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

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Abstract

Driver somatic mutations for aldosterone excess have been found in approximately 90% of aldosterone-producing adenomas (APAs) using an aldosterone synthase (CYP11B2)-guided sequencing approach. In the present study, we identified a novel somatic *CACNA1H* mutation (c.T4289C, p.I1430T) in an APA without any currently known aldosterone-driver mutations using CYP11B2 immunohistochemistry-guided whole exome sequencing. The *CACNA1H* gene encodes a voltage dependent T-type calcium channel alpha-1H subunit. Germline variants in this gene are known as a cause of familial hyperaldosteronism IV. Targeted next-generation sequencing detected identical *CACNA1H* variants in two additional APAs in a cohort of the University of Michigan, resulting in a prevalence of 4% (3/75) in APAs. We tested the functional effect of the variant on adrenal cell aldosterone production and *CYP11B2* mRNA expression using the human adrenocortical HAC15 cell line with a doxycycline-inducible *CACNA1H*^{I1430T} mutation. Doxycycline treatment increased *CYP11B2* mRNA levels as well as aldosterone production, supporting a pathologic role of the *CACNA1H* p.I1430T mutation on the development of primary aldosteronism. In conclusion, somatic *CACNA1H* mutation is a genetic cause of APAs. Although the prevalence of this mutation is low, this study will provide better understanding of molecular mechanism of inappropriate aldosterone production in APAs.

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DISCLOSURES

S.A. Tomlins is co-founder of, equity holder in, and current employee of Strata Oncology.

Keywords

Aldosterone; Primary aldosteronism; Aldosterone-producing adenoma; CYP11B2; Mutation; Calcium channel

INTRODUCTION

Somatic mutations that cause excess aldosterone production have been identified in aldosterone-producing adenomas (APAs). The affected genes include *KCNJ5*[1], *CACNA1D*[2, 3], *ATP1A1*[4], and *ATP2B3*[4]. Somatic mutations in these genes alter intracellular ion homeostasis, resulting in increased aldosterone synthase (*CYP11B2*) expression and enhanced aldosterone production. CYP11B2 is a steroidogenic enzyme that is required for the final steps of aldosterone biosynthesis. Because of the critical role of CYP11B2 in aldosterone production, immunohistochemistry (IHC) for this enzyme allows identification of cells involved in physiologic or pathologic production of aldosterone. To improve the accuracy for detection and subsequent analysis of APA, we recently developed a CYP11B2 IHC-guided targeted next-generation sequencing (NGS) method using formalin-fixed, paraffin-embedded (FFPE) material. Using this approach, somatic mutations in aforementioned aldosterone-driver genes were identified in nearly 90% of APAs[5, 6]. However, there are still some mutation negative populations of tumors that are confirmed to be APA by CYP11B2 IHC.

In the present study, we performed whole exome sequencing (WES) on a mutation-negative APA by CYP11B2 IHC-guided targeted NGS. WES identified a somatic mutation in *CACNA1H*, encoding voltage-dependent T-type calcium channel subunit alpha-1H, in which germline variants have been identified in early-onset patients with primary aldosteronism (PA)[7]. We further investigated the pathophysiologic role of the *CACNA1H* variant using human adrenocortical cell line HAC15 cells.

MATERIALS AND METHODS

The authors declare that all supporting data are available within the article and its online-only Data Supplement.

Patients

PA patients who underwent unilateral adrenalectomy at the University of Michigan were studied. The diagnosis of PA was made according to the institutional consensus available at the time or the Endocrine Society Clinical Practice guideline[8]. Patients for mutational analysis were selected based on the availability of archival formalin-fixed paraffin-embedded (FFPE) blocks of resected adrenals. This study was approved by Institutional Review Boards at the University of Michigan. A waiver of informed consent was granted for the use of specimens for the experiments in this study (HUM00083056).

