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# Exceptional Response to Temsirolimus in a Metastatic Clear Cell Renal Cell Carcinoma With an Early Novel *MTOR*-Activating Mutation

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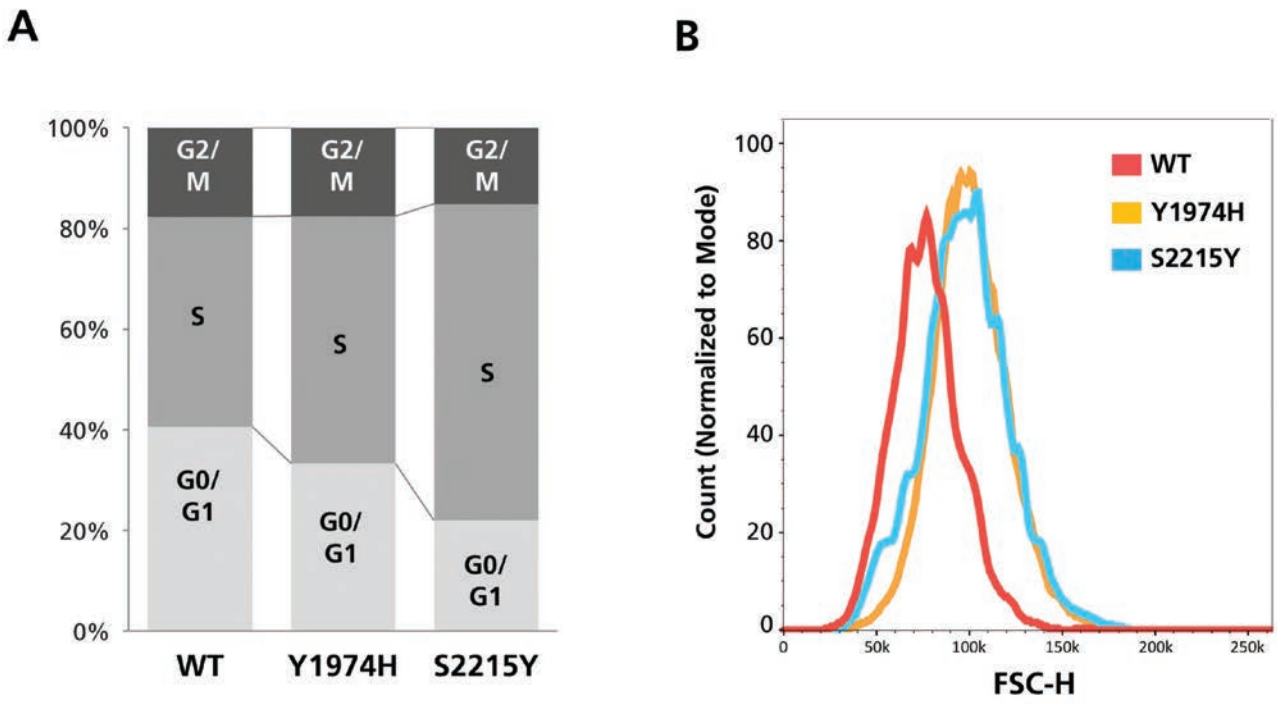


**eFigure 1.** *MTOR* mutation effects on cell cycle and forward scatter. HEK293 cells transfected with pCDNA3-FLAG-mTOR (wild-type, Y1974H or S2215Y) were subjected to serum starvation. After trypsinization, the cells were fixed and cellular DNA stained with propidium iodide. The transduced cells, which were found to be FLAG-positive, were analyzed on a FACS CANTO II for (A) DNA content using propidium iodide and (B) forward scatter pattern.

**eTable 1. Nonsynonymous Coding and LOF Variants Detected in the Tumors of the Patient (Primary Tumor and Metastasis) and Absent in the Blood**

