Published in final edited form as:

Clin Endocrinol (Oxf). 2016 December ; 85(6): 845–851. doi:10.1111/cen.13132.

Functional characterization of two novel germline mutations of the *KCNJ5* gene in hypertensive patients without Primary Aldosteronism but with ACTH-dependent aldosterone hypersecretion

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Abstract

Background—Germline mutations of the *KCNJ5* gene encoding Kir3.4, a member of the inwardly rectifying K⁺ channel, have been identified in "normal" adrenal glands, patients with Familial Hyperaldosteronism (FH) type III, aldosterone-producing adenomas (APAs) and sporadic cases of primary aldosteronism (PA).

Objective—To present two novel *KCNJ5* gene mutations in hypertensive patients without PA, but with ACTH-dependent aldosterone hypersecretion.

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Disclosure Statement:

The authors have nothing to disclose.

Design and Patients—Two hypertensive patients without PA, who exhibited enhanced ACTH-dependent response of aldosterone secretion, underwent genetic testing for the presence of the *CYP11B1/CYP11B2* chimeric gene and *KCNJ5* gene mutations. Genomic DNA was isolated from peripheral white blood cells and the exons of the entire coding regions of the above genes were amplified and sequenced. Electrophysiological studies were performed to determine the effect of identified mutation(s) on the membrane reversal potentials. Structural biology studies were also carried out.

Results—Two novel germline heterozygous *KCNJ5* mutations, p.V259M and p.Y348N, were detected in the two subjects. Electrophysiological studies showed that the Y348N mutation resulted in significantly less negative reversal potentials, suggesting loss of ion selectivity, while the V259M mutation did not affect the Kir3.4 current. In the mutated structural biology model, the N348 mutant resulted in significant loss of the ability for hydrogen bonding, while the M259 mutant was capable of establishing weaker interactions. The *CYP11B1/CYP11B2* chimeric gene was not detected.

Conclusions—These findings expand on the clinical spectrum of phenotypes associated with *KCNJ5* mutations, and implicate these mutations in the pathogenesis of hypertension associated with increased aldosterone response to ACTH stimulation.

Keywords

KCNJ5 mutations; aldosterone; ACTH; hypertension

Introduction

Primary aldosteronism (PA) is the most common cause of secondary hypertension, with an estimated prevalence of up to 30% within the hypertensive population (1–4). Patients with inappropriate aldosterone secretion have PA (5). Early diagnosis and treatment is of fundamental importance, given that patients with PA have significantly higher incidence of stroke, myocardial infarction and atrial fibrillation than age-, sex-, and blood pressure (BP)-matched patients with essential hypertension (6, 7).

PA can be sporadic or familial. The sporadic forms include the bilateral adrenal hyperplasias (BAH) and aldosterone-producing adenomas (APAs), and together account for 90% of all cases of PA (6). The familial forms include Familial Hyperaldosteronism (FH) types I, II and III, which differ in clinical presentation and molecular basis (8). FH-I, also known as glucocorticoid-remediable aldosteronism (GRA), is an autosomal dominant condition characterized by early onset hypertension, elevated ACTH-dependent aldosterone concentrations, suppressed plasma renin activity and increased urinary excretion of the hybrid steroids 18-hydroxy-cortisol and 18-oxo-cortisol (9, 10). The molecular basis of FH-I is attributed to a chimeric *CYP11B1/CYP11B2* gene encoding a hybrid enzyme that synthesizes aldosterone throughout the *zona fasciculata*, under the control of ACTH (6, 9).

FH-III is an autosomal dominant condition characterized by early onset hypertension, non-glucocorticoid-remediable aldosteronism and hypokalemia. The clinical spectrum of the condition is broad, ranging from mild or moderate hypertension responsive to medical

therapy, to more severe and refractory to medical treatment hypertension that may require bilateral adrenalectomy (11–18). The molecular basis of FH-III is attributed to mutations in the *KCNJ5* gene, which encodes the selectivity filter of the G protein-activated inward rectifying potassium channel Kir3.4 (*GIRK4* or *KCNJ5*) (11–14). K^+ channels play a central role in regulating the membrane potential of glomerulosa cells. Most of the reported somatic and germline mutations are localized in the selectivity filter of the channel, the region of the channel pore that allows the specific transport of K^+ , and the exclusion of other causes. Mutations within the filter allow Na^+ to enter the mutation-bearing cells, depolarize the membrane and result in Ca^{2+} mobilization and consequent activation of the calcium signaling cascade, ultimately leading to increased aldosterone synthesis and cell proliferation (11, 19).

We have recently reported a cohort of patients with apparent essential hypertension without PA, who exhibited ACTH-dependent aldosterone hypersecretion, and responded to treatment with mineralocorticoid receptor antagonists (5). These findings indicated that chronic physical and/or psychological stress might sensitize adrenocortical cells to ACTH (5, 20). Genetic studies carried out in twenty-six patients of this cohort revealed two novel mutations of the *KCNJ5* gene in two subjects (5).

In this study we present the clinical manifestations of these two patients and the functional characterization of the two novel mutations of the *KCNJ5* gene.