Whole exome sequencing on DNA from FFPE

Based on our somatic mutation prevalence study using CYP11B2-IHC guided targeted NGS approach[5, 6], whole exome sequencing (WES) was conducted on DNA from a mutation negative APA paired with matched adjacent normal tissue to identify somatic variants. In brief, APA (identified by CYP11B2 IHC[9]) and adjacent normal adrenal FFPE tissue slides were scraped separately using disposable scalpels. Genomic DNA (gDNA) was isolated using QIAGEN AllPrep DNA/RNA FFPE kit (QIAGEN). WES was performed using standard protocols in our Clinical Laboratory Improvement Amendments (CLIA) compliant sequencing lab[10, 11]. 500ng of gDNA was sheared using a Covaris S2 to a peak target size of 250 base pairs (bp). Fragmented DNA was concentrated using AMPure beads, followed by end-repair, A-base addition, ligation of the Illumina indexed adapters, and size selection on 3% Nusieve agarose gels (Lonza). Fragments between 300 to 350 bp were recovered, amplified using Illumina index primers, and purified by AMPure beads. One microgram of the library was hybridized to the Agilent SureSelect Human All Exon V4. The targeted exon fragments were captured and enriched following the manufacturer's protocol (Agilent). Paired-end whole-exome libraries were analysed by an Agilent 2100 Bioanalyzer and DNA 1000 reagents and sequenced using an Illumina HiSeq HiSeq 2500 (Illumina Inc). The primary base call files were converted into FASTQ sequence files using the bcl2fastq converter tool bcl2fastq-1.8.4 in the CASAVA 1.8 pipeline.

Ion torrent targeted NGS

To validate the variants identified by WES, we have developed a new Ampliseq multiplex PCR based panel for targeted NGS using Ion Torrent Sequencing (panel v3). The panel targets the complete coding sequence of the following genes: *KCNJ5, CACNA1D, ATP1A1, ATP2B3 CACNA1H and PRKACA.* Known oncogenic hotspots in *GNAS* and *CTNNB1* were also targeted. For *CACNA1H*, the amplicons were redesigned for increased specificity compared to our previous panel, and p.I1430T was better targeted due to the previous version having common SNPs in the binding site. The methods for library preparation and sequencing were performed as described previously[9]. Somatic variants were identified using our validated pipelines as described previously[5, 12]. The *CACNA1H* p.I1430T variant was further confirmed by direct Sanger sequencing as described previously[13]. The primer sequence is available in online-only Data supplement.

Lentiviral transduction and cell experiments

To assess the effect of newly identified *CACNA1H* variant p.I1430T, the variant was cloned into the doxycycline-inducible lentiviral vector pCLX-pTF- R1-DEST-R2-EBR65[14] via the shuttle vector pENTR1A-GFP-N2 (FR1)[15] to generate the lentivector pCLX-pTF-Blast-*CACNA1H*^{I1430T}. HAC15 cells[16] were transduced with lentivirus (HAC15-dox-CACNA1H^{I1430T}) followed by antibiotic selection with blasticidin (1 ¼g/mL). Since the transduction efficiency was relatively low even after antibiotic selection, several clones were isolated using cloning cylinders. A doxycycline-responsive clone (clone 6) was used for further cell experiments. Cells were cultured in DME/F12 medium containing 10% Cosmic Calf Serum, ITS+ Premix, and antibiotics. The detail of cell-based experimental protocols,

including quantitative real-rime RT-PCR[17–19] and aldosterone quantification by LC-MS/MS (Table S1 and Table S2) are described in online-only Data Supplement.

Statistical analysis

Results of cell-based experiments are given as means ± S.E.M. The data were analyzed with unpaired t test for comparison of samples from two groups. One-way ANOVA method was applied for comparison of samples from three groups. Results were considered significantly different when the *P* value was less than 0.05. GraphPad Prism ver. 8.0.0 was used for the analyses.

RESULTS

Identification of novel somatic mutations in APA

We performed WES on FFPE DNA from an APA that was found to be negative for somatic mutations previously shown to cause APA using CYP11B2 IHC-guided targeted NGS. This case was reported as Case 5 in our previous publication[9]. The tumor had distinct heterogeneity in CYP11B2 expression within the tumor. Genomic DNA was selectively isolated from CYP11B2-expressing region and matched adjacent normal adrenal DNA was also used for somatic variant call. For the tumor sample, a total of 103,256,358 reads (85.7% alignment to hg19 reference genome) were obtained for an effective average coverage of 114x per base; for the matched normal sample, a total of 72,114,926 reads (89.6% alignment to hg19 reference genome) provided an effective coverage of 137x per base. No major copy-number variation was observed (Figure S1). One novel somatic mutation in *CACNA1H* (c.T4289C, p.I1430T, NM_001005407) was identified only in the tumor (Table 1). Although germline *CACNA1H* variants have been reported as a cause of familial hyperaldosteronism IV (FH-IV)[7, 20], somatic mutation in this gene has not been documented in APA.

