Germline Mutations in the CDKN2B Tumor Suppressor Gene Predispose to Renal Cell Carcinoma


ABSTRACT
Familial renal cell carcinoma (RCC) is genetically heterogeneous and may be caused by mutations in multiple genes, including VHL, MET, SDHB, FH, FLCN, PTEN, and BAP1. However, most individuals with inherited RCC do not have a detectable germline mutation. To identify novel inherited RCC genes, we undertook exome resequencing studies in a familial RCC kindred and identified a CDKN2B nonsense mutation that segregated with familial RCC status. Targeted resequencing of CDKN2B in individuals (n = 82) with features of inherited RCC then revealed three candidate CDKN2B missense mutations (p.Pro40Thr, p.Ala23Glu, and p.Asp86Asn). In silico analysis of the three-dimensional structures indicated that each missense substitution was likely pathogenic through reduced stability of the mutant or reduced affinity for cyclin-dependent kinases 4 and 6, and in vitro studies demonstrated that each of the mutations impaired CDKN2B-induced suppression of proliferation in an RCC cell line. These findings identify germline CDKN2B mutations as a novel cause of familial RCC.

SIGNIFICANCE: Germline loss-of-function CDKN2B mutations were identified in a subset of patients with features of inherited RCC. Detection of germline CDKN2B mutations will have an impact on familial cancer screening and might prove to influence the management of disseminated disease. Cancer Discov; 5(7): 723–9. © 2015 AACR.

INTRODUCTION
The identification of the genetic basis for familial cancer syndromes can provide important insights into the molecular mechanisms of familial and sporadic tumorigenesis. Furthermore, improved understanding of the molecular factors involved in tumorigenesis has provided a basis for the rational development of targeted therapies. This is exemplified in inherited renal cell carcinoma (RCC; MIM#144700) in which the identification of mutations in the VHL tumor suppressor

© 2015 American Association for Cancer Research.
gene (TSG) as the cause of von Hippel–Lindau (VHL) disease (MIM#193300) led to the recognition that the VHL TSG is somatically inactivated in the majority of sporadic clear-cell RCC. Subsequently, elucidation of the role of the VHL gene product in the regulation of hypoxia response pathways enabled the development of novel targeted treatments that have revolutionized the clinical management of RCC (1).

About 3% of all cases of RCC occur in individuals with familial RCC (2). A number of genetic mechanisms have been implicated in RCC predisposition. Thus, genome-wide association studies have identified several common genetic variants that predispose to RCC, and at least two loci are linked to pVHL-regulated hypoxic gene response pathways [at 2p21 (rs11894252) and 11q13.3 (rs7105934) close to EPAS1 (HIF2A) and CCND1 (Cyclin D1), respectively; ref. 3]. In familial RCC, in addition to VHL, germline mutations in MET, FLCN, FH, SDHB, PTEN, and BAP1 have all been associated with inherited RCC [2 (and references within), 4–6]. Constitutional translocations, particularly involving chromosome 3, can also cause inherited RCC (7). However, overall less than 20% of patients with features of nonsyndromic inherited RCC have a detectable mutation in a known familial RCC gene (2, 4–6, 8, 9). To identify novel inherited RCC genes, we undertook exome sequencing and targeted resequencing studies in individuals with features of inherited RCC and no detectable mutation in known RCC predisposition genes.

**RESULTS**

**Identification of CDKN2B Mutations in Inherited RCC**

Analysis of exome resequencing in a male proband diagnosed with familial clear-cell RCC (see Fig. 1A) at age 57 years revealed no candidate mutations in RCC predisposition genes (VHL, SDHB, FLCN, FH, PTEN, or BAP1). Further bioinformatic analysis was undertaken for the presence of pathogenic mutations in genes linked to cancer. A candidate nonsense mutation in CDKN2B was highlighted for further investigation, and the CDKN2B c.103G>T (p.Glu35Stop) mutation was confirmed by Sanger sequencing (see Fig. 1B). Two of his eight siblings had been diagnosed with clear-cell RCC (two brothers diagnosed at ages 56 and 60 years, respectively), and his mother had been diagnosed with RCC at age 60 years. DNA was available from an unaffected sibling (Fig. 1A). The c.103G>T CDKN2B nonsense mutation was present in one of his unaffected siblings (Fig. 1A).

To identify novel inherited RCC genes, we undertook exome sequencing and targeted resequencing studies in individuals with features of inherited RCC and no detectable mutation in known RCC predisposition genes.

**Figure 1.** A, family pedigree for familial RCC kindred in which exome sequencing was performed. B, electropherogram showing heterozygous nonsense mutation c.103G>T(p.E35X) and corresponding normal sequence; C, electropherogram showing heterozygous missense mutation c.68C>A p.Ala23Glu (A23E) and evolutionary conservation of residue; D, electropherogram showing heterozygous missense mutation c.118C>A p.Pro40Thr (P40T) and evolutionary conservation of residue; E, electropherogram showing heterozygous missense mutation c.256G>A p.Asp86Asn (D86N) and evolutionary conservation of residue.
To further investigate the potential role of CDKN2B mutations in inherited renal cancer, mutation screening was performed in 50 individuals with features of nonsyndromic inherited RCC [either familial RCC (n = 37) or multiple RCC aged <50 years (n = 13)], and three candidate missense mutations were detected (Fig. 1C–E). A rare sequence variant (c.256G>A) predicted to cause a pathogenic missense substitution, p.Asp86Asn, was detected in a proband who was diagnosed with clear-cell RCC at age 35 years. No other family members (her mother had died from RCC) were available for testing. In addition, a novel candidate mutation [c.118C>A (p.Pro40Thr)] was identified in an individual who developed bilateral RCC at age 42 years. No other family members were available for testing. Mutation analysis of CDKN2B in a cohort of 34 individuals with features of possible inherited RCC (familial RCC, multicentric RCC, or young onset RCC) revealed candidate heterozygous missense substitutions [c.68C>A (p.Ala23Glu)] in one case. Two of the three candidate CDKN2B missense mutations (p.Ala23Glu and p.Pro40Thr) were absent from publicly available large exome/genome sequencing sets comprising >12,000 CDKN2B alleles, and the p.Asp86Asn variant occurred with a frequency of <1 in 200 individuals (11). All three substitutions occurred at evolutionarily conserved residues (see Fig. 1C–E) and were predicted to be pathogenic by Polyphen and SIFT algorithms (12, 13).

**Structural Predictions**

To understand the potential impact of the mutations on CDKN2B, the mutations were analyzed in the context of the apo (unbound) structure, and in complex with cyclin-dependent kinases (CDK) 4 or 6. The overall structure of CDKN2B is very similar to CDKN2D and is expected to bind through its concave face to the cleft between the N- and C-terminal domains of CDK4 and CDK6. Binding of CDKN2B prevents productive binding of ATP to the CDKs, and potentially inhibiting movement of the PLSTIRE helix to its active conformation (14).

The three variants identified were spread across the protein (Fig. 2A), with p.Ala23 and p.Asp86 located close to the surface, and along the interface with CDK4 and CDK6, whereas p.Pro40 is a buried residue distal to the interaction interface. CDKN2B p.Pro40 is located on a hydrogen-bonded turn leading into the sheet of the first ankyrin repeat and forming interactions with helices in the first and second ankyrin repeats (Fig. 2B). The first two ankyrin repeats mediate the majority of the interactions with the CDKs (14). Mutation to threonine relaxes this loop, altering the packing of the helices and the overall stability of CDKN2B, as predicted by mCSM and DUET (Table 1).

