Standard TAIPAI protocol and Aldosteronism Consensus in Taiwan

Patients were enrolled from the following hospitals:

This study included two medical centers (National Taiwan University Hospital (NTUH), Taipei, Taiwan; Taipei University Hospital, Taipei, Taiwan) and five regional hospitals (Cardinal Tien Hospital, New Taipei City, Taiwan; Taipei Tzu Chi Hospital, New Taipei City, Taiwan; Yun- Lin Branch of NTUH, Douliou City, Taiwan; Hsin-Chu Branch of NTUH, Hsin-Chu City, Taiwan; Zhongxing Branch of Taipei City Hospital, Taipei, Taiwan) (Wu et al., 2014).

Material and methods

Ethical approval (approval number 200611031R) was obtained from the institutional review board of the National Taiwan University Hospital. Written informed consent for clinical data collection and research use was obtained from all participants before enrollment in the study.

Adrenalectomy

Adrenalectomy was performed via lateral transperitoneal laparoscopic approach by experienced surgeons. Adrenal tumors removed via the surgery were fresh-frozen and stored at −80°C until further examination.

Our standard protocol to identify primary aldosteronism (PA) and functional lateralization:

The diagnosis of primary aldosteronism was established in hypertensive patients on the basis of the following criteria (Wu et al., 2009, Kuo et al., 2011, Wu et al., 2011a) (Fig S1):

Confirmation

Fulfillment of the following three conditions confirmed a diagnosis of PA:
(1) autonomous excess aldosterone production evidenced with an aldosterone-renin ratio (ARR) > 35; (2) a TAIPAI score larger than 60% (Wu et al., 2011b); (3) seated post-saline loading PAC > 16 ng/dL (Wu et al., 2019), or PAC/PRA > 35 (ng/dL)/(ng/mL/h) shown in a post-capotopril/losartan test (Wu et al., 2017b).

(Abbreviations: PAC, plasma aldosterone concentration; PRA, plasma renin activity).

The probability of PA (TAIPAI score) was equal to:

\[ P = 1 + e^{-\beta} \]

where \( \beta = (\text{PAC} [\text{ng/dl}] \times [0.063]) + \text{PRA} [\text{ng/ml/h}] \times [-0.205]) + ([\text{ARR} \times 0.001] \text{BMI} [\text{kg/m}^2] \times [0.067]) + (\text{Male} \times [-0.738] + \text{SK} [\text{mmol/l}] \times [-1.512]) + (\text{eGFR} [\text{ml/min/1.73 m}^2] \times [0.017]) + ([\text{propensity score}] \times [-0.539] + [1.851]) \) (Wu et al., 2011b)

Clinical APA

Clinical APA (aldosterone producing adenoma) was identified on the basis of the following four criteria (Wu et al., 2017b): (1) Confirmed PA; (2) an adrenal adenoma or hyperplasia evidenced with a CT or MRI scan; (3) lateralization of aldosterone secretion with adrenal vein sampling (AVS) or during dexamethasone suppression NP-59 SPECT/CT (Yen et al., 2009) on the side of the imaging finding; APA was further confirmed after adrenalectomy, and (4) pathologically proven CYP11B2-positive adenoma or
aldosterone-producing cell clusters at immunohistochemistry after adrenalectomy (Wu et al., 2017c), and subsequent emergence of biochemical correction (Wu et al., 2012, Kuo et al., 2011).

**Selectivity and lateralization indices of AVS**

The selectivity index (SI) is defined as the ratio of the sampled cortisol concentration of each adrenal vein to that of the peripheral vein. The lateralization index (LI) is defined as the ratio of the aldosterone/cortisol concentration on the dominant side to that on the contralateral side. Successful AVS is defined as an SI value ≥2.0 bilaterally. After confirming successful bilateral AVS, lateralization of the PA was determined by an LI value ≥2.0. (Chang et al., 2017)

**Measurements**

All anti-hypertensive medications were discontinued for at least 21 days before performing these tests, although diltiazem and/or doxazosin were used to manage high blood pressure when needed. Serum cortisol levels were measured via a chemiluminescent enzyme immunoassay (Archeitect, Abbott, VA, USA) and ACTH levels via an electrochemiluminescent immunoassay (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA, USA).

Plasma aldosterone concentration and plasma renin activity were measured using radioimmunoassay commercial kits manufactured by Biochem Immunosystems, Bologna, Italy, and Stillwater, MN, USA, respectively.