Prevalence of somatic *CACNA1H* mutations in APA

To validate the significance of somatic mutations in *CACNA1H*, targeted NGS using our newly developed panel was performed on eight mutation negative APA samples from eight white patients[5]. Of them, two had the identical somatic *CACNA1H* p.1430T mutation (Table 2), resulting in an overall prevalence of 4% (3/75) in APA from PA patients (Table S3). Baseline clinical characteristics of patients with *CACNA1H*-mutated APA are summarized in Table S4. The histologic characteristics of *CACNA1H*-mutated APA are shown in Figure 1A–1D. Interestingly, two of them showed distinct heterogeneity of tumor CYP11B2 expression. One is shown in Figure 1 and the other was reported in our previous study as Case 5[9]. The CYP11B2 expressing cells were mainly composed of lipid poor compact cells and mostly negative for CYP17A1, which is required for cortisol synthesis in normal physiology. The *CACNA1H* variant was identified only in the CYP11B2-positive region of the tumor (Figure 1E and 1F).

Effect of *CACNA1H* mutation on adrenal cell aldosterone production and *CYP11B2* mRNA expression

To assess the effect of the *CACAN1H* p.I1430T mutation on aldosterone production, we developed a human adrenocortical cell line using HAC15 cells with doxycycline-mediated

conditional expression of *CACNA1H*^{I1430T}. Doxycycline treatment successfully induced expression of the *CACNA1H* transcript carrying the mutation (Figure 2A). Induction of *CACNA1H*^{I1430T} increased *CYP11B2* mRNA expression by 3.6-fold vs basal (no doxycycline treatment) ($P<0.05$ vs. basal, Figure 2B). LC-MS/MS analysis revealed that the induction of *CACNA1H*^{I1430T} gene led to increased aldosterone accumulation (1.6-fold vs basal, $P<0.01$, Figure 2C). *CACNA1H*^{I1430T} enhanced angiotensin II stimulation of HAC15 cell *CYP11B2* mRNA expression (Figure S2A). However, the trend for *CACNA1H*^{I1430T} enhancement of angiotensin II stimulated aldosterone production did not reach statistical significance due to a large experimental variation in the aldosterone responses between independent experiments (Figure S2B). The results support the concept that the *CACNA1H* variant is a cause of excess aldosterone production.

DISCUSSION

The role of calcium signaling on the regulation of adrenal aldosterone production has been well studied [21]. Most of PA-related somatic and germline mutations affect intracellular calcium homeostasis, supporting the importance of calcium signaling pathways in pathologic settings as well as adrenal physiology. Somatic mutations in APA have been found in approximately half of APA using conventional hot-spot Sanger sequencing of DNA isolated from pathology-provided tumor tissue without consideration of its CYP11B2 expression [22]. We have recently developed a more targeted method of capturing CYP11B2 expressing tumor cells followed by deep sequencing. This approach led to a nearly 90% detection rate of aldosterone-driver mutations in APAs[5, 6]. A CYP11B2-guided approach is very important in the analysis of APAs because a discordance between imaging and immunohistochemical findings has been observed in a subset of PA cases [23]. In this study, we were able to perform CYP11B2 IHC-guided WES, which identified a novel somatic *CACNA1H* mutation (p.I1430T). The identical mutation was found in two additional APAs.

The *CACNA1H* gene encodes voltage-gated T-type calcium channel alpha subunit $Ca_v3.2$ which expresses in the adrenal zona glomerulosa[7]. Several germline *CACNA1H* variants have been reported in PA patients[7, 20]. This condition is currently considered as FH-IV (OMIM #617027). Scholl *et al.*[7] initially reported recurrent gain-of-function *CACNA1H* mutation (p.M1549V) in young-onset PA patients. The CACNA1H p.M1549V variant is located in the S6 segment of repeat III of $Ca_v3.2$. Impaired channel inactivation and a shift of activation to less depolarizing potentials were observed in whole-call patch clamp experiments using HEK293T cells transfected with *CACNA1H*^{M1549V}. This leads to increased calcium influx in adrenal cells. *In vitro* studies using human adrenocortical HAC15 cells conditionally expressing *CACNA1H*^{M1549V} further revealed increased *CYP11B2* mRNA levels and enhanced aldosterone production [24].

