Somatic *CACNA1H* mutation as a cause of aldosterone-producing adenoma

Online-only Data Supplement

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Supplemental methods

Immunohistochemistry

IHC was performed using following primary antibodies as described previously[1]: CYP11B2 (Millipore, #MABS1251, mouse monoclonal, RRID: AB_2650562) and CYP17A1 (LifeSpan Biosciences, #LS-B14227, rabbit polyclonal, RRID: AB_2088387).

Direct Sanger sequencing

Direct Sanger sequencing was performed on gDNA from FFPE as described previously[2]. Following primers for *CACNA1H* gene were used for PCR: forward 5'-GCCATAGATGACTGCAGTG-3'; reverse 5'-TGATGAAGAAGGCGCAGCAG-3'.

Cell experimental protocols

For RNA analysis, cells were plated in triplicate in 48-well cell culture dishes at a density of 0.1 x 10^6 cells per well in 1 mL per well. After 48 hours, the growth medium was replaced with 1 mL per well of low serum medium containing 0.1% Cosmic Calf Serum with or without 1 µg/mL of doxycycline. After an additional 24 hours, the medium was replaced with fresh low serum medium with or without 1 µg/mL of doxycycline. An agonist treatment with angiotensin II (10 nM) was also performed as a comparison. Cells were harvested after 24 hours of treatment for RNA analysis.

For steroid analysis, cells were plated in duplicate in 6-well cell culture dishes at a density of 0.9 x 10^6 cells per well in 3 mL per well of growth medium. After 48 hours, the growth medium was replaced with low serum medium containing 0.1% Cosmic Calf Serum. After an additional 24 hours, the cell culture medium was replaced with 1.3 mL low serum medium with or without 1 µg/mL of doxycycline. As a comparison, treatment with or without angiotensin II (100 nM) for each group was also performed. After 48 hours of treatment, cell culture media was collected and the cells were frozen for future protein extraction. Cell protein was extracted using mammalian protein extraction reagent and protein concentration was determined by the bicinchoninic acid protein assay.

Quantitative real-time RT-PCR

Cell RNA isolation and cDNA generation were performed as described previously[3]. PCR was performed using ABI StepOnePlus Real-Time PCR systems (Applied Biosystems). Primer/probe mixture for the amplification of *CYP11B2* was prepared as previously described[4] and ones for *CACNA1H* (Hs01103527_m1) and *PPIA* (Hs99999904_m1) were purchased from Thermo Fischer Scientific. The *PPIA* transcript was used for sample loading normalization. To calculate relative mRNA expression, the delta-delta threshold cycle method was used[5].

Aldosterone quantification by LC-MS/MS

Aldosterone was quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Unlabeled and deuterium-labeled steroids were obtained from Cerilliant, and Sigma-Aldrich respectively (**Table S1**). 120 µL of deuterated aldosterone internal standard of known concentration was combined with 1.3 mL of cell media, followed by addition of 1 mL of acetonitrile for deproteinization in a 5 mL tube. The suspension was vortexed for 5 minutes and centrifuged for 5 minutes at 3000 rpm. For extraction of aldosterone, the supernatant was mixed with 6 mL methyl-t-butyl ether (MTBE) and 1mL water for 30 minutes. The MTBE phase was then collected and dried down. 1.5 mL ethyl acetate and 375 µL of 10% sodium chloride was

added to the dried residue and suspension was again mixed for 10 minutes and then allowed to stand for 5 minutes. The organic phase was separated and evaporated under nitrogen. The dried residue of each sample was resuspended in 60 µL methanol/deionized water (1:1), transferred to a 0.20 mL vial insert and stored at −20°C until LC-MS/MS analysis.

Samples (20 µL) were injected via autosampler and resolved with a pair of Agilent 1260/1290 binary pump HPLCs via 2D liquid chromatography, first on a 10 mm x 3 mm, 3 µm particle size Hypersil Gold C4 loading column (Thermo Scientific, Waltham, Massachusetts) followed by a Kinetex 150 mm x 2.1 mm, 2.6 µm particle size biphenyl resolving column (Phenomenex, Torrance, CA). The mobile phases consisted of 0.2 mmol/L aqueous ammonium fluoride (A) and methanol with 0.2 mmol/L ammonium fluoride (B). Aldosterone was eluted using gradient specifications as described in **Table S2**. The column effluent was directed into the source of an Agilent 6495 triple quadrupole mass spectrometer using electrospray ionization in positive ion mode. Quantitation was accomplished by comparing ion currents for the monitored ions with weighted (1/x) 10-point linear external calibration curves (r2 was >0.995) and corrected for specimen concentration and recovery of internal standards using ChemStation and MassHunter software (Agilent, Santa Clara, CA). The lower limit of detection (signal-to-noise ratio of 3) and lower limit of quantitation (signal-to-noise ratio of 5) for aldosterone were 12 and 20 pg/mL respectively. The results were normalized to protein concentration and shown as fold change over basal.

