Supplemental online content for:

Whole-Exome Sequencing in Two Extreme Phenotypes of Response to VEGF-Targeted Therapies in Patients With Metastatic Clear Cell Renal Cell Carcinoma

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• eAppendix 1: Supplemental Methods
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Patients and Samples
Formalin-fixed, paraffin-embedded (FFPE) samples from tumor and normal tissue from 20 patients with metastatic renal cell carcinoma (RCC) who met the inclusion criteria were retrieved. Slides were reviewed by an expert genitourinary pathologist (S.S.) and tumor cores were punched for DNA extraction. This project was approved by the Dana-Farber/Harvard Cancer Center (DF/HCC) Institutional Review Board (IRB) and the Peter MacCallum Cancer Center Ethics Committee or the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects previous to clinical data collection and molecular analysis.

FFPE Sequencing

FFPE DNA Extraction
Paraffin is removed from FFPE sections and cores using CitriSolv (Fisher Scientific, Fair Lawn, NJ) followed by ethanol washes, then tissue is lysed overnight at 56°C. Samples are then incubated at 90°C to remove DNA crosslinks, and extraction is performed using QIAGEN’s QIAamp DNA FFPE Tissue Kit (QIAGEN, Valencia, CA).

Library Construction
Initial genomic DNA input into shearing was reduced from 3 mcg to 10 to 100 ng in 50 mcL of solution. For adapter ligation, Illumina paired-end adapters were replaced with palindromic forked adapters, purchased from Integrated DNA Technologies, with unique 8-base molecular barcode sequences included in the adapter sequence to facilitate downstream pooling. With the exception of the palindromic forked adapters, the reagents used for end repair, A-base addition, adapter ligation, and library enrichment polymerase chain reaction (PCR) were purchased from Kapa Biosystems in 96-reaction kits. In addition, during the postenrichment solid-phase reversible immobilization (SPRI) bead cleanup, the elution volume was reduced to 20 mcL to maximize library concentration, and a vortexing step was added to maximize the amount of template eluted from the beads. Any libraries with concentrations less than 40 ng/mcL, as measured by a PicoGreen assay automated on an Agilent Bravo, were considered failures and reworked from the start of the protocol.

In-Solution Hybrid Selection
Also performed as previously described with the following modifications to the hybridization reaction: before hybridization, any libraries with concentrations greater than 60 ng/mcL as determined by PicoGreen were normalized to 60 ng/mcL, and 8.3 mcL of library was combined with blocking agent, bait, and hybridization buffer. Any libraries with concentrations between 50 and 60 ng/mcL were normalized to 50 ng/mcL, and 10.3 mcL of library was combined with blocking agent, bait, and hybridization buffer. Any libraries with concentrations between 40 and 50 ng/mcL were normalized to 40 ng/mcL, and 12.3 mcL of library was combined with blocking agent, bait, and hybridization buffer. Regardless of library concentration range, the same volume of blocking agent and bait previously described were used, and hybridization buffer volume was adjusted to equal the combined volume of library, blocking agent, and bait. Finally, the hybridization reaction was reduced to 17 hours with no changes to the downstream capture protocol.

Preparation of Libraries for Cluster Amplification and Sequencing: After postcapture enrichment, libraries were quantified using PicoGreen (automated assay on the Agilent Bravo), normalized to equal concentration on the Perkin-Elmer Minijanus, and pooled by equal volume on the Agilent Bravo Automated Liquid Handling Platform. Library pools were then quantified using quantitative PCR (kit purchased from Kapa Biosystems) with probes specific to the ends of the adapters; this assay was automated using Agilent’s Bravo Automated Liquid Handling Platform. Based on quantitative PCR (qPCR) quantification, libraries were normalized to 2 nM, and then denatured using 0.2 N of NaOH on the Perkin-Elmer Minijanus. After denaturation, libraries were diluted to 20 pM using hybridization buffer purchased from Illumina.
Cluster Amplification and Sequencing: Cluster amplification of denatured templates was performed according to the manufacturer’s protocol (Illumina) HiSeq v3 cluster chemistry and flowcells, as well as Illumina’s Multiplexing Sequencing Primer Kit. Flowcells were sequenced using HiSeq 2000 v3 Sequencing-by-Synthesis Kits, then analyzed using RTA v.1.12.4.2 or later. Each pool of whole-exome libraries was run on paired 76-bp runs, and 8-base index sequencing read was performed to read molecular indices, across the number of lanes needed to meet coverage for all libraries in the pool.

Analysis and Interpretation

DNA Assembly and Quality Control

Sequence Data Processing: Exomes sequence data processing was performed using established pipelines at the Broad Institute. A BAM file was produced with the Picard pipeline (http://picard.sourceforge.net/), which aligns the tumor and normal sequences to the hg19 human genome build using Illumina sequencing reads. The BAM was uploaded into the Firehose pipeline (http://www.broadinstitute.org/cancer/cga/Firehose), which manages input and output files to be executed by GenePattern. Whole-exome sequencing BAM files for data from this study cases will be deposited in dbGAP (phs001018).

Sequencing Quality Control: Quality control modules within Firehose were applied to all sequencing data for comparison of the origin for tumor and normal genotypes and confirm fingerprinting concordance. Cross-contamination of samples was estimated using ContEst41 to confirm that neither tumor nor germline sample had more than 3% contamination. Single-nucleotide polymorphism fingerprints from each lane of a tumor/normal pair were crosschecked to confirm concordance, and nonmatching lanes were removed from analysis. Somatic alteration identification and annotation The MuTect algorithm was applied to identify somatic single-nucleotide variants in targeted exons. Indelocator (http://www.broadinstitute.org/cancer/cga/indelocator) was applied to identify small insertions or deletions. Annotation of identified variants was performed using Oncotator (http://www.broadinstitute.org/cancer/cga/oncotator). Rearrangements were identified using dRanger (http://www.broadinstitute.org/cancer/cga/dranger). Copy ratios were calculated for each hybrid capture bait by dividing the tumor coverage by the median coverage obtained in a set of reference normal samples. The resulting copy ratios were segmented using the circular binary segmentation algorithm. Genes in copy ratio regions with segment means of greater than log2(4) were evaluated for focal amplifications given the potential clinical significance of a large focal event. Genes in regions with segment means of less than log2(0.5) were evaluated for hemizygous or homozygous deletions, because either broad or focal deletions may involve genes with clinical relevance. RefSeq was used to identify the genes that reside in the chromosomal coordinates demarcated by the segment start and end points.