The *CACNA1H* p.I1430T variant, which was recurrently identified in our cohort, lies in the S5 segment of repeat III (Figure 3). HAC15 cells with induced expression of CACNA1H p.I1430T displayed increased aldosterone production as well as *CYP11B2* mRNA expression, supporting a pathologic role of this variant. Functional characterization of any newly identified variant is very important. Of note, many of the somatic mutations thus far identified in APAs have not been functionally validated. Specifically, somatic mutations in

CACNA1D are widely spread throughout the channel and their impact on aldosterone production is not clear. For example, a germline *CACNA1D* p.V401L variant has also been identified in a patient with autism spectrum disorders and epilepsy without any endocrine abnormality[25]. In addition, extensive electrophysiological analysis of the gating properties of *CACNA1D* p.M1354I, which was previously identified in an APA[2], demonstrated an absence of any gain-of-function effect, suggesting non-pathogenic role of the variant on aldosterone production[26].

The prevalence of somatic *CACNA1H* mutations in APA appears to be relatively low with similar prevalence to that seen for *ATP2B3* mutations. Interestingly, two out of three *CACNA1H*-mutated APAs showed distinct heterogeneity of CYP11B2 and CYP17A1 expression and the somatic *CACNA1H* mutation was observed only in CYP11B2-expressing area of the tumor, suggesting a unique developmental mechanism of *CACNA1H*-mutated APA. The effect of the *CACNA1H* mutation on cell proliferation or apoptosis is also of great interest and will need to be addressed in future studies

In conclusion, we identified a recurrent somatic *CACNA1H* mutation in a subset of APA. The *CACNA1H* p.I1430T mutation was functionally characterized as having the ability to increase *CYP11B2* mRNA expression and adrenal cell aldosterone production. The significance of somatic *CACNA1H* mutation will need to be further validated in other cohorts.

PERSPECTIVES

A recurrent somatic *CACNA1H* mutation was found in APAs without any other known aldosterone-driver gene mutation. The pathologic role of the variant in enhanced aldosterone production was confirmed by *in vitro* studies using a mutant gene inducible cell model. Although *CACNA1H*-mutated APA represents a small subset of APAs, the identification of somatic *CACNA1H* mutations provides better understanding of molecular pathogenesis of APA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

What Is New?

- A recurrent somatic mutation in *CACNA1H*, which encodes a voltage dependent T-type calcium channel alpha-1H subunit, was identified in a subset of aldosterone-producing adenomas.
- *In vitro* studies suggest that the *CACNA1H* p.I1430T variant has a pathologic potential to increase aldosterone production and *CYP11B2* expression.

What Is Relevant?

- Genetic alterations in the *CACNA1H* gene can cause not only familial hyperaldosteronism type IV (germline mutation) but also aldosterone-producing adenoma (somatic mutation).

Summary

- Somatic *CACNA1H* mutation is a genetic cause for a rare subset of aldosterone-producing adenomas. Our study expands our understanding of molecular mechanisms leading to the inappropriate aldosterone production in aldosterone-producing adenomas.