CDKN2B p.Ala23 is located on an α-helix at the periphery of the interface among CDKN2B and CDK4 and CDK6, where it makes a series of inter- and intramolecular weak hydrophobic interactions (Fig. 2C). This space is not sufficient to accommodate the larger, charged glutamate residue in the mutant, leading to a reduced affinity for the CDKs as predicted by mCSM-PPI (Table 1). CDKN2B p.Asp86 is also located on an α-helix and makes strong intermolecular ionic/hydrogen bonds with CDK4 and CDK6 (Fig. 2D). In the variant, the asparagine side chain is unable to make these

![Figure 2.](image-url)
interactions, leading to a significant reduction in affinity as predicted by mCSM-PPI (Table 1).

**Functional Effects of Germline CDKN2B Variants**

To evaluate the pathogenicity of the candidate germline mutations detected in inherited RCC cases, we undertook in vitro studies of growth suppressor activity. Thus, the effects on growth of the two RCC cell lines were compared for (i) wild-type CDKN2B, (ii) vector only [empty vector (EV)], and (iii) three candidate missense substitutions: p.Pro40Thr, p.Ala23Glu, and p.Asp86Asn. Initially, the effects of transfecting a vector expressing wild-type CDKN2B and an EV into the SKRC47 (wild-type CDKN2B) and KTCL26 (VHL-null) RCC cell lines were compared with the colony formation assay. For both cell lines, there were significantly fewer colonies after transfection with wild-type CDKN2B compared with the EV control. The effects of the three candidate missense mutations (p.Pro40Thr, p.Ala23Glu, and p.Asp86Asn) were then studied, and each missense mutation impaired in vitro growth suppressor activity of CDKN2B (Fig. 3).

**DISCUSSION**

We identified germline-inactivating CDKN2B mutations in approximately 5% (95% confidence interval, 0.21%–9.43%) of patients with features of nonsyndromic inherited RCC analyzed. Thus, the frequency of germline CDKN2B mutations is approximately similar to that which can be attributed to germline FLCN and SDHB mutations (8, 9) but larger than that accounted for by germline BAP1 mutations (5, 6). These mutations lead to the deregulation of CDK4 and CDK6 by CDKN2B, by altering the binding interfaces or overall stability of CDKN2B. The identification of CDKN2B as an inherited RCC gene will facilitate the diagnosis and management of this disorder. In addition to a nonsense mutation that segregated with RCC in familial RCC kindred, we identified three rare missense mutations in individuals with features of inherited RCC. Two of these missense mutations were novel, but one (NM_004936 c.256G>A p.Asp86Asn) had previously been reported in a patient with parathyroid adenoma and in a patient with a metastatic pancreatic endocrine tumor (15, 16). The p.Asp86Asn substitution occurs at a highly conserved residue and has an allele frequency of approximately 0.2% in Single Nucleotide Polymorphism database (dbSNP) build 137 (17), and, consistent with the *in silico* structural predictions reported here, Costa-Guda and colleagues (15) demonstrated that the p.Asp86Asn substitution inhibited binding of CDKN2B/p15 to CDK6.

CDKN2B encodes the p15<sub>INK4B</sub> protein, which binds to and inhibits CDK4 and CDK6 (18). In the absence of CDKN2B, CDK4- and CDK6-dependent phosphorylation of pRb inhibits the interaction between pRb and E2F and promotes cell growth. Overexpression of p15<sub>INK4B</sub> causes cell-cycle arrest in G<sub>1</sub> phase and redistribution of CDK4 from cyclin D–CDK4 complexes to CDK4–p15<sub>INK4B</sub> complexes, leading to degradation of unbound cyclin D by the ubiquitin-dependent proteasome degradation pathway (18). Interestingly, cyclin D1 is a downstream target of pVHL, inactivation of the VHL TSG is associated with HIF2-dependent upregulation of cyclin D1 (19), and genomewide association studies identified genetic variation linked to CCND1 expression with RCC susceptibility (3). The CDKN2B gene maps close to the CDKN2A locus which encodes two tumor suppressor proteins (p16INK4a and p14ARF), and many deletions found in human cancer encompass both CDKN2B and CDKN2A. However, experiments in mice demonstrated that Cdkn2b also possesses critical tumor suppressor activity (20). Though somatic CDKN2B mutations are uncommon in human neoplasia, somatic inactivation of CDKN2B by allele loss and/or promoter hypermethylation has been reported in a variety of human cancers, most notably hematologic neoplasms, including leukemia, lymphoma, and myelodysplastic syndrome (21). Cytogenetic studies have identified chromosome 9p deletions in approximately 15% of clear-cell RCC, array-based studies of copy-number abnormalities have confirmed that the 9p critical region contains the CDKN2A/CDKN2B loci, and Girgis and colleagues (22) found that a subset of RCC exhibited hypermethylation of CDKN2A and CDKN2B (13% and 21%, respectively).

Previously, CDKN2B mutations have been linked to predisposition to endocrine tumors (15, 16, 23). Thus, in addition to p.Asp86Asn mutation that was reported in individuals with a parathyroid adenoma (wild-type allele loss was detected in the tumor tissue) and metastatic pancreatic endocrine tumor (15, 16), two further missense mutations (p.Asn41Asp and p.Leu64Arg) were reported in patients with suspected multiple endocrine neoplasia: one with primary hyperparathyroidism (3 parathyroid tumors), skin schwannoma, meningioma, and liver hemangioma, and one with primary hyperparathyroidism (3 parathyroid tumors), Zollinger–Ellison syndrome, adrenal

---

### Table 1. Predicted effects of mutations upon CDKN2B stability and protein interactions

<table>
<thead>
<tr>
<th>Variant</th>
<th>Secondary structure</th>
<th>Solvent accessibility</th>
<th>CDKN2B stability—mCSM (ΔΔG kcal/mol)</th>
<th>CDKN2B stability—DUET (ΔΔG kcal/mol)</th>
<th>CDKN2B-CDK4 affinity (ΔΔG kcal/mol)</th>
<th>CDKN2B-CDK6 affinity (ΔΔG kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.Pro40Thr</td>
<td>h-bonded turn</td>
<td>5.3% (buried)</td>
<td>-1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.6</td>
<td>-0.6</td>
</tr>
<tr>
<td>p.Asp86Asn</td>
<td>α-helix</td>
<td>27.1% (partially accessible)</td>
<td>-0.9</td>
<td>-0.8</td>
<td>-1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>p.Ala23Glu</td>
<td>α-helix</td>
<td>44.9% (partially accessible)</td>
<td>-1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Highly destabilizing.*
mass, and prostate cancer. Both p.Asn41Asp and p.Leu64Arg substitutions impaired binding to CDK6 (23). The finding of multiple phenotypes with mutations in a single gene is not uncommon and may reflect variable modes of ascertainment and/or genotype-phenotype correlations. Thus, germline BAP1 mutations were initially described in association with uveal melanoma, mesothelioma, and other tumors before being recognized as a cause of familial RCC (5, 6), and, recently, mutations in FH were detected in individuals with pheochromocytoma/paraganglioma without features of hereditary leiomyomatosis-RCC (Reed syndrome; refs. 24, 25). In our study, none of the paraganglioma without features of hereditary leiomyomatosis–renal tubular adenomatosis (HLR-TA) or von Hippel–Lindau disease (VHL) were detected in individuals with pheochromocytoma/paraganglioma. The finding that germline CDKN2B mutations predispose to RCC is consistent with the observation that VHL inactivation is associated with disordered cell-cycle regulation and provides a basis for investigating the role of cell-cycle inhibitors (e.g., palbociclib) for the treatment of advanced RCC—particularly in patients with germline CDKN2B mutations. Thus, inclusion of CDKN2B mutation analysis in the routine investigation of individuals could facilitate the management of both affected individuals and their at-risk relatives.

