ccRCC.

Gene expression profiling has been successfully used to find genes that are differentially expressed among RCC subtypes, correlated with chromosomal abnormalities, or correlated with deregulated oncogenic pathways (8–10). However, finding therapeutic targets from those lists of genes is still challenging. Genes whose expression varies with clinical parameters such as tumor grade, stage, or survival duration may be more helpful in identifying molecular targets, but they require additional validation (11, 12).

The discovery of RNA interference (RNAi) and the advance of technologies for using RNAi on large sets of genes in mammalian cells—either siRNA or short hairpin RNA (shRNA)—allow us to systematically interrogate gene functions at high throughput (13–16). Such an approach has proven successful in the discovery of genes that were components of the p53 tumor-suppressor pathway (13), of genes that when knocked down sensitize resistant cells to chemotherapeutic agents (15, 16), and of genes essential to the proliferation of human mammary and colon cancer cells (17, 18).
Here, we expand upon previous partial kinase library screening of ccRCC (19) by examining the effects on cell proliferation within the whole library of kinases and phosphatases. In addition, we used expression profiling data carried out on 90 ccRCC samples for which we had tumor grade information. Combining these results identified a class of cell-cycle kinases, such as Polo-like kinase 1 (PLK1), as potential therapeutic targets for ccRCC.

Materials and Methods

Tissue collection and gene expression profiling
Gene expression profiles from 90 ccRCC tumors and 13 normal kidney samples were produced as previously described (10, 20). This data has been deposited at the Gene Expression Omnibus (GSE 17895). The kidney samples were obtained from the Cooperative Human Tissue Network, and approval was obtained from the Van Andel Research Institute Institutional Review Board to study the samples. Gene expression analysis was conducted as previously described (21, 22).

The genes differentially expressed between the weakly aggressive group of tumors (grades I and II) and the highly aggressive tumors (grade IV) were identified using the Fisher test (raw P value) or elim Fisher test (adjusted P value) as stated in the limma package (23).

For Gene Ontology (GO) gene enrichment analysis, significant GO terms were calculated using the Fisher test (raw P value) or elim Fisher test (adjusted P value) as stated in the top GO package (24).

Survival analysis
Gene expression profiles from 179 ccRCC tumors, with following survival duration after surgery, were used to carry out survival analysis as previously described (25).

Statistical analysis
For all statistical analyses, values were expressed as mean ± SD, except that the relative gene expression values from microarrays were expressed as median ± median absolute deviation (MAD; ref. 26). Values were compared using Student’s t-test; P < 0.05 was considered significant.

siRNA library screening and target identification
Custom siRNA human kinase and phosphatase library sets were designed and synthesized as previously described (15). Our validation data showed greater than 93% transfection efficiency in ccRCC cell lines, and this library has been observed to induce, on average, greater than 80% knockdown for each mRNA target species in our previously published study (15) and this article.

786-O cells (WT2 or RC3) were seeded onto 96-well plates at 1,000 cells per well. Twenty-four hours later, 10 μL of siRNA and lipid (Oligofectamine; Invitrogen) mix was added to each well. Cell viabilities were analyzed 4 days after transfection using the CellTiter 96 AQueous assay (MTS assay; Promega).

For proliferation analyses, B-score normalization was applied to primary screening MTS OD values to eliminate plate-to-plate variability and well-position effects (26).

Cells and cell culture
The A498, ACHN, Caki-1, and 786-O RCC cell lines were obtained from the American Type Culture Collection. SN12C cells were kindly provided by Dr. George Vande Woude (Van Andel Research Institute). 786-O cells (WT2, VHL−) and 786-O cells (RC3, VHL−) were kindly provided by Dr. Michael Ohh (University of Toronto). The cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) in a humidified incubator containing 5% CO2 at 37°C.

Viability staining
786-O cells were plated at 2,000 cells per well in a 96-well plate, and to 4 different wells were added 1 of 4 siRNAs targeting a single gene. At 24 hours after transfection, the cells were trypsinized and then transferred to a 6-well plate, where the cells were cultured for another 8 to 10 days. Finally, the cells were fixed and stained with 1.5% (w/v) crystal violet (15).

Cell proliferation assay
Cells were plated under the same conditions as the primary screening. Twenty-four hours later, cells were either transfected with siRNAs or treated with BI 2536. Cells were further cultured for another 4 days and then cell proliferation was assessed using either the MTS assay or the CellTiter-Glo Luminescent assay (Promega).