| Gene | Variant | Ref. Allele | Alt. Allele | Primary T1_AF | Primary T2_AF | Primary T3_AF | Metastasis_AF | Effect | Amino_Acid_Change |
|---|---|---|---|---|---|---|---|---|---|
| **ACACB** | 12:109670522 | C | A | 0.31 | 0.20 | 0.22 | 0.33 | MISSENSE | S1280R;S1350R;S1350R |
| **ADAL** | 15:43643188 | C | A | 0.36 | 0.18 | 0.32 | 0.31 | MISSENSE | D274E;D274E |
| **ADAM28** | 8:24181384 | G | A | 0.17 | | | | MISSENSE | G253D |
| **ADAMTSL4** | 1:150526429 | G | A | 0.20 | | | | MISSENSE | G321E;G321E;G321E |
| **AJAP1** | 1:4772367 | C | T | | 0.17 | | | MISSENSE | P146L;P146L |
| **AK2** | 1:33487194 | C | T | 0.17 | | | | MISSENSE | M110I;M110I;M110I |
| **ALS2CR11** | 2:202358161 | C | T | 0.20 | | | | MISSENSE | G968E |
| **ANKRD18B** | 9:33541216 | G | C | 0.33 | 0.23 | 0.24 | | MISSENSE | G294R |
| **AP001468.1** | 21:47613570 | T | C | 0.45 | 0.31 | | | MISSENSE | H35R |
| **APOBEC3C** | 22:39411749 | G | A | | 0.22 | | | MISSENSE | R56Q |
| **ARHGAP22** | 10:49658586 | C | A | | 0.27 | | | MISSENSE | C529F;C535F;C545F |
| **ARID2** | 12:46245092 | G | T | 0.36 | 0.28 | 0.41 | 0.28 | MISSENSE | Q1062H |
| **ARL14EP** | 11:30352649 | G | A | 0.23 | | | | MISSENSE | G52R |
| **ARMC4** | 10:28250610 | C | A | | 0.29 | 0.24 | 0.14 | MISSENSE | D425Y |
| **ARX** | X:25025243 | C | G | 0.64 | 0.48 | 0.42 | 0.57 | MISSENSE | S478T |
| **ATHL1** | 11:289880 | C | G | 0.44 | 0.33 | 0.51 | 0.38 | MISSENSE | L22V;L22V |
| **ATP9B** | 18:76886357 | AG | A | 0.26 | | 0.21 | 0.21 | FRAME_SHIFT | |
| **BAP1** | 3:52437801 | CTTT | CTTTT | 0.34 | 0.16 | 0.28 | 0.26 | FRAME_SHIFT | |
| **BCL11B** | 14:99641956 | G | A | 0.27 | | | | MISSENSE | T212M;T335M;T406M |
| **BSCL2** | 11:62472862 | C | A | 0.39 | 0.18 | 0.19 | 0.40 | MISSENSE | L105F;L105F;L105F;L41F;L41F;L41F;L41F |
| **C4orf21** | 4:113539720 | G | A | 0.18 | | | | MISSENSE | S493F |
| **C7orf55-LUC7L** | 7:139092020 | G | T | 0.26 | 0.16 | 0.25 | 0.30 | MISSENSE | R204I;R270I |
| **CARD16** | 11:104915222 | C | A | | 0.18 | | | MISSENSE | L57F;L57F;L57F |
| **CCNG1** | 5:162868187 | G | A | 0.13 | | | | MISSENSE | R123Q;R123Q |
| **CDC14B** | 9:99296337 | A | T | 0.38 | 0.22 | 0.24 | 0.51 | MISSENSE | F273Y;F273Y;F273Y |
| **CELF2** | 10:11259399 | G | A | | | 0.22 | | MISSENSE | V64I;V64I;V64I;V64I;V64I;V64I |
| **CFH** | 1:196715027 | G | A | 0.20 | | | | MISSENSE | A1131T |
| **CHUK** | 10:101950628 | T | A | 0.42 | 0.29 | 0.34 | 0.34 | MISSENSE | M736L |
| **CIC** | 19:42795216 | G | T | 0.15 | | | | MISSENSE | G1675C;G766C;G766C |
| **CILP2** | 19:19656719 | C | T | | | 0.16 | | MISSENSE | P1122L;P1128L |
| **CKAP5** | 11:46786695 | C | A | 0.20 | | | | NONSENSE | E1175* |
| **CLSPN** | 1:36219445 | T | G | 0.39 | 0.24 | 0.30 | 0.20 | MISSENSE | K558Q;K558Q;K558Q |
| **CNKSR1** | 1:26511636 | G | A | | 0.15 | | | MISSENSE | V423M;V430M |
| **CPNE2** | 16:57144689 | C | T | 0.57 | 0.32 | 0.29 | 0.19 | MISSENSE | A12V;A12V;A12V |
