The prevalence of CTNNB1 mutations in primary aldosteronism and consequences for clinical outcomes

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Constitutive activation of the Wnt pathway/β-catenin signaling may be important in aldosterone-producing adenoma (APA). However, significant gaps remain in our understanding of the prevalence and clinical outcomes after adrenalectomy in APA patients harboring CTNNB1 mutations. The molecular expression of CYP11B2 and gonadal receptors in adenomas were also explored. Adenomas from 219 APA patients (95 men; 44.2%; aged 50.5 ± 11.9 years) showed a high rate of somatic mutations (n = 128, 58.4%). The majority of them harbored KCNJ5 mutations (n = 116, 52.9%); 8 patients (3.7%, 6 women) had CTNNB1 mutations. Patients with APAs harboring CTNNB1 mutations were older and had shorter duration of hypertension. After adrenalectomy, CTNNB1 mutation carriers had a higher possibility (87.5%) of residual hypertension than other APA patients. APAs harboring CTNNB1 mutations have heterogeneous staining of β-catenin and variable expression of gonadal receptors and both CYP11B1 and CYP11B2. This suggests that CTNNB1 mutations may be more related to tumorigenesis rather than excessive aldosterone production.

Primary aldosteronism (PA), which is characterized by hyperaldosteronism, affects 20% of patients with resistant hypertension1. Somatic mutations in the selectivity filter of the potassium channel, GIRK4 (encoded by KCNJ5) in aldosterone-producing adenoma (APA) result in a loss of potassium selectivity and entry of sodium and membrane depolarization2. Further somatic mutations in a subunit of an L-type voltage-gated Ca2+ channel, Cav1.3 (encoded by CACNA1D) and in 2 ATPases (Na+/K+ -ATPase alpha subunit and Ca2+ -ATPase 3, encoded by ATP1A1 and ATP2B3, respectively) have also been identified3–5. The resultant Ca2+ influx and activation of the Ca2+ signaling pathway leads to the increase in CYP11B2 gene transcription and an increase in aldosterone biosynthesis2. CTNNB1 (β-catenin) mutations have been reported in APAs4 and in cortisol-secreting or non-functional adenomas7. Constitutive activation of β-catenin in the adrenal cortex of transgenic mice resulted in progressive steroidogenesis, adrenal hyperplasia and late development of malignant characteristics and excessive secretion of aldosterone4. Moreover, overexpression of β-catenin in adrenal cortical carcinoma (ACC) has been correlated with a worse prognosis5. Recently, cases of APAs harboring activating mutations of β-catenin, which expressed high levels of the gonadal receptors LHCG and GNRHR, were described in three women (two during pregnancy and one postmenopausal) where Wnt activation caused adrenocortical cells to de-differentiate toward an adrenal-gonadal precursor cell6. The aim of this study was to determine the prevalence of the CTNNB1 mutations in APA patients and to correlate the mutation status with clinical outcomes in order to determine the outcomes on patients who harbor these mutations.

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Material and Methods

Ethics Declaration. This study has been approved, supervised, and monitored by the institutional review board of National Taiwan University Hospital, Taipei, Taiwan (No. 200611031R). It complied with the Declaration of Helsinki. All participants signed the informed consent before they were included in the study.

PA Identification. The present study was based on the Taiwan Primary Aldosteronism Investigation (TAIPAI) database and tissue bank. The TAIPAI database was constructed from June 2008 to March 2011 for quality assurance, including two medical centers, three affiliated hospitals and two regional hospitals in different cities in Taiwan. All antihypertensive medications were discontinued for at least 21 days before confirmatory and lateralizing tests. Doxazosin and/or diltiazem were administered to control markedly high blood pressure where required.

The diagnosis and subtype identification of PA were established and performed according to the standard protocol of TAIPAI, including saline infusion test, adrenal venous sampling and NP-59 scintigraphy with SPECT-CT imaging (supplementary file and Figure S1).

Adrenalectomy. The adrenalectomy was performed via the lateral trans-peritoneal laparoscopic approach by experienced surgeons, and adrenal tumors removed during the surgery were freshly frozen and stored at −80°C.

Sequencing. Nucleic acid extraction. Genomic DNA was extracted from 219 paired adenoma and its peritumoral normal adrenal cortices. Tumor DNA was extracted via a QiAamp DNA mini kit (Qiagen, Hilden, Germany); total RNA was isolated from frozen tissue using Trizol (Invitrogen, Carlsbad, CA, USA) and then cleaned-up by using the GENEzol TriRNA Pure Kit (Geneaid, New Taipei City, Taiwan). After D Nasel treatment (Invitrogen, Carlsbad, CA, USA), 500 ng of total RNA was reverse-transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (M–MLV RT; Promega, Madison, WI, USA) and random hexamers (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Relative gene expression in relation to GAPDH was calculated with the formula: 2−ΔΔCt of target gene−ΔCt of GAPDH.

Sequencing of somatic mutations. The coding area of the genomic DNA was investigated by exon sequencing. The entire coding sequence and flanking regions of candidate mutations were amplified and sequenced using gene-specific primers as previously reported. Accordingly, the PCR primers used to amplify fragments for direct sequencing of CTNNB1/ATP1A1/ATP2B3/CTNNB1 and CACNA1D also followed previous reports (listed in supplement Table S1). The annealing temperature was 58°C. Direct sequencing of PCR products was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) with the Magna-amp Pure DNAClean-Up Reagent (Thermo-Fisher) containing a protease inhibitor cocktail (Roche). A 30 μg sample of protein from each specimen was separated using SDS-PAGE and transferred onto PVDF membranes (Millipore). Visinin-like 1 (VSNL1) is upregulated in aldosterone-producing adenomas (APAs) compared with normal adrenals. Western blotting analysis. Human adrenal specimens were homogenized in T-PER tissue protein extraction reagent (Thermo-Fisher) containing a protease inhibitor cocktail (Roche). A 30 μg sample of protein from each specimen was separated using SDS-PAGE and transferred onto PVDF membranes (Millipore). Visinin-like 1 (VSNL1) is upregulated in aldosterone-producing adenomas (APAs) compared with normal adrenals. CTNNB1 exon 3 mutations could be involved in APA formation due to accumulation of β-catenin and increased expression of Cyclin D1. The canonical (Wnt/β-catenin-mediated) signaling functionally interacts with GATA425, a marker of gonadal differentiation that is crucial in adrenal development. Therefore, the primary antibodies used were as follows: mouse monoclonal anti-β-catenin (Anti-ABC) (05–665, Millipore), rabbit polyclonal antibody for CYP11B2 (a kind gift from Professor Celso Gomez-Sanchez), rabbit monoclonal antibody for CYP11B1 (a kind gift from Professor Celso Gomez-Sanchez), rabbit monoclonal anti-Cyclin D1 (ab134175, Abcam), rabbit polyclonal anti-VSNL1 (GTX115039, GeneTex), and rabbit polyclonal anti-GATA4 (GTX113194, GeneTex). Levels of proteins were detected using chemiluminescent detection reagents (Millipore) and visualized using a UVP Biospectrum 810 imaging system (Ultra Violet Products Ltd, Cambridge, UK).

Western blotting analysis. Human adrenal specimens were homogenized in T-PER tissue protein extraction reagent (Thermo-Fisher) containing a protease inhibitor cocktail (Roche). A 30 μg sample of protein from each specimen was separated using SDS-PAGE and transferred onto PVDF membranes (Millipore). Western blot analysis revealed the expression of VSNL1, β-catenin, GATA4, and CYP11B2 in the adrenal cortex. Visinin-like 1 (VSNL1) is upregulated in aldosterone-producing adenomas (APAs) compared with normal adrenals. CTNNB1 exon 3 mutations could be involved in APA formation due to accumulation of β-catenin and increased expression of Cyclin D1. The canonical (Wnt/β-catenin-mediated) signaling functionally interacts with GATA4, a marker of gonadal differentiation that is crucial in adrenal development. Therefore, the primary antibodies used were as follows: mouse monoclonal anti-β-catenin (Anti-ABC) (05–665, Millipore), rabbit monoclonal antibody for CYP11B2 (a kind gift from Professor Celso Gomez-Sanchez), rabbit monoclonal anti-Cyclin D1 (ab134175, Abcam), rabbit polyclonal anti-VSNL1 (GTX115039, GeneTex), and rabbit polyclonal anti-GATA4 (GTX113194, GeneTex). Levels of proteins were detected using chemiluminescent detection reagents (Millipore) and visualized using a UVP Biospectrum 810 imaging system (Ultra Violet Products Ltd, Cambridge, UK).