**KCNJ5 mutation**

DNA was purified from adrenal tumor tissue using a QIAamp DNA mini kit (Qiagen, Hilden, Germany). PCR was performed using Platinum Taq high fidelity (Invitrogen, Carlsbad, CA, USA) in a final volume of 50 µl. After DNase I treatment, 500 ng of total RNA were reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) and random hexamers (Promega, Madison, WI, USA). The entire coding sequence (exons 2–3) and flanking regions of KCNJ5 were amplified and sequenced using gene-specific primers. Direct sequencing of PCR products was performed using The BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, USA) with a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

**Tissue immunohistochemistry**

Immunohistochemistry was performed using mouse monoclonal antibody for CYP11B2, rat monoclonal antibody for CYP11B1, (generous gifts from Professor Celso Gomez-Sanchez(Gomez-Sanchez et al., 2014)) and polyclonal antibody for KCNJ5 (Atlas, Bromma, Sweden). Sections of paraffin-embedded adrenal tumor and surrounding tissues were stained using the polymerized horseradish peroxidase (HRP)-anti-mouse conjugate method (Novolink; Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) according to the manufacturers’ protocol. In brief, deparaffinization, antigen retrieval, blocking of endogenous peroxidase (Peroxidase Block, Novolink commercial kit), incubating with primary antibody, incubating with secondary antibody, application of horseradish peroxidase (HRP)-linked conjugate detection (Novolink Polymer, Novolink commercial kit), developing peroxidase activity and counterstaining
with Hematoxylin were conducted. Images were acquired using an Olympus BX51 fluorescence microscope with a built-in Olympus DP72 camera and processed using cellSens Standard 1.14 software (Olympus, Germany).

**H-score analysis**

All the staining areas of adenoma were judged by 2 investigators, KY Peng and S Wu, independently, and they were blinded to the KCNJ5 grouping when scoring, as well as blinded to patient outcomes throughout the analysis. CYP11B1 and CYP11B2 staining in APA specimens were categorized according to the intensity of staining in the tumor core; ranking from highly positive (3+), positive (2+), slightly positive (1+), and negative (0) and the percentage of area stained was calculated using an algorithm developed for the National Institutes of Health software Image J with IHC profiler plugin as previously described (Varghese et al., 2014). H-scores were calculated for each core by multiplying the intensity score by the percentage of core staining.

**Electrophysiology study**

Preparing for the electrophysiology study, the HEK293T cells (Chi et al., 2017) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, and incubated at 37 °C in a humidified atmosphere with 10% CO₂. A total of 2 μg of pIRES-green fluorescent protein (GFP) empty vector, pIRES-GFP-wild-type hKir3.4 or IRES-GFP-hKir 3.4-Gly387Arg plasmid mixed with Lipofectamine 2000 according to the manufacturer’s instructions were added to a 35-mm dish grown with HEK293T cells. Transfected cells were confirmed by visualization of GFP expression. Recordings were performed within 48 hours of the transfection.

The recording procedure was described previously (Chi et al., 2017). In brief, a cell was incubated in HBSS buffer (in mM, 137.9 NaCl, 5.33 KCl, 0.49 MgCl₂, 0.41 MgSO₄, 0.44 KH₂PO₄, 0.34 Na₂HPO₄, 4.17 NaHCO₃, 1.26 CaCl₂, 5.56 glucose, pH 7.2 with KOH, 300 mOsm/Kg) and patched in the whole-cell mode. The pipette solution consisted of (in mM) 120 K-Aspartate, 5 MgCl₂, 0.1 EGTA, 40 Hapes, 2 ATP, 0.3 GTP, pH 7.2 with KOH, 310 mOsm/kg. The recordings were performed at room temperature with an EPC-10 amplifier and were controlled by the Pulse program (HEKA Elektronik, Lambrecht/Pfalz, Germany). The membrane potential was held at 0 mV and step-depolarized to various potentials for 100 ms with an interval of 10 seconds, which is long enough to return the conformation of the channels to the state at 0 mV.