As the inventory of genetic and epigenetic changes described in RCC enlarges, the challenge of differentiating “driver” and “passenger” events increases. Identification of the genetic basis of inherited cancer provides a powerful strategy for highlighting key pathways in oncogenesis. Thus, though the VHL TSG is frequently somatically inactivated in sporadic RCC, other inherited RCC genes, such as FH, FLCN, and SDHD, are infrequently implicated in RCC but, like pVHL, have been linked to regulation of hypoxic gene response pathways. The finding that germline CDKN2B mutations predispose to RCC is consistent with the observation that VHL inactivation is associated with disordered cell-cycle regulation and provides a basis for investigating the role of cell-cycle inhibitors (e.g., palbociclib) for the treatment of advanced RCC—particularly in patients with germline CDKN2B mutations. Thus, inclusion of CDKN2B mutation analysis in the routine investigation of individuals could facilitate the management of both affected individuals and their at-risk relatives.
METHODS

Patients
Whole-exome sequencing was undertaken in the proband from a kindred with familial RCC (see Fig. 1A). Following the identification of a candidate germline CDKN2B mutation in the proband, mutation analysis of CDKN2B was performed in other family members and in a further 50 unrelated probands with (i) a diagnosis of RCC and a family history of one or more close relatives with a history of RCC (n = 37, 19 males and 18 females; mean age, 52.9 years; range, 29–71 years) or (ii) two or more primary RCC before age 50 years (n = 13, 7 males and 6 females; mean age, 41 years; range, 17–49 years). In addition, a further 32 DNA samples were tested from an anonymized cohort of individuals with evidence of possible inherited predisposition to RCC [i.e., a diagnosis of RCC plus one of (i) positive family history or (ii) multiple primary RCC or (iii) early onset RCC (<40 years)]. All subjects gave consent for genetic studies; the investigations were approved by the South Birmingham Research Ethics committee and were conducted in accordance with the Declaration of Helsinki.

Molecular Genetic Analysis
Exome sequencing was performed at the Biomedical Research Centre at King’s College London as described previously (28). Briefly, after extraction of DNA from peripheral blood lymphocytes using standard techniques, exon capture was performed using the Agilent SureSelect All Exon 50 Mb Target Enrichment System. The Illumina Analyzer IIA with 76 base paired ends and reads was used for sequencing, and the depth of sequence coverage was calculated using the BedTools software package (29). Sequence alignment, identification of SNPs, and small deletions were identified by the SAMTools software package (30). The ANNOVAR software package (31) was used to filter data for variants identified in dbSNP and common SNPs in the 1,000 genomes data. Furthermore, data were compared with 250 control exomes sequenced by the same method.

Sanger Sequencing
Sanger sequencing was performed using standard techniques. The following primer pairs were used:
CDKN2B exon 1: 5′-AAGAGTGTCTGTTAAGTTACG-3′ and 5′-ACA TCCGGCCTAGCTTCCA-3′.
CDKN2B exon 2: 5′-TGAATATAAACCAGGTTG-3′ and 5′-GGGT GGGAAATTGGGTAAG-3′.

The following conditions were used: (i) 95°C for 5 minutes, (ii) 95°C for 45 seconds, (iii) 58°C for 45 seconds, decreasing by 1°C per cycle, (iv) repeat steps (i) to (iii) for 4 cycles, (v) 95°C for 45 seconds, (vi) 54°C for 45 seconds, (vii) 72°C for 45 seconds, (viii) repeat (v) to (vii) 30 times, (viii) incubate at 72°C for 5 minutes.

Colony Formation Assays
The CDKN2B clone was obtained from Cambridge Bioscience (True ORF Gold RC204895). The sequence of the clone was confirmed by direct sequencing. Site-directed mutagenesis was performed on the CDKN2B clone using the Quik Change II Site Directed Mutagenesis Kit (Stratagene; obtained from Agilent Technologies; primer details available on request). Mutant plasmids were transformed into competent cells (Bioline) were transformed with mutant plasmid, and the plasmid was amplified to a stock concentration of 1 μg/μL using the Endotoxin-Free Maxi Spin Kit (Qiagen). The VHL-null cell line KTCL26 and the VHL–wild-type cell line SKRC47 were plated at a density of 6 × 10⁴/mL in 6-well plates on day 1. Transfection was carried out on day 2 using 2 μM plasmid diluted in 200 μL of OptiMem (Gibco) and 6 μL of Fugene HD (Promega) per well. A serial dilution was performed in KTCL26 cells treated with EV and wild-type using the following dilutions: undiluted, 1:5, 1:10, 1:20. Dilutions (1:5) were used for subsequent experiments. The KTCL26 and SKRC47 cell lines were obtained from a collaborator prior to 2006, and no authentication was performed apart from confirming a VHL mutation [c.370 C>T p.Gln124Stop] in KTCL26 and the absence of a VHL mutation in SKRC47 (32).

Molecular Modeling
Models of CDKN2B in complex with CDK4 or CDK6 were based on the X-ray crystal structure of CDK6 in complex with CDKN2D (PDB code: 1BLX; ref. 14) and generated using Modeller (33) and MacroModel (Schrodinger). The models were then minimized using the MMFF94s forcefield in Sybyl-X 2.1.1 (Cetrara L.P.). The resulting models were then aligned to the nuclear magnetic resonance (NMR) structure of CDKN2B (PDB code 1D9S; ref. 34) and showed minimal variation in the overall structure [root mean square difference (r.m.s.d) over Cα of 1.6 Å]. The difference is mainly due to a small long-range bending of the molecule. The model of CDK4 was also compared with the CDK4 apo structure (PDB code: 3G33. r.m.s.d over Cα of 1.1 Å). The effects of the mutations on the stability of CDKN2B were analyzed by mCSM (35) and DUET (36) using both the models and the apo NMR structure (PDB code: 1D9S). The effects of the mutations on the affinity of CDKN2B for CDK4 and CDK6 were analyzed by mCSM-PPI (35) using the models of the complexes. DUET and mCSM are novel machine-learning algorithms that use the three-dimensional structure in order to quantitatively predict the effects of point mutations on protein stability and protein-protein and protein–nucleic acid affinities.

Statistical Analysis
T tests were performed using SPSS Statistics 21 (IBM software). To allow for multiple comparisons in Fig. 3 (wild-type and four mutants), a P value of <0.01 was taken to be statistically significant.

Disclosure of Potential Conflicts of Interest
T.L. Bundell is a deputy chair trustee at the Institute of Cancer Research; President and Chair of the Science Council; and a consultant/advisory board member for Astex Therapeutics and BBSRC; has received a commercial research grant from UCB Celltech; and has provided expert testimony for Isogenica, Pfizer, and Syntaxin. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Acknowledgments
The authors thank the patients, families, and referring clinicians for their assistance.
Grant Support

M. Jafari is in receipt of a Medical Research Council Clinical Research Fellowship. D.B. Ascher is supported by a National Health and Medical Research Council CJ Martin Fellowship. D.E.V. Pires is supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)—Brazil. M.A. Simpson thanks the National Institutes for Health Research (NIHR) Biomedical Research Centre based at Guy’s and St. Thomas’ NHS Foundation Trust and King’s College London for financial support. E.R. Maher thanks the Association for International Cancer Research (AICR; now Worldwide Cancer Research), The Eveson Charitable Trust, and the NIHR Biomedical Research Centre based at Cambridge for financial support.

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

Received September 18, 2014; revised April 1, 2015; accepted April 9, 2015; published OnlineFirst April 14, 2015.

REFERENCES

Germline Mutations in the CDKN2B Tumor Suppressor Gene Predispose to Renal Cell Carcinoma

Mariam Jafri, Naomi C. Wake, David B. Ascher, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-14-1096

Cited articles
This article cites 34 articles, 4 of which you can access for free at:
http://cancerdiscovery.aacrjournals.org/content/5/7/723.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerdiscovery.aacrjournals.org/content/5/7/723.full#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.