Measure of outcomes. For the first 3 months post-operatively the patients were followed monthly, and every three months subsequently. Evaluation of ‘cured’ hypertension has been described previously. Hypertension was considered as ‘cured’ if 75% of the recorded systolic BP was < 140 mmHg and the diastolic BP was < 90 mmHg without taking antihypertensive medications at least 1 year after adrenalectomy.

We further studied the differences in patients who had CTNNB1, KCNJ5 mutations or no-identified mutation (wild-type; WT), and defined them as the ‘enrolled group’. Given the differences in the baseline characteristics among the ‘enrolled group’ patients during the statistical analysis, we attempted to match each patient in the CTNNB1 mutation group with 3 patients in the KCNJ5 mutation group and 3 patients in the WT group (matched group), based on nearest neighbor matching without replacement, using age, sex and mean blood pressure (MBP).
Table 1. The clinical characteristics of CTNNB1 mutations. Categories of antihypertensive drugs. Abbreviations: APA, aldosterone-producing adenoma; AVS, arterial venous sampling; F, female; M, male; L, left; OP, operation; Pt, patients; R, right. NP-59 (SPECT/CT), I131–6b-iodomethyl-19-norcholesterol/SPECT/CT.

| Pt ID | Mutation | Sex | Age | AVS | Number of nodules | Aldosterone (ng/dL) | Renin (ng/mL/hr) | Potassium mmol/dL | Categories before OP* | Categories after OP* |
|-------|----------|-----|-----|-----|------------------|-------------------|----------------|-----------------|----------------------|----------------------|
| Pt1   | S45F     | F   | 50  | L   | —                | 1                 | 28.9           | 0.31            | 3.7                  | 1                    | 1                    |
| Pt2   | S45P     | F   | 54  | L   | —                | 1                 | 26.1           | 0.04            | 4.2                  | 2                    | 1                    |
| Pt3   | S45P     | F   | 51  | L   | lateralization   | 1                 | 86.0           | 0.34            | 4.5                  | 3                    | 0                    |
| Pt4   | S45P     | F   | 86  | R   | lateralization   | 1                 | 27.7           | 0.09            | 3.2                  | 3                    | 2                    |
| Pt5   | S45P     | M   | 62  | R   | lateralization   | 1                 | 40.5           | 0.27            | 3.7                  | 3                    | 3                    |
| Pt6   | S45F     | F   | 58  | L   | lateralization   | 1                 | 195.8          | 0.24            | 3.4                  | 3                    | 2                    |
| Pt7   | S45F     | F   | 62  | R   | lateralization   | 2                 | 22.1           | 0.66            | 4.1                  | 1                    | 1                    |
| Pt8   | S45F     | M   | 67  | R   | —                | 1                 | 24.8           | 0.35            | 4.0                  | 1                    | 1                    |

Statistical analysis. All data were expressed as the mean ± standard deviation (SD). A p-value of < 0.05 was considered significant.

Statistical analyses were performed with R software, version 2.8.1 (Free Software Foundation, Inc., Boston, MA, U.S.A.). Aldosterone and ARR were log-transformed to normal distribution. Logistic regression analysis with a stepwise variable selection procedure was applied using available variables to identify important factors associated with post-operative residual hypertension. The goodness-of-fit (GOF) of the fitted multiple logistic regression model was assessed by the estimated area under the receiver operating characteristic (ROC) curve, the Hosmer-Lemeshow GOF test, and the adjusted generalized R².

Results

Patient characteristics. Demography of patients and distribution of genetic alterations. Among 219 APA patients (95 men; 44.2%) who underwent adrenalectomy, the rate of somatic mutations was 58.4% (n = 128). Targeted sequencing for the reported mutations in APAs of KCNJ5, CACNA1D, ATP1A1, ATP2B3 and CTNNB1 exons was performed from the adenomas. CTNNB1 mutations were found in 8 of the 219 patients (3.7%) with APAs, of which there were 3 with S45F and 5 with S45P mutations. The detected mutations occurred in conserved serine/threonine residues in exon 3 (Table 1, Figures s2 and s3). The absence of CTNNB1 mutations in all peripheral blood DNA samples and in paired peri-tumoral adrenal cortices confirmed the somatic nature of the genetic alteration.

The majority of the somatic mutations were positive for KCNJ5 mutations (n = 116, 52.9%) (Figure s4). Sequencing of adenoma samples revealed the occurrence of the following somatic KCNJ5 mutations: p.Gly151Arg (c.451 G > A or c.451 G > C) (n = 64), p.Leu168Arg (c.503 T > G) (n = 48), p.Ile157del (c.470_472delTCA) (n = 1), and p.Thr158Ala (c.472 A > G) (n = 3) mutations in the heterozygous state (Fig. 1).

Besides the 128 somatic mutations, we also identified that there were two APA patients noted to have germ-line glucocorticoid remediable aldosteronism (GRA) mutations from two different families among the 219 APA patients investigated.

Enrolled group and matched group. We further studied the ‘enrolled group’, defined as those patients without mutated adenoma (WT group) and those with CTNNB1 or KCNJ5 mutations (n = 213). CTNNB1 mutation carriers were older (p < 0.001), had higher serum potassium and creatinine levels compared with those with KCNJ5 mutations. The duration of hypertension is shorter among CTNNB1 mutation carriers than KCNJ5 mutation carriers or WT APA patients (all p < 0.001). The majority of patients harboring CTNNB1 mutations were female (75%, n = 6), however the sex ratio is not significantly different from patients harboring KCNJ5 mutations or WT. Furthermore, the tumor size and ratio of parental hypertension were not significantly different among patients who had CTNNB1 mutations, KCNJ5 mutations or WT (Table 2).

Factors predicting post-operative residual hypertension. Hypertension was considered ‘cured’ in 144 (67.6%) of APA patients, defined as taking no antihypertensive agents at one year after adrenalectomy. Most (n = 109, 75.7%) cured patients became normotensive within 6 months after surgery; 27 patients (18.8%) became normotensive after 9 months, and 8 took up to 1 year (6.3%) (Table 3).

Compared with the KCNJ5 mutation carriers (12.5% vs. 79.3%, p < 0.001) and WT group (12.5% vs. 57.3%, p = 0.018), the CTNNB1 mutation carriers had a much lower chance of recovery from hypertension even after one-year follow-up. This result remained the same after matching — CTNNB1 mutation carriers had a significantly lower cure rate for hypertension (12.5% vs. 66.%, p = 0.011 compared with matched KCNJ5 mutation carriers; 12.5% vs. 54.2%, p = 0.047 and with matched WT patients).

Even after adjustment for possible variables when compared with all those who had KCNJ5 mutations, being a CTNNB1 mutation carrier was an independent factor to predict post-operative residual hypertension [odds ratio (OR) = 18.9, p = 0.010] (Table 2). When the CTNNB1 mutation carriers were compared with all WT APA patients, the chance of having residual hypertension was not significant (p = 0.051). However, after matching for the effects of age, sex and blood pressure, APA patients who had CTNNB1 mutations had significantly higher...
risk of post-operative residual hypertension than either KCNJ5-mutation carriers (OR = 18.2, \( p = 0.046 \)) or WT patients (OR = 14.5, \( p = 0.028 \)).

The final multiple logistic regression model had a high discriminatory power and fitted the observed binary data well before matching (adjusted generalized \( R^2 = 0.290 \) and Hosmer-Lemeshow goodness-of-fit (GOF) test \( p = 0.536 \)). After matching, the adjusted generalized \( R^2 = 0.564 \), and Hosmer-Lemeshow GOF test \( p = 0.620 \) which showed the model fitted with the data.

**mRNA and protein expressions in investigated APA.** The results of mRNA expression from real-time PCR showed that CTNNB1 mutated adenoma had similar density of CYP11B2 expression as WT patients; however, both groups of adenoma had less CYP11B2 expression than those with KCNJ5 mutations (all \( p < 0.01 \); Fig. 2a). However, there was no difference in CYP11B1 mRNA expression levels among the three groups. (Fig. 2b). VSNL1 and CYP11B2 are overexpressed in APAs compared with normal adrenals, especially in those with KCNJ5 mutation in Western blots (Fig. 3). Cyclin D1 expression was high in both CTNNB1 and KCNJ5 mutations. However, the GATA4 expression was not predominant in those with CTNNB1 mutation. CYP11B2 expressed diffusely on adenomas harboring CTNNB1 mutations and showed mottled staining on adenomas harboring KCNJ5 mutations (Fig. 4).

**Histologic expression of investigated APA.** Adenoma harboring CTNNB1 mutations displayed heterogeneous cytoplasmic, membranous and nuclear expression of active \( \beta \)-catenin. The CTNNB1 mutants displayed higher, diffuse active \( \beta \)-catenin expression than KCNJ5 mutation carriers or WT, especially in adenomas from female patients; and showed prominent nuclear staining. Adenomas with mutant CTNNB1 and all investigated adenomas revealed an unremarkable expression of GATA4.

GnRHR showed diffuse cytoplasmic, membranous and nuclear expressions on adenomas, and was especially enhanced in adenoma harboring CTNNB1 mutations from female patients. GnRHR was attenuated in KCNJ5 mutated adenomas. LHCGR was diffusely expressed in adrenal tissues and was prominent in adenoma harboring CTNNB1 mutations. The expression of GnRHR were non-specifically and diffusely stained both in areas with CYP11B1 and CYP11B2 expression (Fig. 4).

**Discussion**

About 3.7% (8 adenomas) of our 219 APA patients were found to harbor somatic CTNNB1 mutations, and their molecular expressions and clinical outcomes were reported. This low prevalence is similar to the 5.1% (10/189) reported in APAs39, but much lower than those reported in 15–26.9% of various types of adrenal adenomas and up to 30.8% of adrenocortical carcinomas20–31.

Of great interest, we found that patients who harbor CTNNB1 mutations had a higher likelihood of residual hypertension after adrenalectomy, when compared with wild-type APA patients or KCNJ5 mutation carriers. This is in contrast to the first case report of CTNNB1 mutation in a female APA patient who had her hypertension cured after adrenalectomy30. Although CTNNB1 mutation carriers had a shorter duration of hypertension, their average age was higher than the other groups. One of the possible explanations of the higher post-adrenalectomy residual hypertension among the patients who harbor CTNNB1 mutations could be that age-related essential hypertension played an important role in hypertension observed for these patients. Their shorter hypertensive latency and older ages could indicate that their hypertension is not only a reflection of the severity of excessive aldosterone related vascular remodeling. Several other mechanisms could explain residual hypertension after adrenalectomy, such as vascular damage, endothelial dysfunction, and arteriolosclerosis30,31. Age could give a high predictive power and represents a significant independent risk factor for effecting hypertension cure rate33.

![Figure 1. The distribution of known mutation of aldosterone producing adenoma in the cohort (n = 219).](image-url)
| CTNNB1 (n = 8) | Before matching | WT (n = 89) | p1 | After matching | WT (n = 24) | p3 |
|---------------|----------------|-------------|----|---------------|-------------|----|
| Gender, male (%) | 2 (25) | 48 (41.4) | 45 (50.6) | 0.302 | 0.155 | 7 (29.2) | 0.602 | 0.207 |
| Age (years) | 60.4 ± 8.7 | 46.4 ± 10.6 | 55.8 ± 11.1 | <0.001 | 0.261 | 56.1 ± 4.5 | 60.2 ± 6.0 | 0.078 | 0.946 |
| MBP (mmHg) | 101.1 ± 14.3 | 110.9 ± 20.3 | 107.6 ± 18.1 | 0.213 | 0.355 | 110.5 ± 13.0 | 105.7 ± 14.2 | 0.111 | 0.454 |
| Tumor size (cm) | 2.1 ± 1.2 | 1.7 ± 0.5 | 1.7 ± 0.7 | 0.076 | 0.162 | 1.7 ± 0.6 | 1.7 ± 0.7 | 0.072 | 0.185 |
| Duration of HTN (years) | 1.1 ± 2.1 | 5.9 ± 5.4 | 8.7 ± 8.7 | <0.001 | <0.001 | 10.2 ± 5.2 | 10.3 ± 8.1 | <0.001 | <0.001 |
| Family history of HTN (%) | 2 (25) | 57 (49.4) | 40 (44.9) | 0.278 | 0.676 | 14 (18.3) | 10 (41.7) | 0.220 | 0.676 |
| BMI (kg/m²) | 24.1 ± 3.5 | 25.2 ± 4.3 | 25.3 ± 0.9 | 0.515 | 0.450 | 24.7 ± 3.1 | 24.3 ± 2.8 | 0.675 | 0.888 |
| Diabetess (%) | 1 (12.5) | 11 (9.6) | 20 (22.5) | 0.571 | 0.448 | 3 (12.5) | 4 (16.7) | 0.705 | 0.633 |
| Proteinuria (%) | 5 (62.5) | 55 (47.4) | 35 (39.3) | 0.477 | 0.268 | 11 (45.8) | 9 (37.5) | 0.685 | 0.412 |
| Smoker (%) | 0 (0) | 19 (16.5) | 14 (15.7) | 0.250 | 0.273 | 5 (20.5) | 3 (12.5) | 0.211 | 0.408 |
| Serum K (mmol/L) | 3.9 ± 0.4 | 3.1 ± 0.6 | 3.7 ± 0.5 | <0.001 | 0.432 | 3.2 ± 0.6 | 3.7 ± 0.4 | 0.009 | 0.359 |
| Serum Cre (mg/dL) | 1.28 ± 1.20 | 0.9 ± 0.2 | 0.97 ± 0.35 | 0.007 | 0.081 | 0.95 ± 0.29 | 0.92 ± 0.19 | 0.223 | 0.155 |
| Phe | 7.41 ± 0.03 | 7.44 ± 0.04 | 7.41 ± 0.05 | 0.255 | 0.944 | 7.44 ± 0.03 | 7.40 ± 0.05 | 0.175 | 0.652 |
| HCO3 | 24.9 ± 3.3 | 25.8 ± 6.0 | 24.3 ± 4.3 | 0.165 | 0.581 | 25.1 ± 3.5 | 22.7 ± 3.6 | 0.365 | 0.929 |
| Log PAC (ng/dL) | 1.59 ± 0.35 | 1.73 ± 0.30 | 1.62 ± 0.30 | 0.197 | 0.807 | 1.70 ± 0.27 | 1.56 ± 0.25 | 0.371 | 0.817 |
| PRA (ng/mL/hr) | 0.29 ± 0.19 | 0.42 ± 0.77 | 0.98 ± 2.34 | 0.633 | 0.403 | 0.19 ± 0.12 | 0.11 ± 0.38 | 0.231 | 0.557 |
| Log ARR (ng/dL per (ng/mL/hr)) | 2.24 ± 0.50 | 2.56 ± 0.77 | 2.19 ± 0.75 | 0.248 | 0.829 | 2.68 ± 0.74 | 2.32 ± 0.83 | 0.136 | 0.823 |

Table 2. Clinical and biochemical characteristics of study patients during screening. *KCNJ5 vs CTNNB1. ||WT vs. CTNNB1. Abbreviations: ACEI/ARB, Angiotensin Converting Enzyme Inhibitors/Angiotensin Receptor Blockers. ARR, aldosterone to renin ratio (ng/dL per ng/mL/hr); APA, aldosterone producing adenoma; eGFR, estimated glomerular filtration rate; EH, essential hypertension; K, potassium; MBP, mean blood pressure; PAC, plasma aldosterone concentration; PRA, plasma renin activity. Data are shown as the mean values ± standard deviation. Note: To convert potassium in mmol/L to mEq/L, multiply by 1; PAC in ng/dL to nmol/L, multiply by 0.02774; PRA in ng/mL/hr to ng/L/s, multiply by 0.2778; ARC in ng/dL to pmol/L, multiply by 0.0361. 

Future studies are needed to determine the long-term cardio-vascular events in patients with or without mutations, especially focusing on the effects of variable somatic mutations.

Our study found that not only were CTNNB1 mutations more prevalent in women with APA29, but also that these women were diagnosed at their menopausal or postmenopausal ages. This is in contrast to a previous study, where female patients were identified during pregnancy or during childbearing age. We performed a literature systematic review of all CTNNB1 mutations in adrenal APAs and showed that out of 16 cases formerly reported and our 8 cases described herein, 75% of them were women.3,10,29,30,34.

In addition to confirming the previous reports which showed that APAs harboring CTNNB1 mutation could display CYP11B1 or CYP11B2 heterogeneous expression30, or in both CYP11B2-positive and CYP11B2-negative regions, but also existed in CYP11B1-positive areas. Diverse staining of CYP11B1 and CYP11B2 were previously documented only in 2 cases29 and here we further demonstrated these results in our 6 female cases, with a total of 8 cases to reinforce this finding. All these findings, together with the reported higher prevalence of CTNNB1 mutations among other adrenal adenomas60 and adrenal cancers66, suggest that CTNNB1 mutations may be more related to tumor cell growth (tumorigenesis), rather than to actual aldosterone production. It is also consistent with our result that Wnt/β-catenin pathway drives down-streamed cyclin D1 transcription, a gene involved in cell growth17 in adenomas with CTNNB1 mutations compared with wild-type APA adenomas.

We observed the existence of CTNNB1 mutations in APAs seemed mutually exclusive to the mutations in KCNJ5, ATP1A1 and ATP2B3. This might indicate the possibility that aberrantly activated β-catenin signals for adrenal tumor formation8. Recently, activated Wnt/β-catenin signaling has been reported in 70% of APAs and as a contributor to adrenal tumorogenesis6. In APAs harboring CTNNB1 mutations, the nuclear and/or cytoplasmic accumulation of active β-catenin protein increased, especially for female patients. It is proposed that CTNNB1 mutations stabilize β-catenin and increase the activity of the finely tuned Wnt signaling pathway, leading to tumor formation39. The positive nuclear β-catenin staining indicates the active components of Wnt/β-catenin signaling.


could lead to β-catenin protein accumulation. The accumulation of β-catenin protein and increased expression of cyclin D1, VSNL1 and the aberrantly active Wnt signaling could be involved in APA proliferation and anti-apoptosis.

Most of our CTNNB1 mutations were identified at S45P (62.5%), which is slightly lower than previously reported (80%)\(^{29}\). Phosphorylation with GSK-3 regulates β-catenin degradation and mutations with altered serine/threonine residues in the GSK-3 binding domain decrease β-catenin degradation\(^{39}\).

Although not statistically significant, there was a trend for the APA patients harboring CTNNB1 mutations to have larger tumor sizes, higher serum potassium and creatinine levels, which implicate that there could be other factors than just aldosterone alone to affect the underlying etiologies and severity of hypertension among these patients. This finding is different to a previous report where patients harboring CTNNB1 mutations had larger adenomas but did not have higher aldosterone compared to patients with no mutations\(^{29}\).

Constitutive activation of the Wnt signaling pathway in zona glomerulosa-like adenomatous cells could lead to de-differentiation toward their common adrenal-gonadal precursor cell type, and lead to aberrant expression of gonadal receptor LHCGR and/or GnRHR\(^{10}\). In a subset of non-pregnant PA patients from our cohort, the aberrant GnRHR was expressed and several of these patients had increased aldosterone secretion.
Figure 3. A representative western blot (30μg/well) for tissue lysate. The expression levels of ABC, CYP11B2, CyclinD1, VSNL1 and GATA4 in adenoma from patients harboring CTNNB1 mutation, KCNJ5 mutation, wild type and controlled adrenal gland were determined by western blot analysis. Abbreviations ABC, active β–catenin; APA, aldosterone producing adenoma, VSNL1, Visinin-like 1, WT, wild type.

Figure 4. Histologic expression in patients harboring CTNNB1 and KCNJ5 mutations and in WT patients. (A) H&E stain of investigated adenoma. The WT was from adrenal cortex. (B) CYP11B2 expressed diffusely on adenoma harboring CTNNB1 mutation and mottled expression on adenoma harboring KCNJ5 mutation. (C) However, CYP11B1 stating showed strong expression at there with low CYP11B2 expression. (D) Adenomas displayed weak nuclear and cytoplasmic active β–catenin expression, especially in female patients harboring CTNNB1 mutation. Bar, 50 mm (X40) and (E) in a high magnification view (X400). (F) LHGHR was diffusely expressed in the adrenal tissue and was also prominent in adenomas harboring CTNNB1 mutation. (G) GnRHR showed diffuse cytoplasmic, membranous and nuclear expression in adenomas, and was most enhanced in adenomas harboring CTNNB1 mutation or wild type. (H) The tissue expression of GATA4 in adrenal tissue was not significant. Abbreviations ABC, active β–catenin; GnRHR, gonadotropin-releasing hormone receptor, HE, hematoxyline and eosin. LHGHR, luteinizing hormone-chorionic gonadotropin receptor.
The GnRHR staining identified in normal and APA adrenal tissues in this study was consistent with previous reports. Although there was increased expression of GnRHR and LHCGR in wild-type APA patients and less in KCNJ5 mutation adenoma, we further showed patients harboring CTNNB1 mutations over-expressed GnRHR and LHCGR compared to adenomas with KCNJ5 mutation in both genders. Prior studies have reported the over-expression of GnRHR (55%) and LHCGR (41.7%) in APAs, however the status of CTNNB1 was not evaluated.

The over-expression of GnRHR and LHCGR in a high proportion of APAs is probably a consequence of events other than an activating mutation in CTNNB1. Interestingly, we observed that CTNNB1 mutated APAs with diffuse GnRHR expression occurred both in areas with CYP11B1 and CYP11B2 expression. As mentioned earlier, all six of our female patients with CTNNB1 mutated APAs were discovered either at menopause or postmenopausal ages. Although gonadotropin-releasing hormone, through stimulated GnRHR might regulate aldosterone production in rare cases of APA, most APA patients with CTNNB1 mutations were not identified during pregnancy.

Our findings also confirm that there is high prevalence of KCNJ5 mutations among APA patients in Taiwan, and the prevalence of APA tumors harboring other specific mutations (e.g. ATP, CACNA, CTNNB1) is considerably low. The current cohort and others from Asia (ranging from 59.5 to 76.8%) reported a higher prevalence of APAs harboring KCNJ5 somatic mutations (52.9% in this cohort). This finding differs to related reports from Western countries (ranging from 12.5 to 46.3% of KCNJ5 mutations among APAs), and might suggest the presence of certain genetic and epidemiological differences between Asian and Western populations.

There were some strengths and limitations to our study. Using the standard diagnostic implementation criteria and with patients possessing the same ethnic background, enrollment and sample collection for this study was standardized and unified across all participating centers. Under the standard methods among the hypertensive patients evaluated, the higher rate of KCNJ5 somatic mutations for APAs is unlikely to be related to differences in diagnosis and treatment methods.

Searching for further “sleeper or dormant” somatic mutations that are silent until triggered by some specific identifiable events could shed more light on the study of APAs in the future. Although unilateral adrenalectomy represents the treatment of choice for lateralized PA, further investigations on somatic mutations of APAs may disclose some interesting new drug targets for some subgroups of APAs not eligible or not amenable for surgery, especially in the area of higher prevalence of somatic mutation-carriers. Further studies to identify these somatic mutation patients without analysis of the tumor DNA specimen is challenging, but could save these patients from undergoing surgery with substantial long-term benefits.

Conclusions

In summary, we described CTNNB1 somatic mutation prevalence among our APA patients, along with its phenotype and clinical outcomes, and identified a female gender dominance and higher risk for post-adrenalectomy residual hypertension. APAs harboring CTNNB1 mutations have variable expressions of CYP11B1 and CYP11B2, and heterogeneous expressions of gonadal receptors. All these points suggest the possibility that CTNNB1 mutations in APAs may be more related to tumorigenesis rather than aldosterone production.

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Author Contributions
V.C.W., S.C.C., C.G.S. and R.C. wrote the main manuscript text K.D.W., Y.H.H., J.J.W. and Y.H.L conceived and designed the experiments. S.M.W, S.Y.Y., K.H.H., K.H.H., K.Y.P. collected the specimens.

Additional Information
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