**Cell culture, transient transfection, ELISA and Western blot**

The HAC15 cells (Parmar et al., 2008) were cultured in DMEM/F12 medium (Gibco, Carlsbad, CA) as we previously described (Peng et al., 2018). The plasmids expressing the L168R mutation was constructed by introducing the indicated mutation into the wild-type KCNJ5 plasmid using PCR-assisted site-directed mutagenesis (Pfu DNA Polymerase, Thermo Scientific, Waltham, MA, USA). The successful introduction of the L168R mutation was confirmed by a PCR-based direct sequencing method. The HAC15 cells were transiently transfected with 2 μg pIRES-EGFP empty vector, pIRES-GFP-wild-type hKir3.4, IRES-GFP hKir 3.4-Gly387Arg, or IRES-GFP-hKir3.4-Leu168Arg. Transfections were performed by Lipofectamine and Plus reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Complementary DNAs encoding human Kir3.4 (GenBank accession number NM_000890) were used.

The transfected wild-type KCNJ5, G387R mutant, L168R mutant or pIRES-GFP empty vector HAC15
cells were homogenized with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM NaF, 1 mM Na3VO4), and protease inhibitor cocktail (Complete EDTA-free tablets; Roche Diagnostics, Mannheim, Germany). The medium of the cultured cells was collected for aldosterone detection. The concentrations of aldosterone were measured by radioimmunoassay (RIA) with commercial kits (ALDO-RIACT RIA kit, Cisbio Bioassays, Codolet, France). Aldosterone measurements were normalized using protein concentrations of cell lysates. In Western blot analysis, protein from whole cell extracts were separated through 10% SDS-PAGE gels and electrophoretically transferred to PVDF membranes. The membranes were then blocked by incubating in the blocking buffer (5% nonfat dry milk, 150 mM NaCl, 10 mM Tris–HCl, pH 7.5) for 1 hr and then incubated with blocking buffer containing various antibodies overnight at 4°C. Following extensive washing in Tris-buffered saline containing 0.1% Tween-20 (TBST) buffer, the transfer membranes were further incubated for 1.5 hours in 5% nonfat dry milk blotting buffer that contained HRP-conjugated secondary antibodies. Enhanced chemiluminescent reagent (Thermo Scientific) was applied in a 1:1 ratio. Proteins were normalized to GAPDH as indicated. Levels of proteins were detected using chemiluminescent detection reagents (Millipore) and visualized using a UVP Biospectrum 810 imaging system (Ultra Violet Products Ltd).

Figure legends of supplementary materials

Figure S1. The diagnostic and treatment protocol of TAIPAI. (Kuo et al., 2011, Wu et al., 2012, Wu et al., 2017a). Reprinted from Journal of the Formosan Medical Association, Volume 116/Issue 12, Vin-Cent Wu, Ya-Hui Hu, Leay Kiaw Er, Ruoh-Fang Yen, Chia-Hui Chang, Ya-Li Chang, Ching-Chu Lu, Chin-Chen Chang, Jui-Hsiang Lin, Yen-Hung Lin, Tzung-Dau Wang, Chih-Yuan Wang, Shih Te Tu, Shih-Chieh Jeff Chueh, Ching-Chung Chang, Fen-Yu Tseng, Kwan-Dun Wu, TAIPAI group, Case detection and diagnosis of primary aldosteronism - The consensus of Taiwan Society of Aldosteronism, Pages 993 to 1005, 2017, with permission from Elsevier.

Patients < 35 years with adrenal adenoma >1 cm can also receive AVS if clinically indicated.

Abbreviations: APA, aldosterone-producing adenomas; ARR, aldosterone-to-renin ratio; AVS, adrenal venous sampling; CT, computed tomography; NP-59- SPECT, I-131-6-beta-iodomethyl-19-norcholesterol single-photon emission computed tomography.

Figure S2. The EKG of six KCNJ5-G387R mutant patients

The EKG of six KCNJ5-G387R mutant patients did not show long QTc syndrome. The 6 APA patients were examined in detail by our cardiologist who concluded no evidence of long QTc syndrome.

Figure S3. The KCNJ5 mutation on G387R in adrenal adenoma and peripheral blood cells. (A) The guanine-to-cytosine substitution at nucleotide 1159 (c.1159 G>C) in the KCNJ5 gene was identified in 6 primary aldosteronism patients in adrenal tumors and peripheral blood cells.
Figure S4. The expression of CYP11B2 after administration of angiotensin II. Angiotensin II, an aldosterone secretagogue, was administered to transfected HAC15 cells. (A) The CYP11B2 production did not show a significant difference between the G387R mutant and wild type HAC 15 cells. KCNJ5 protein expressed in G387R mutant cells represented the successful transfection. (B) The aldosterone concentrations in the culture medium did not demonstrate a significant difference between the G387R mutant and wild type HAC 15 cells. The data was presented as the means ± SEM of three independent experiments.

Reference:


