SUPPLEMENTARY METHODS AND MATERIAL

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Samples. Previously archived operative specimens were reviewed by an expert genitourinary pathologist to confirm the diagnosis and to identify separate areas of ≥ 70% tumor content and healthy kidney tissues. Macrodissection was performed for each area of interest, and DNA was extracted using the DNeasy tissue kit (Qiagen). DNA was quantified using the Thermo Scientific NanoDropTM 1000 Spectrophotometer and samples with an A260/A280 ratio of 1.8-2.0 and concentration of 135 ng/μL or greater were considered acceptable for further analysis.

Next-generation sequencing. DNA from tumors and matched normal was subjected to an analysis by two next-generation sequencing platforms. The IMPACT assay (Integrated Mutation Profiling of Actionable Cancer Targets) a customized targeted-exome capture assay of 230 cancer-associated genes with ultra-deep sequencing coverage (>500x) using Illumina HiSeq 2000 and whole-exome capture assay with standard sequencing coverage (~85x) using the Agilent SureSelect XT HumanAllExon 50Mb. Single-nucleotide variants, small insertions and deletions, and copy number alterations (CNA) were interrogated.

The IMPACT Assay. Ultra-deep targeted sequencing of key cancer-associated genes was performed using the IMPACT assay (We designed target-specific probes to capture all protein-coding exons of 230 genes of interest for hybrid selection (Agilent SureSelect or Nimblegen SeqCap) as previously described1. This list included commonly implicated oncogenes, tumor suppressor genes, and components of pathways deemed actionable by current targeted therapies (Supplementary Table 1 for complete target list).
Two protocols were followed during the course of the study. For 10 samples, barcoded sequence libraries (Illumina TruSeq) were prepared using 500 ng of input tumor or matched normal DNA according to the manufacturer’s instructions. Libraries were pooled at equimolar concentrations (100 ng per library) for a single exon capture reaction (Agilent SureSelect) as previously described. For the remaining samples, barcoded sequence libraries were prepared using 250 ng of input DNA using a hybrid protocol based on the NEBNext DNA Library Prep Kit (New England Biolabs). Manufacturer’s instructions were followed with two substitutions: we used NEXTflex barcoded adapters (Bio Scientific) and HiFi DNA polymerase (Kapa Biosystems). Libraries were pooled at 100 ng per tumor library and 50 ng per normal library for a single exon capture reaction (Nimblegen SeqCap). To prevent off-target hybridization in all capture reactions, we spiked in a pool of blocker oligonucleotides complementary to the full sequences of all barcoded adaptors (to a final total concentration of 10 μM). Hybridized DNA was sequenced on an Illumina HiSeq 2000 to generate paired-end 75-bp reads.

Data were demultiplexed using CASAVA, and reads were aligned to the reference human genome (hg19) using the Burrows-Wheeler Alignment tool. Local realignment and quality score recalibration were performed using the Genome Analysis Toolkit (GATK) according to GATK best practices. We achieved mean exon sequence coverage of 570x (600x for all tumor samples). Deep sequencing ensured sensitivity for detecting mutations in multiclonal and stroma-admixed samples and enabled accurate determination of mutation allele frequencies.

Sequence data were analyzed to identify three classes of somatic alterations: single-nucleotide variants, small insertions/deletions (indels), and copy number alterations. Single-nucleotide variants were called using muTect (Cibulskis et al., manuscript in preparation) and retained if the variant allele frequency in the tumor was >5 times that in the matched normal.
Indels were called using the SomaticIndelDetector tool in GATK. All candidate mutations and indels were reviewed manually using the Integrative Genomics Viewer\(^5\). The mean sequence coverage was calculated using the DepthOfCoverage tool in GATK and was used to compute copy number as described previously\(^6\).

**Whole-exome capture sequencing.** Between 1.9 and 3 µg of high quality genomic DNA was captured by hybridization using the SureSelect XT HumanAllExon 50Mb (Agilent). Samples were prepared according to the manufacturer instructions. PCR amplification of the libraries was carried out for 6 cycles in the pre-capture step and for 10 cycles post capture. Samples were bar-coded and run on a Hiseq 2000 in a 75bp/75bp Paired end run, using the TruSeq SBS Kit v3 (Illumina). Two samples were pooled in a lane, the average number of read pairs per sample was 69 million.

All reads were aligned to the reference human genome (NCBI build 37.1 hg19). Exome reads were aligned with BWA\(^3\) which does a gaped alignment for the detection of small indels, as described in below. Samples mapping to the reference genome which mapped uniquely (MAPQ>0) were retained and then converted to SAM format\(^2\) for subsequent analyses and for visualization in the Integrative Genomics Viewer\(^5\). Single-nucleotide variants were determined in regions of sufficient coverage. We first removed duplicate reads (using Picard MarkDuplicates) from further analysis, defined here as any read chromosome, start position, strand, and color-space sequence matched another aligned read. Indel realignment, base quality recalibration, variant detection, and variant annotation were performed with the GATK framework\(^4,7\). Specifically, after base quality recalibration for color-space reads, variant detection in exome data was performed with the UnifiedGenotyper. For high-coverage exome
experiments, variants were excluded if their variant quality was <30, genotype quality <5, or if they were associated with either homopolymer runs or excessive strand bias. Novel variants, those not previously identified in either dbSNP ver. 132, were required to be derived from base-space reads not duplicated from non-duplicate color-space reads, were not resident exclusively in higher-error base positions (positions 38-50) and had evidence of the variant allele in reads mapping to both strands.

Candidate somatic mutations were those with a variant genotype in the tumor and reference genotype in the normal sample with minimum coverage of \( \geq 10 \) and 6 reads respectively. Additionally, we required that the tumor variant frequency was \( \geq 10\% \), and each variant was detected in 4 or more tumor reads. Our pipeline for small insertion and deletion (indel) detection was as follows. Gapped alignment of exome sequencing reads was performed with BWA. The alignment output was sorted and duplicate reads removed with the Picard pipeline and BAM files created and indexed with Samtools. Interval detection, local realignment, indel genotyping, and post-processing were performed with the GATK framework after base quality recalibration, as described above. Retained indels were those with sufficient quality and coverage and not associated with homopolymer runs of 5bp or greater.

**Sanger Sequencing.** Bidirectional Sanger sequencing for validation was performed for all mTOR pathway mutations using standard techniques with primers provided by the Geoffrey Beene Translational Oncology Core (available upon request).
Supplementary Materials

Primers.

Primers for PCR-mutagenesis are:

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>E919V</td>
<td>GTC AGC CTG TCA GTA TCC AAG TCA AGT C</td>
</tr>
<tr>
<td>Q2223K</td>
<td>GGA AAA ACC TCA GCA TCA AGA GAT ACG CTG TC</td>
</tr>
</tbody>
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Plasmids. Plasmids containing cDNA encoding myc-epitope-tagged S6K (pcDNA3-myc-S6K) and Flag-epitope-tagged Wild-type mTOR (pcDNA3-Flag mTOR) were obtained from Addgene (#26610 and #26603). To generate individual mTOR mutations, a corresponding nucleotide change was introduced via QuikChange site-directed mutagenesis (Stratagene), producing pcDNA3-Flag mTOR mutants.

Cell Culture, transfection, and immunoblots. HEK293T cells were cultured in DMEM with 10% fetal bovine serum, glutamine, non-essential amino acids, and antibiotics. To assay mTOR activity, pcDNA3-Flag mTOR was transfected alone or with Myc-S6K into HEK293T cells using Lipofectamine 2000, according to manufacturer instructions (Invitrogen). Twenty-four hours after transfection, cells were treated as indicated. Lysates were measured for protein concentration (Pierce BCA assay), and equal amounts of protein were resolved by PAGE and subjected to immunoblotting using the following antibodies against phospho-S6K1(T389), total S6K, phospho-S6 (S235/236), phospho-AKT (S473) (Cell Signaling #9205, #9202, #4858, #9271), β-Actin (Sigma, AC-15), Flag (Sigma, M2), and c-Myc (Santa Cruz, SC-40).
Computational modeling of mTOR kinase domain. The model was built by HMM-HMM (hidden markov-model) comparison, using the HHpred server (http://toolkit.tuebingen.mpg.de) and the x-ray crystal structure of PIK3C3 (pdb: 3ls8) as a template.

REFERENCES