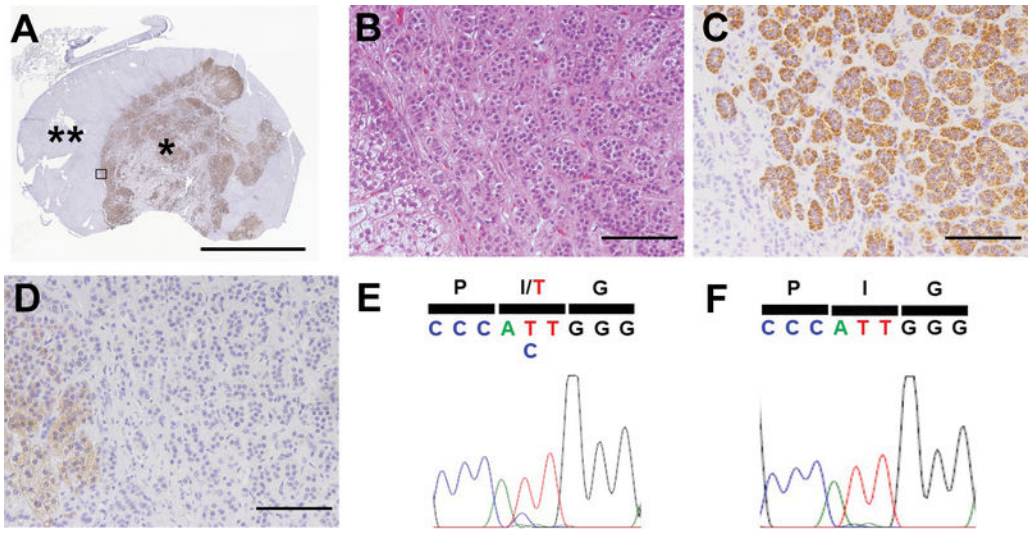

Figure 1. Histologic and genetic heterogeneity of an aldosterone-producing adenoma with somatic *CACNA1H* mutation

A. Low magnification image showing CYP11B2 IHC for the resected adrenal tumor from a patient with primary aldosteronism (brown, CYP11B2). Boxed area indicates the region where high magnification pictures were captured. Scale bar, 5 mm. **B-D.** High magnification micrographs of the tumor. Scale bars, 100 ¼m. **B.** Hematoxylin and eosin staining. **C.** CYP11B2 IHC. **D.** CYP17A1 IHC. **E and F.** Results of Sanger sequencing of CYP11B2-expressing tumor region (*, indicated in **A**) and CYP11B2-negative tumor region (**, indicated in **A**), respectively. IHC, immunohistochemistry.

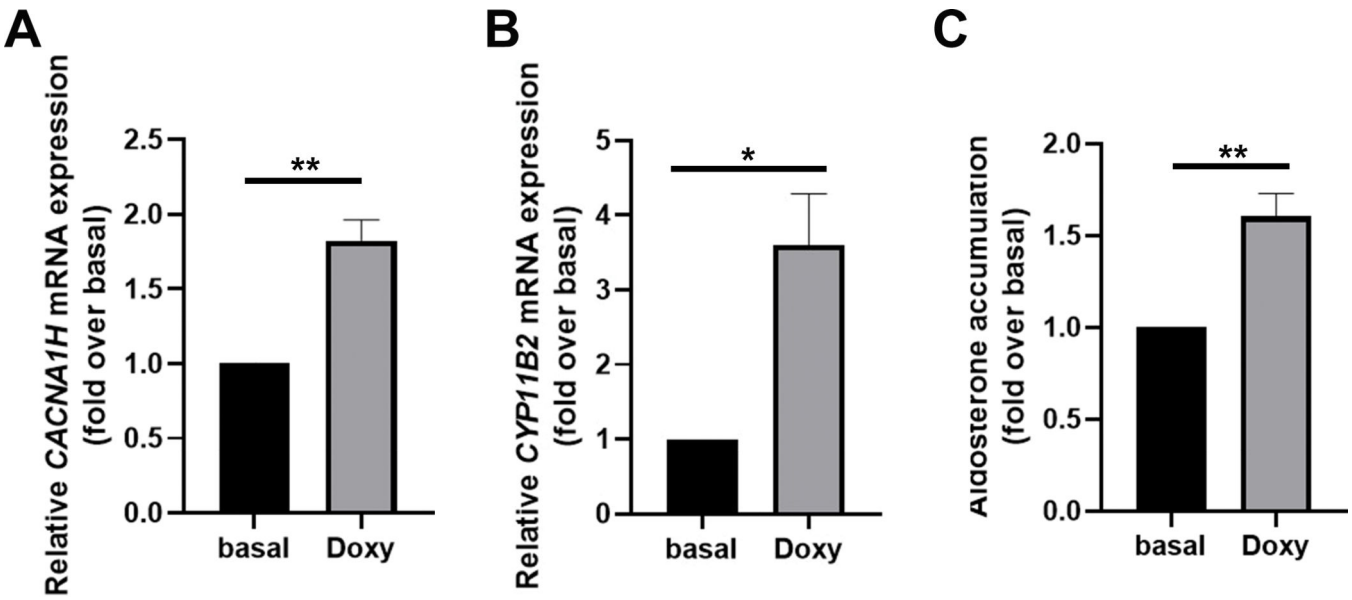

Figure 2. Effect of *CACNA1H*^{I1430T} on aldosterone production and *CYP11B2* expression in HAC15 cells