Quantitative reverse transcriptase PCR
Total RNA was isolated from 786-O cells 48 hours after transfection, followed by reverse transcription to cDNA using universal primers and a TaqMan Gene Expression Cells-to-CT kit (Applied Biosystems). The reverse transcriptase PCR (RT-PCR) reaction was carried out as previously described (15).

Cell-cycle analysis
Cells were incubated with either 10, 20, or 40 nmol/L BI 2536: 1:1,000 dilution of 0.1 N HCl (vehicle); or were left treatment naïve for 24 hours. Cell-cycle profiles were determined by flow cytometric analysis as previously described (27). Nocodazole (0.1 μmol/L) was used as a positive control for cell-cycle arrest at the G2-M phase.

Immunofluorescence of cultured cells
Cell lines were grown on coverslips (Nunc Lab-Tek II Chamber Slide) for 24 hours and treated the same as in cell-cycle analysis. The cells were stained with mouse anti-α-tubulin antibody (1:500; Sigma) followed by addition of a Rhodamine Red-X-conjugated (1:200) antibody to mouse IgG.

4',6-Diamidino-2-phenylindole (DAPI; 300 nmol/L) was used to highlight DNA. Fluorescently labeled cells were visualized using a confocal or fluorescence microscope.

Cell lysate and Western blotting analysis
Cell lysates were prepared by washing cells with PBS and then following published methods (28). Western blotting was conducted as previously described (28), except that the
primary antibody was mouse antiphosphorylated histone H3 (Ser10).

Xenograft models
All animal studies were in compliance with VARI Institutional Animal Care and Use Committee (IACUC) policies. Six-week-old female BALB/c nu/nu nude mice (Charles River) were given subcutaneous injections of 3 x 10^6 786-O, A498, Caki-1, or SN12C cells in the right flank. Tumor size was measured as previously described (27).

Tumor growth study
When tumors had grown to an average volume of 200 to 300 mm^3, mice were separated into 2 groups of 5 animals. For 1 treatment cycle, 1 group received an intratumoral injection of BI 2536 either once or twice per day on 2 consecutive days per week, at a dosage of 50 mg/kg per injection; the vehicle control group received 0.1 N HCl. The mice received 3 to 4 treatment cycles before sacrifice.

Target modulation study
When tumors had grown to an average volume of 400 to 500 mm^3, tumor-bearing mice were separated into 2 groups of 5 animals. Then, the mice were treated twice on the first day and once on the following day by intratumoral injection of BI 2536 (50 mg/kg per injection) or of 0.1 N HCl. Six hours after the third treatment, the mice were sacrificed.

Immunohistochemical staining for phosphorylated histone H3 (Ser10) was carried out as described (20). Phosphorylated histone H3 (Ser10) antibody (Cell Signaling) was used at a dilution of 1:50. The quantification of proliferation index was also carried out as previously described (20).

Results

Genes associated with aggressive ccRCC
Gene expression profiling was carried out on 90 ccRCC patient samples for which tumor grade information was available (Supplementary Table S1). To identify individual genes whose expression is associated with tumor aggressiveness, we first filtered out genes that did not show much difference among the patients, using 1.414 (log_2 value <0.5) as the cutoff for interquartile expression levels. This left about half the genes (9,824/18,185) as candidates for further analysis. Then, we classified the patients into 2 groups, weakly aggressive (grades I and II) and highly aggressive (grade IV). A linear model was used to identify genes differentially expressed between these 2 groups. We identified 1,159 genes as being significantly associated with aggressiveness when highly expressed [False Discovery Rate (FDR) <0.2 and fold change >1; Fig. 1A and Supplementary Table S2].

High expression of cell-cycle–related kinases is associated with tumor aggressiveness
To place the individual genes into biological context, a gene enrichment analysis approach based on GO was used (29). When the Biological Process GO categories were examined, 6 out of the top 10 GO terms were associated with the cell cycle, for example, the GO terms "mitosis" (GO:0007067, raw P = 4.30 x 10^{-13}, adjusted P = 4.30 x 10^{-13}) and "cell-cycle phase" (GO:00022403, raw P = 1.60 x 10^{-13}, adjusted P = 0.024; Supplementary Table S3 and Supplementary Fig. S1). The GO term "cell cycle" (GO:0007049) was the highest level of parent node for these 6 related significant nodes, and it was itself also significantly related to aggressiveness (raw P = 2.3 x 10^{-12}, adjusted P = 0.11; Supplementary Fig. S1 and Supplementary Table S3). Within the GO term "cell cycle," 117 genes were identified as being associated with aggressiveness, representing 10.1% (117/1,159) of the aggressiveness-related genes (Fig. 1A). Thus, genes associated with the cell cycle were strongly associated with ccRCC aggressiveness.