References

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Table S1. Aldosterone measurement by LC-MS/MS

Steroid	Steroid Manufacturer	Precursor/ Product Ions (m/z[a])	RT (min)	Internal standard	Internal standard Manufacturer
Aldosterone	Cerilliant	361.2/ 315.1	9.8	Aldosterone-d_7	Sigma-Aldrich

[a]m/z, mass to charge ratio; RT, retention time

Table S2: Gradient specifications of the Agilent 1260/1290 two dimensional liquid chromatography system

(A) 1260 HPLC Pump - C4 loading column

Mobile Phase A: 0.2 mmol/L Ammonium fluoride (NH_4F)
Mobile Phase B: Methanol + 0.2 mmol/L NH_4F

Time (min)	A (%)	B (%)	Flow (mL/min)	Pressure (bar)
0.00	65	35	0.20	400
0.80	65	35	0.20	400
0.85	0	100	0.20	400
1.20	0	100	0.20	400
6.00	0	100	0.20	400
6.10	65	35	0.20	400

(B) 1290 HPLC Pump - Biphenyl resolution column

Mobile Phase A: 0.2 mmol/L Ammonium fluoride (NH_4F)
Mobile Phase B: Methanol + 0.2 mmol/L NH_4F

Time (min)	A (%)	B (%)	Flow (mL/min)	Pressure (bar)
0.00	65	35	0.20	800
0.50	65	35	0.20	450
2.00	65	35	0.20	450
2.01	40	60	0.20	500
5.00	40	60	0.20	500
5.01	40	60	0.15	500
10.20	11	89	0.15	500
10.40	11	89	0.15	500
10.41	0	100	0.50	800
11.20	0	100	0.50	800
11.21	65	35	0.50	800
13.20	65	35	0.50	800

Table S3. Prevalence of somatic mutations in APAs from white patients

Affected gene	APAs from men	APAs from women	Total
KCNJ5	11 (24%)	21 (70%)	32 (43%)
ATP1A1	10 (22%)	3 (10%)	13 (17%)
ATP2B3	3 (7%)	0	3 (4%)
CACNA1D	15 (33%)	1 (3%)	16 (21%)
CACNA1H	2 (4%)	1 (3%)	3 (4%)
CTNNB1	0	2 (7%)	2 (3%)
Mutation negative	4 (9%)	2 (7%)	6 (8%)
Total	45	30	75

Determination of *CACNA1H* mutation prevalence includes tumor samples analyzed in our previous publication[6].

Table S4. Clinical characteristics of patients with *CACNA1H*-mutated APA

Age	Sex	Race	NGS ID of APA	BP (mmHg)	Number of anti-hypertensive medication	Serum potassium (mEq/L)	Potassium supplementation	PAC (ng/dL)	PRA (ng/mL/h)	Tumor size on imaging study (mm)	Side of adrenal tumor	Side of aldosterone excess by AVS
40*	Female	White	APA3	148/102	6	3.4	No	27	0.1	23	Left	not done
56	Male	White	APA_UM60	130/80	4	not available	Yes	20.9	0.15	not detected	-	Left
48	Male	White	APA_UM90	151/91	7	3.7	No	87.9	<0.1	25	Left	Left

BP, blood pressure; AVS, adrenal venous sampling.
*, This case was described as Case 5 in our previous study[1].