| **CTR9** | 11:10781764 | A | G | 0.37 | 0.20 | 0.18 | 0.24 | MISSENSE | K213E |
| **CTSH** | 15:79224788 | T | G | | | 0.14 | | MISSENSE | S140R |
| **CXorf23** | X:19971111 | C | T | 0.20 | | | | MISSENSE | E542K;E542K;E542K |
| **CYP2A6** | 19:41355828 | C | T | 0.12 | | | | MISSENSE | V80M |
| **DDX46** | 5:134131842 | T | A | 0.17 | | | | SPLICE_SITE_DONOR | |
| **DEF6** | 6:35286030 | C | T | | 0.21 | | | MISSENSE | R333W |
| **DENND3** | 8:142185453 | G | T | 0.42 | 0.22 | 0.22 | 0.37 | MISSENSE | K730N;K810N |
| **DHRS4** | 14:24424266 | C | T | 0.25 | | | | MISSENSE | R51C |
| **DHX30** | 3:47891435 | T | A | 0.46 | 0.33 | 0.42 | 0.56 | MISSENSE | L1098Q;L1137Q |
| **DHX40** | 17:57679913 | G | T | 0.18 | | | | MISSENSE | G613C |
| **DIDO1** | 20:61512104 | G | A | 0.20 | | | | MISSENSE | P1735L;P1735L |
| **DNAH6** | 2:85011924 | A | T | 0.43 | 0.24 | 0.34 | 0.32 | MISSENSE | N3648I;N3648I |
| **DOCK11** | X:117676803 | C | T | 0.17 | | | | MISSENSE | S73F;S73F |
| **EEA1** | 12:93226564 | A | T | 0.18 | | | | MISSENSE | N326K |
| **EIF4G3** | 1:21268389 | C | T | 0.18 | | | | MISSENSE | E364K;E364K;E370K;E370K |
| **EML4** | 2:42510010 | C | T | 0.19 | | | | MISSENSE | P222L;P280L;P291L |
| **FCGBP** | 19:40424297 | A | T | 0.36 | 0.20 | 0.30 | 0.34 | MISSENSE | F636I |
| **FER1L6** | 8:124975587 | T | C | 0.29 | 0.25 | 0.34 | 0.23 | MISSENSE | V49A;V49A |
| **GAK** | 4:861013 | G | A | 0.20 | | | | MISSENSE | P868L |
| **GLT6D1** | 9:138516493 | G | A | 0.23 | 0.24 | 0.32 | | MISSENSE | P94L |
| **GOLPH3L** | 1:150620846 | C | G | 0.61 | 0.27 | 0.37 | 0.47 | MISSENSE | S270T |
| **HAS1** | 19:52216790 | C | T | 0.19 | | | | MISSENSE | A542T;A543T;A550T |
| **HCAR3** | 12:123200354 | G | A | | 0.69 | | 0.38 | MISSENSE | R311C |
| **HLA-DOA** | 6:32975808 | G | A | 0.25 | 0.36 | 0.33 | 0.39 | MISSENSE | R105C |
| **HLA-DRB1** | 6:32549540 | C | T | 0.20 | 0.21 | 0.15 | | MISSENSE | S149N |
| **HLA-DRB1** | 6:32549531 | T | C | | | 0.15 | | MISSENSE | Y152C |
| **HLA-DRB1** | 6:32549525 | C | G | | | 0.15 | | MISSENSE | G154A |
| **HNRNPLL** | 2:38812862 | C | T | 0.35 | | 0.32 | 0.37 | MISSENSE | G157E;G157E |
| **HS6ST2** | X:131842613 | A | G | | | 0.14 | | MISSENSE | M1T |
| **HSD17B14** | 19:49316771 | T | C | 0.33 | 0.19 | 0.27 | 0.38 | MISSENSE | E194G |
| **IGHMBP2** | 11:68676003 | G | T | | 0.15 | | | MISSENSE | A151S |
| **IGHV3-35** | 14:106845586 | A | C | 0.43 | 0.14 | | | MISSENSE | L30V |
| **IGHV3-35** | 14:106845579 | T | C | 0.30 | | | | MISSENSE | Q32R |
| **INPP4B** | 4:143235912 | G | A | 0.19 | | | | NONSENSE | R126*;R126*;R126*;R126* |
| **IRAK1** | X:153284141 | G | A | 0.14 | | | | MISSENSE | S213L;S213L |
| **KLHL20** | 1:173744950 | C | T | 0.21 | 0.21 | 0.14 | 0.27 | MISSENSE | P536L |
| **KLHL28** | 14:45414636 | C | T | 0.28 | 0.19 | 0.25 | 0.28 | MISSENSE | A166T |

*(continued)*

**eTable 1. Nonsynonymous Coding and LOF Variants Detected in the Tumors of the Patient (Primary Tumor and Metastasis) and Absent in the Blood (cont.)**

| Transcript_ID |
|---|
| ENST00000377854;ENST00000338432;ENST00000377848 |
| ENST00000422466;ENST00000562188 |
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| ENST00000375706;ENST00000375704;ENST00000525374 |
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| ENST00000262992;ENST00000308502;ENST00000508116;ENST00000513000 |
| ENST00000393687;ENST00000369980 |
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| ENST00000396128 |

## eAppendix 1. Supplementary Material

### Material and DNA Extraction

Samples available for the study included 3 different regions of the primary tumor obtained from the nephrectomy before temsirolimus treatment; tissue from a single bone metastasis that appeared 7 years after temsirolimus treatment initiation and that was surgically removed; and from the peripheral blood from the patient. DNA was isolated from the formalin-fixed paraffin-embedded (FFPE) tumor tissues using DNeasy Blood & Tissue Kit (QIAGEN) and from the peripheral blood using the FlexiGene DNA Kit (QIAGEN).

The project was approved by the Instituto de Salud Carlos III Review Board, and the patient provided written informed consent to participate in the study.

### Whole-Exome Sequencing

Whole-exome sequencing (WES) was performed on the 3 regions of the primary tumor and on the metastasis and blood of the patient at the National Centre for Genomic Analysis, as described previously.[1] Briefly, the Covaris S2 System (Covaris, Woburn, MA) was applied for DNA fragmentation, and exome capture was performed using the SureSelect XT HumanAllExon V5 kit (Agilent Technologies, Santa Clara, CA). Exome sequencing was performed using 100-base paired-end technology in a HiSeq2000 (Illumina, San Diego, CA). The mean depth of coverage was >80x in all samples. Tumor variants were defined as those detected in the tumor DNA but not in the blood DNA. Metastatic-specific tumor mutations were those detected in the metastasis DNA but not in the primary tumor. An allele frequency threshold of 0.10 was applied. Variants were manually curated and relevant mutations were confirmed by Sanger sequencing with an ABI PRISM 3700 DNA Analyzer capillary sequencer (Applied Biosystems, Foster City, CA). In addition, *MTOR* introns 41–46 were sequenced by Sanger in the primary tumor and metastatic sample. Primers are available on request.

### Experimental Analysis

A wild-type human *MTOR* expression construct (pCDNA3-Flag-mTOR) was acquired from Addgene (Cambridge MA; plasmid #26603). Site-directed mutagenesis was performed in pCDNA3-Flag-mTOR to generate Y1974H and S2215Y point mutations (DNA Express Inc, Montreal, Quebec, Canada).

Functional assessment of mTOR pY1974H variant was performed in HEK293 cells. Cells were transfected with pCDNA3-Flag-mTOR, pCDNA3-Flag-mTOR-Y1974H, or pCDNA3-Flag-mTOR-S2215Y using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, and 24 hours after transfection cells were serum-starved for 24 hours, and then stimulated with 1% or 10% fetal bovine serum for 2 hours. To block the mTOR pathway, cells were treated with 2.5, 12.5, and 100 nM of rapamycin (Apollo Scientific, Bredbury, Stockport, UK) for 6 hours. Cells were lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO) with Phosphatase Inhibitor Cocktail 2 and 3 (Sigma-Aldrich, P5726 and P0044) and Protease Inhibitor Cocktail (P8340). Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) and 30 mcg were resolved in an SDS-PAGE gel and subjected to immunoblotting using antibodies for Thr389 phospho-S6K1, total S6K1, GAPDH (Cell Signaling Technology, Danvers, MA; 9234, 9202, and 2118, respectively), and anti-Flag (Sigma-Aldrich, F1804).

For cell-cycle experiments, cells were transfected with the different *MTOR* constructs and serum-starved. After trypsinization, cells were fixed with 70% ethanol and intracellularly stained with an anti-FLAG antibody (Sigma-Aldrich), followed by a secondary antimouse Alexa Fluor 647 (Thermo Fisher Scientific), so to identify the positive transfected cells. After the intracellular staining, cells were resuspended in 50 mcg/mL of propidium iodide (Sigma-Aldrich) solution containing 20 mg/mL of ribonuclease A. Cells with the secondary AF647 only were used as staining controls. The cells were analyzed using a FACSCanto II (BD Biosciences, San Jose, CA), pulse processing was used to exclude cell aggregates, and at least 10,000 single cell events were collected. All data were analyzed using FlowJo v10.1 (Tree Star, Ashland, OR).

### Histologic Studies

Hematoxylin-eosin–stained and immunohistochemically stained tumor sections were evaluated via

visual examination under a microscope. Immunohistochemistry was performed on tumor tissue sections using antibodies against Ser235/236 phosphoribosomal protein S6 (p-S6) and Thr202/Tyr204 phospho-ERK (p-ERK) (Cell Signaling Technology; 2211 and 9101, respectively) following standard protocols. The intensity of the staining was evaluated by an experienced pathologist (L.B.).

## Reference

1. Apellániz-Ruiz M, Lee MY, Sánchez-Barroso L, et al. Whole-exome sequencing reveals defective CYP3A4 variants predictive of paclitaxel dose-limiting neuropathy. Clin Cancer Res 2015;21:322–328.