When the analysis was conducted using the Molecular Function GO categories, one of the significant related nodes was the GO term "kinase activity" (GO:0016301; raw P = 4.14 x 10^{-3}, adjusted P = 0.19), which is the parent node of GO terms of "protein kinase activity" (GO:0004672, P = 5.97 x 10^{-3}, adjusted P = 0.38) and "protein serine/threonine kinase activity" (GO:0004674, raw P = 3.35 x 10^{-3}, adjusted P = 3.35 x 10^{-3}). Another significant enriched GO term was "ATP binding" (GO:0005524, raw P = 3.61 x 10^{-3}; adjusted P = 4.30 x 10^{-3}), which includes 661 kinases (Supplementary Table S4 and Supplementary Fig. S2). Seventy-two genes in the GO term "kinase activity" were identified as being associated with aggressiveness, representing 6.21% (72/1,159) of the aggressiveness-related genes (Fig. 1A). These results highlight the importance of kinases in ccRCC aggressiveness.

There was an overlap of 101 genes between the "cell cycle" and the "kinase activity" GO terms. Of the overlapped genes, 19.8% (20/101) were significantly related with disease aggressiveness, and 85% (17/20) of those were serine/threonine kinases (Supplementary Fig. S3). In particular, PLK1, Aurora kinase B (AURKB), and cyclin-dependent kinase 1 (CDK1) are 3 representatives of these 20 cell-cycle–related kinases (CCRK; Fig. 1B). The significance of these kinases is supported by patient survival analysis using another cohort of 179 patients (Fig. IC and Supplementary Fig. S4).

Importance of cell-cycle kinases for ccRCC cell line proliferation in vitro
We used a cell-based assay to identify genes that were essential to ccRCC cell proliferation, and we carried out a high-throughput siRNA screen targeting the whole kinome and phosphatome using an isogenic pair of ccRCC cell lines, 786-O (RC3, VHL–) and 786-O (WT2, VHL ); ref. 30), trying to find targets specific required for VHL-mutated cell proliferation. Each cell line was transfected with 4 unique siRNA duplexes targeting each of 678 known and putative kinases and 198 known and putative phosphatases in a 1-gene/1-well format on 96-well microtiter plates. The transfection efficiency was greater than 93% (Supplementary Fig. S5). At 96 hours after transfection, cell proliferation was measured using an MTS assay, which measures the activity of reductase enzymes in living cells (31). Raw absorbance values were normalized to internal reference control samples on each plate to permit plate-to-plate comparisons, and each well was assigned a
B-score after normalization of the position effects (26). Although we started with the idea of finding synthetic lethal genes, we did not find such targets except for the genes which have been reported before (19). Next, we used the average B-score from these 2 cell lines to choose positive hits regardless of VHL status (Supplementary Table S5).

In this screen, siRNAs having an average B-score of less than −3 were defined as proliferation genes for ccRCC (Fig. 2A). Overall, we identified 35 kinases, including PLK1, nonmetastatic cells 3 (NME3), CCRK, NAD kinase (NADK), and PCTAIRE protein kinase 3 (PCTK3), as well as 2 phosphatases, protein phosphatase 1 regulatory (inhibitor) subunit 3F (PPP1R3F) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2). To get a functional overview of these data, the genes were classified according to function; the majority of hits were metabolic and cell-cycle kinases and phosphatases (Fig. 2B).

A subset of these genes was selected for further study. To rule out potential off-target and cell line effects of the siRNA screen, each gene was targeted with 4 different siRNAs individually in the 786-O, A498, ACHN, Caki-1, and SN12C cell lines. To rule out potential assay effects, proliferation rates were measured by quantitation of ATP levels (CellTiter-Glo) rather than by reductase activity (32). All ccRCC cell lines showed a similar requirement, regardless of VHL status, for the NME3, PLK1, CCRK, NADK, PCTK3, deoxyguanosine kinase (DGUOK), and glucokinase (GCK) genes for cell survival in vitro (Fig. 2C and Supplementary Fig. S6A).

To confirm these results, 786-O cells were transfected with 4 siRNAs individually targeting 1 of 5 different kinases. The cells were transferred to 6-well plates and allowed to grow for 8 to 10 days and then were stained with crystal violet to visualize the relative number of cell colonies (Fig. 2D and Supplementary Fig. S6B). At the same time, the mRNA levels for the kinases were measured using quantitative PCR (Q-PCR) following knockdown by single siRNAs (Fig. 2E and Supplementary Fig. S6C). The results were consistent, showing that the effective siRNAs were targeting their specific targets and that a decrease of candidate gene expression was associated with decreased cell proliferation. In summary, we identified 37
genes, including PLK1, that are important to ccRCC cell line proliferation in vitro (Table 1).

**Inhibition of PLK1 function suppresses ccRCC cell proliferation in vitro**

The combined results of the aggressiveness analysis and the cell line proliferation screening indicated that cell-cycle-related genes were important to both disease progression and cell proliferation. Specifically, we found that 3 kinases (AURKB, PLK1, and JAK3) were identified in both data sets (Supplementary Fig. S7). We previously examined the role of the Aurora kinases in ccRCC, showing that inhibition of this class of kinases suppresses the growth of ccRCC (25). Here, we used the small-molecule inhibitor of PLK1, BI 2536, which has recently become available, to examine the effects of PLK1 inhibition on ccRCC.