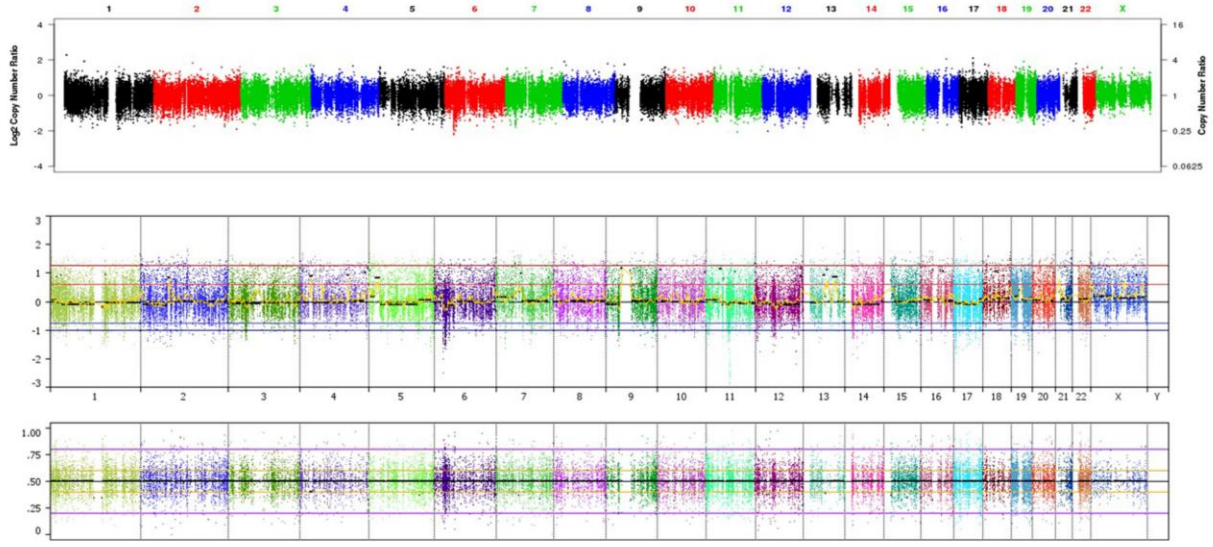

Figure S1. Copy number plot of the APA sample harboring *CACNA1H* mutation. X-axis represents chromosomal loci linearly arranged from chr1 to 23 followed by chrX (colored for easy visualization); the y-axis represents copy number level measured as ratio of APA tissue versus patient matched normal adjacent tissue (Log2 ratio on the left axis, and linear ratio on the right axis).

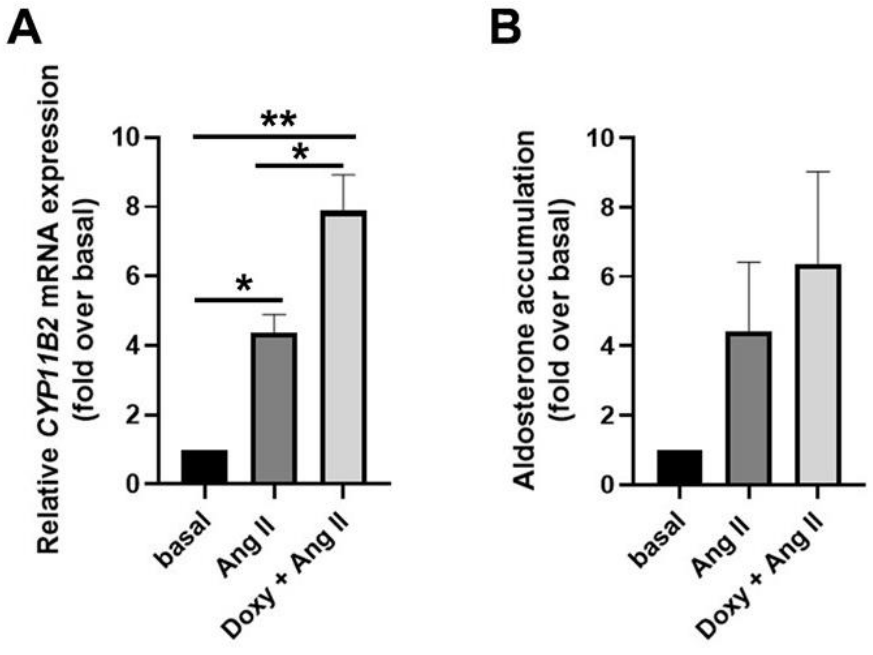

Figure S2. Effect of angiotensin II (AngII) on *CACNA1H*^{I1430T} induced HAC15 cells
A. Relative *CYP11B2* transcript levels in basal (untreated) cells and cells treated with AngII (10 nM) alone or with AngII plus doxycycline (Doxy) (1 µg/mL). **B.** Relative aldosterone production in basal (untreated) cells and cells treated with AngII (100 nM) alone or along with doxycycline (1 µg/mL). *CACNA1H*^{I1430T} enhanced AngII stimulated *CYP11B2* expression. However, the trend for *CACNA1H*^{I1430T} enhanced AngII aldosterone production did not reach statistical significance due to a large variation of the independent cell-based experiments. Experimental data represent a minimum of three independent experiments. Statistical analyses were performed using one-way ANOVA followed by Turkey's multiple comparison test. *P <0.05, ** P <0.001.