A. Induction of *CACNA1H*^{I1430T} in HAC15 cells after treatment of 1 ¼g/mL doxycycline. **B.** Effect of the induction of *CACNA1H*^{I1430T} on *CYP11B2* mRNA expression. **C.** Effect of the induction of *CACNA1H*^{I1430T} on HAC15 cell aldosterone production. Results represent means ± SEM. Experimental data represent a minimum of three independent experiments. Statistical analyses were performed using unpaired t test. **P* <0.05 vs basal, ** *P* <0.01 vs basal.

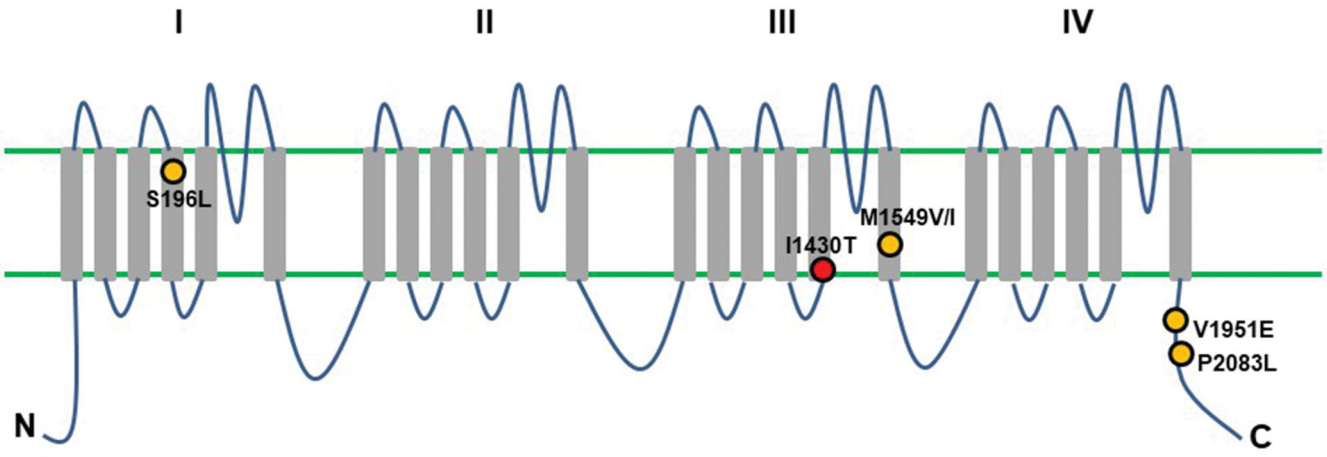

Figure 3. *CACNA1H* mutations identified in patients with primary aldosteronism

Transmembrane structure of $Ca_v3.2$ is described. $Ca_v3.2$ contains four homologous repeats (I-IV). Each domain contains six transmembrane segments (S1-S6). Orange circles indicate previously reported gain-of-function germline *CACNA1H* variants[7, 20]. The red circle indicates the somatic *CACNA1H* mutation identified in this study. The p.I1430T mutation is located in S5 of repeat III.

Table 1.

Results of whole exome sequencing

NGS ID	Gene	Type	Base changes	Amino Acid changes	Exon	Ref seq	VF (Tumor)	VF (Normal)
APA3	*CACNA1H*	Missense	c.T4289C	p.I1430T	22	NM_001005407	54/150(36%)	0/123(0%)

VF, variant allele frequency

Table 2.

Targeted ion torrent NGS results

NGS ID	Gene	Reference allele	Variant allele	Amino Acid change	FDP	VF (%)
APA3	*CACNA1H*	T	C	p.I1430T	1999	34
APA_UM60	*CACNA1H*	T	C	p.I1430T	1994	36
APA_UM90	*CACNA1H*	T	C	p.I1430T	1999	24

There was no evidence of the variants in adjacent normal adrenal tissue or CYP11B2-negative tumor regions by Sanger sequencing or targeted NGS. FDP, flow-corrected read depth; VF, variant allele frequency.