BI 2536 suppressed the proliferation of the ccRCC cell lines 786-O, A498, ACHN, Caki-1, and SN12C were treated for 96 hours with concentrations of BI 2536 from 5 to 80 nmol/L (Fig. 3A). These concentrations were previously reported to inhibit PLK1 kinase activity in vitro (33, 34). BI 2536 suppressed the proliferation of the ccRCC cell lines.
<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Symbol</th>
<th>Description</th>
<th>B-score</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Metabolic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_002513</td>
<td>NME3</td>
<td>Nonmetastatic cells 3</td>
<td>-14.46</td>
<td></td>
</tr>
<tr>
<td>NM_023018</td>
<td>NDK</td>
<td>NAD kinase</td>
<td>-6.23</td>
<td></td>
</tr>
<tr>
<td>NM_001929</td>
<td>DGUOK</td>
<td>Deoxyguanosine kinase</td>
<td>-4.90</td>
<td></td>
</tr>
<tr>
<td>NM_000162</td>
<td>GCK</td>
<td>Glucokinase</td>
<td>-4.39</td>
<td></td>
</tr>
<tr>
<td>NM_182471</td>
<td>PKM2</td>
<td>Pyruvate kinase, muscle</td>
<td>-4.23</td>
<td></td>
</tr>
<tr>
<td>NM_006212</td>
<td>PFKFB2</td>
<td>6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 2</td>
<td>-3.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Cell cycle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_005030</td>
<td>PLK1</td>
<td>Polo-like kinase 1 (Drosophila)</td>
<td>-13.33</td>
<td></td>
</tr>
<tr>
<td>NM_002596</td>
<td>PCTK3</td>
<td>PCTAIRE protein kinase 3</td>
<td>-7.02</td>
<td></td>
</tr>
<tr>
<td>NM_004217</td>
<td>AURKB</td>
<td>Aurora kinase B</td>
<td>-4.31</td>
<td></td>
</tr>
<tr>
<td>NM_012161</td>
<td>CDK9</td>
<td>Cyclin-dependent kinase 9 (CDC2-related kinase)</td>
<td>-4.08</td>
<td></td>
</tr>
<tr>
<td>NM_003390</td>
<td>WEE1</td>
<td>WEE1 homolog (S. pombe)</td>
<td>-3.77</td>
<td></td>
</tr>
<tr>
<td>NM_003137</td>
<td>SRPK1</td>
<td>SFRS protein kinase 1</td>
<td>-3.64</td>
<td></td>
</tr>
<tr>
<td>NM_003157</td>
<td>NEK4</td>
<td>NIMA (never in mitosis gene a)-related kinase 4</td>
<td>-3.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Lipid signaling pathway</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_020126</td>
<td>SPHK2</td>
<td>Sphingosine kinase 2</td>
<td>-4.21</td>
<td></td>
</tr>
<tr>
<td>NM_004570</td>
<td>PIK3C2G</td>
<td>Phosphoinositide-3-kinase, class 2, gamma polypeptide</td>
<td>-4.12</td>
<td></td>
</tr>
<tr>
<td>NM_005026</td>
<td>PIK3CD</td>
<td>Phosphoinositide-3-kinase, catalytic, delta polypeptide</td>
<td>-3.44</td>
<td></td>
</tr>
<tr>
<td>NM_004779</td>
<td>PIP5K2C</td>
<td>Phosphatidylinositol-4-phosphate 5-kinase, type II, gamma</td>
<td>-3.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>MAPK signaling pathway</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_004759</td>
<td>MAPKAPK2</td>
<td>Mitogen-activated protein kinase-activated protein kinase 2</td>
<td>-3.31</td>
<td></td>
</tr>
<tr>
<td>NM_004579</td>
<td>MAP4K2</td>
<td>Mitogen-activated protein kinase kinase 2</td>
<td>-3.11</td>
<td></td>
</tr>
<tr>
<td>NM_001654</td>
<td>ARAF</td>
<td>v-raf murine sarcoma 3,611 viral oncogene homolog</td>
<td>-3.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Apoptosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB014541</td>
<td>AATK</td>
<td>Apoptosis-associated tyrosine kinase</td>
<td>-5.28</td>
<td></td>
</tr>
<tr>
<td>NM_006712</td>
<td>FASTK</td>
<td>FAST kinase</td>
<td>-3.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Calcium signaling pathway</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_018584</td>
<td>CalMIIalpha</td>
<td>Calcium/calmodulin-dependent protein kinase II</td>
<td>-3.36</td>
<td></td>
</tr>
<tr>
<td>NM_006383</td>
<td>CIB2</td>
<td>Calcium and integrin binding family member 2</td>
<td>-3.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_017449</td>
<td>EPHB2</td>
<td>EPH receptor B2</td>
<td>-4.81</td>
<td>Ephrin signaling pathway</td>
</tr>
<tr>
<td>NM_003215</td>
<td>TEC</td>
<td>Tec protein tyrosine kinase</td>
<td>-4.43</td>
<td>None receptor protein tyrosine kinase</td>
</tr>
<tr>
<td>NM_015112</td>
<td>MAST2</td>
<td>Microtubule-associated serine/threonine kinase 2</td>
<td>-3.64</td>
<td>Microtubule</td>
</tr>
<tr>
<td>NM_006374</td>
<td>STK25</td>
<td>Serine/threonine kinase 25 (STE20 homolog, yeast)</td>
<td>-3.50</td>
<td>Stress</td>
</tr>
<tr>
<td>NM_005246</td>
<td>FER</td>
<td>fer (fps/fes-related) tyrosine kinase (phosphoprotein NCP94)</td>
<td>-3.50</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>NM_005975</td>
<td>PTK6</td>
<td>PTK6 protein tyrosine kinase 6</td>
<td>-3.38</td>
<td>None receptor protein tyrosine kinase</td>
</tr>
<tr>
<td>NM_033215</td>
<td>PPP1R3F</td>
<td>Protein phosphatase 1, regulatory (inhibitor) subunit 3F</td>
<td>-3.38</td>
<td>Unknown</td>
</tr>
<tr>
<td>NM_182948</td>
<td>PRKACB</td>
<td>Protein kinase, cAMP-dependent, catalytic, beta</td>
<td>-3.23</td>
<td>cAMP signaling pathway</td>
</tr>
<tr>
<td>NM_000215</td>
<td>JAK3</td>
<td>Janus kinase 3 (a protein tyrosine kinase, leukocyte)</td>
<td>-3.03</td>
<td>JAK-STAT</td>
</tr>
<tr>
<td>NM_001954</td>
<td>DDR1</td>
<td>Discoidin domain receptor family, member 1</td>
<td>-3.20</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>XM_055866</td>
<td>LMTK3</td>
<td>Lemur tyrosine kinase 3</td>
<td>-3.95</td>
<td>Unknown</td>
</tr>
<tr>
<td>NM_032017</td>
<td>MGC4796</td>
<td>Ser/Thr-like kinase</td>
<td>-3.18</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Table 1. Functional categories of *in vitro* proliferation kinases and phosphatases.
lines in a dose-dependent manner with IC₅₀ values of 2 to 20 nmol/L, which are consistent with the values found for other cell lines (33, 34).

PLK1 has been shown to be critical for mitotic entry, centrosome maturation, and cytokinesis (35). The loss of PLK1 function (via either knockdown or small-molecule inhibitors) results in a metaphase block in which cells fail to assemble a functional bipolar spindle; instead, mitotic chromosomes become arranged in a circular fashion around a monopolar spindle, termed a "Polo" spindle (33, 35, 36). To confirm that BI 2536 was inhibiting PLK1 function at concentrations that inhibited in vitro proliferation, cells were treated for 24 hours with 10, 20, or 40 nmol/L BI 2536, stained with propidium iodide, and analyzed by flow cytometry for effects on the cell cycle (Fig. 3B). Cells treated with nocodazole were used as a control population blocked at G₂–M phase.

As expected, the cells treated with BI 2536 accumulated at G₂–M, which is consistent with the function of PLK1 in early mitotic entry. Sustained evidence indicates that the level of histone H3 phosphorylated on serine 10 (pH3) correlates with proliferation rate and that the intracellular pattern of pH3 staining differentiates between stages of mitosis (37), so next we examined both pH3 levels and the chromosome orientation. Compared with either nontreated or buffer controls, BI...
2536-treated cells had increased levels of pH3, reaching the level found in cells treated with nocodazole (Fig. 3C). Cells were also stained with α-tubulin, followed by secondary antibody conjugated with Rhodamine Red-X, and chromosomes were visualized with DAPI. Confocal images of the cells treated with BI 2536 showed the Polo chromosomal phenotype. The number of cells with the Polo phenotype was substantially increased in the BI 2536 treatment group relative to controls, indicating that the inhibition of PLK1 function results in an M-phase block that is associated with decreased cellular proliferation (Fig. 3D).

Sustained inhibition of PLK1 function inhibits ccRCC tumor growth in vivo

786-O, A498, Caki-1, or SN12C cells were subcutaneously implanted in nude mice and treated with BI 2536. Following the published literature (34), we initially used intravenous injection of 50 mg/kg BI 2536 and a 2-days on, 5 days off weekly dosing schedule; we observed only minor inhibition effects on Caki-1 and SN12C xenograft tumors and no effects on 786-O and A498 tumors (Supplementary Fig. S8).

Then, we used the same treatment schedule, but we applied BI 2536 to 786-O xenografts by intratumoral injection. Under this protocol, tumors were allowed to grow to 200 mm³, and then 50 mg/kg BI 2536 was injected intratumorally according to the dosing schedule. After 2 cycles of treatment, no effect on tumor growth was observed. We speculated that the tumor was not responsive because of the incomplete inhibition of PLK1 function. Thus, in subsequent treatments, 50 mg/kg of BI 2536 was injected twice a day (100 mg/kg total per day), once at 7:00 AM and then at 5:00 PM, for 2 consecutive days. Somewhat surprisingly, the tumors significantly regressed upon this BI 2536 treatment (Fig. 4A), and we repeatedly observed similar effects on both 786-O (data not shown) and A498 ccRCC xenografts (Fig. 4B).

To confirm that BI 2536 inhibited PLK1 function using the twice-a-day schedule in vivo, a target modulation study was carried out. 786-O cells were subcutaneously implanted in nude mice, tumors were allowed to grow to 400 to 500 mm³, and then the mice were treated by intratumoral injection 3 times, twice in the first day and once in the following day, using 50 mg/kg BI 2536 per injection. The mice were sacrificed 6 hours after the final injection. The tumor tissues were collected and examined for pH3 and for chromosome alignment (Fig. 4C and D). Compared with the vehicle control group, pH3 and aberrations in chromosome alignment were

Figure 4. Inhibition of PLK1 by BI 2536 suppressed in vivo tumor growth. A, BI 2536 inhibited the growth of 786-O xenograft tumors. When xenograft tumors were 200 to 300 mm³, they were treated with vehicle (0.1 N HCl, filled squares) or 50 mg/kg BI 2536 (empty triangles) by intratumoral injection (arrows). The treatment cycle was 1 injection per day for 2 consecutive days each week for 2 weeks; it then changed to 2 injections per day for 2 consecutive days each week for another 3 weeks. B, BI 2536 treatment at 2 injections per day for 2 consecutive days inhibited the growth of A498 xenograft tumors. C, when 786-O tumors were 400 to 500 mm³, they were injected (intratumorally) either with vehicle control (0.1 N HCl) or 50 mg/kg BI 2536 3 times, twice the first day and once the following day. The treated tumor tissues were excised 6 hours after the final injection, and either immunostained with antibodies to the mitotic marker phosphorylated histone H3 or stained with hematoxylin and eosin (H&E). Cells with aberrant alignment chromosomes are indicated by arrows. Scale bars, 20 μm. Boxes at the lower left of the bottom images are enlargements of the small box in each image. D, mitotic cells (as described in C) positive for phosphorylated histone H3, were calculated using 5 fields from each of 3 separate tumors. **, P < 0.01.
increased in BI 2536-treated cells. Together, these results showed that sustained inhibition of the PLK1 function in vivo by BI 2536 can suppress ccRCC tumor growth.

Discussion

This study integrated both gene expression profiling and RNAi screening data to identify genes involved in ccRCC development and progression. Consistent with cancer being a disease of uncontrolled proliferation, we found an enrichment of cell-cycle–related processes when genes associated with tumor grade were examined for deregulated biological pathways. However, the appearance of kinase-related genes in the molecular function analysis was not initially expected, because ccRCC renal tumors have not been reported to contain specific kinase-associated pathway activation induced by either gene mutation or focal amplification (33, 38, 39).

On the basis of the gene expression results and the generation of several potent small-molecule kinase inhibitors, we carried out an RNAi screen to identify kinases and phosphatases that were required for the growth of ccRCC cell lines. Both our expression analysis and our siRNA proliferation screen highlighted the importance of cell-cycle–related genes like PLK1 and AURKB in ccRCC. PLK1 is a serine–threonine kinase that plays a key role in mitotic entry, centrosome maturation, and cytokinesis (35). Consistent with our finding that PLK1 is overexpressed in high-grade kidney cancers, PLK1 has been found overexpressed in other solid tumors (40), including breast cancer (41) and colorectal cancer (42). As we report here for ccRCC, preclinical studies have also shown promising results in other solid cancers, including breast cancer and colorectal cancer (34, 43), by either knocking down PLK1 via siRNA/shRNA or inhibiting PLK1 function via small-molecular inhibitors. Several PLK1 inhibitors have been evaluated or ongoing against solid tumors in the clinical setting (44, 45). Our data reported here and previously (25) clearly show that inhibition of PLK1 or AURKB function can suppress ccRCC tumor growth both in vitro and in vivo. However, one of the most notable results of this work was that a twice-a-day schedule is required for the in vivo effects of BI 2536. This result may indicate that for cell-cycle kinase inhibitors to cause ccRCC tumor regression, the function of the kinases needs to be inhibited for a longer duration than is needed by, for example, receptor tyrosine kinase inhibitors. We speculate that to induce cell death or apoptosis in ccRCC, the function of PLK1 needs to be suppressed for a certain period, highlighting the need for careful selection of delivery schedules for this class of drugs in clinical trials for ccRCC.

We also indentified a series of proliferation genes for ccRCC; in particular, NME3 (also known as DR-nm23) seemed to potently suppress the growth of these cells when knocked down. NME3 belongs to the nm23 gene family having a common nucleoside diphosphate kinase activity (46). NME3 has been shown to either inhibit or promote cell differentiation or apoptosis when highly expressed in different cell types (47). Genes from the same family have been shown to be closely related to metastasis (48). Whether the kinase activity of NME3 is required for ccRCC cell proliferation could be a fruitful area for further study.

Disclosure of Potential Conflicts of Interest

The authors declare that no conflicts of interest exist.

Acknowledgments

We thank Lisa DeCamp, Vivarium Operations, Van Andel Research Institute, for help with the animal experiments; Eric Hudson from the Laboratory of Analytical, Cellular, and Molecular Microscopy, Van Andel Research Institute, for help in confocal imaging; Aiko Ooi for scientific discussions; Sabrina Noyes for administrative assistance, and David Nadziejka for reading and correcting the manuscript. We thank the Cooperative Human Tissue Network (CHTN) of the National Cancer Institute for providing samples for analysis. 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 January 10, 2011; revised May 12, 2011; accepted June 01, 2011; published OnlineFirst June 3, 2011.

References


Combined Gene Expression Profiling and RNAi Screening in Clear Cell Renal Cell Carcinoma Identify PLK1 and Other Therapeutic Kinase Targets

Yan Ding, Dan Huang, Zhongfa Zhang, et al.