Patients and Methods

Patient 1

The first patient was a 53 year-old man, who presented with hypertension (mean BP: 150/85 mmHg) while on treatment with nifedipine (Adalat CR 30 mg twice daily). His body mass index (BMI) was 27 kg/m² and his waist circumference 94 cm. Serum sodium (141 mEq/L) and potassium (4.4 mEq/L) concentrations were normal, while urinary potassium excretion was relatively high (96 mEq/24hr). Baseline aldosterone (ALD), renin (REN) and aldosterone to renin ratio (ARR) were 15.3 ng/dL (425 pmol/L), 27μU/mL and 0.58 ng/dL/u/mL (16 pmol/mIU), respectively. The patient underwent an ultra-low dose ACTH (0.03 μg) stimulation test and displayed an increased aldosterone response (Table 1). A computed tomography (CT) scan of the adrenal glands showed normal morphology. Following introduction of a mineralocorticoid-receptor antagonist (Eplerenone 100 mg daily), his BP normalized (129/83 mmHg). His father had hypertension diagnosed at the age of 45 years and died of myocardial infarction at the age of 62 years. He has two sons aged 25 years and 27 years, who do not have hypertension.

Patient 2

The second patient was a 59 year-old woman who presented with hypertension (mean BP: 160/90 mmHg) despite treatment with nebivolol (Nebivol hydrochloride 5mg daily). Her BMI was 33 kg/m² and her waist circumference 99 cm. Serum sodium (143 mEq/L) and potassium (4.1mEq/L) concentrations were normal, while urinary potassium excretion was relatively high (78mEq/24hr). Baseline ALD, REN and ARR were 12.9 ng/dL (357pmol/L),

7 $\mu\text{U/mL}$ and 1.8 ng/dL/ u/mL (51pmol/mIU), respectively. An ultra-low dose ACTH (0.03 μg) stimulation test showed an increased aldosterone response (Table 1), while a CT scan of the adrenal glands excluded adrenal hyperplasia. When treatment was changed to a mineralocorticoid- receptor antagonist (Aldactone 25 mg daily), her BP normalized (130/82 mmHg). Her mother had hypertension diagnosed at the age of 50 years and died at the age of 87 years because of renal failure secondary to diabetes mellitus type 2. Her 45 year old daughter is hypertensive but declined genetic testing.

The study was approved by the local Committee on the Ethics of Human Research. Written informed consent was obtained in both cases.

Genetic studies

Genomic DNA was isolated from peripheral white blood cells using the Maxwell 16 instrument for automated DNA extraction (Promega Corp., Madison, WI, USA) as previously described (13). The presence or absence of the *CYP11B1/CYP11B2* chimeric gene was tested by PCR amplification (21). The coding regions, exons 2 and 3 and their intron-exon junctions of the *KCNJ5* gene (NM_000890.3) were amplified by polymerase-chain reaction (PCR) and bidirectionally sequenced using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3100 sequencer (ABI 3100, Applied Biosystems) (13). The primers used for PCR amplification of the *KCNJ5* gene have been described previously (13).

The mutations detected were confirmed by sequencing on a second PCR product. To exclude the possibility that the mutations detected were polymorphisms, 149 random population samples were sequenced. Three *in silico* programs were used to predict the probable pathogenic effect of the amino acid changes: PolyPhen-2 (Polymorphism Phenotyping v2, (<http://genetics.bwh.harvard.edu/pph2>), SIFT (Sorting Intolerant From Tolerant, <http://sift.jcvi.org/>.) and Mutation Taster (<http://www.mutationtaster.org/>). The mutations detected are described according to the recommendations of the Human Genome Variation Society using the transcript NM_000890.3 as the reference sequence and they have been submitted to LOVD 3.0 database.

Plasmids

The plasmid expressing the wild-type Kir3.4 (*KCNJ5*) was kindly provided by Dr. Richard P. Lifton (Departments of Genetics and Internal Medicine, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06510, USA) (12). Specifically, for the wild-type construct, the *KCNJ5* gene was cloned into the BAMH1 site of the PIRE2-EGFP plasmid (Clontech Laboratories, Mountain View, CA, USA) (13). The plasmids expressing the *KCNJ5*^{Y348N} and the *KCNJ5*^{V259M} were constructed by introducing the indicated mutations into the wild-type Kir3.4 plasmid using PCR-assisted, site-directed mutagenesis (Stratagene, La Jolla, CA). The successful introduction of the two novel mutations, Y348N and V259M, into the wild-type construct was confirmed by Sanger sequencing.

Electrophysiology

Human embryonic kidney (HEK) 293T cells were cotransfected with either wild-type Kir3.4 (KCNJ5) (0.5 µg) or its mutants and Kir3.1 (KCNJ3) (0.5 µg) in 35 mm dishes using Fugene 6 (Roche Applied Science, Indianapolis, IN, USA). An empty vector was used as a control. Recordings were made 24 h after transfection. Coverslips containing transfected HEK 293T cells were submerged in the recording chamber and exposed to a continuous flow of ACSF consisting of the following: 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1.8 mM MgCl₂, 10 mM HEPES. ACSF was perfused with 95% O₂ and 5% CO₂. An infrared differential interference contrast (IR-DIC) videomicroscopy system (Zeiss Instruments, Oberkochen, Germany; Diagnostic Instruments, Sterling Heights, MI, USA) was used to visualize cells. Patch pipettes were pulled from thick-walled borosilicate glass (Warner Instruments, Hamden, CT, USA) to achieve a tip resistance of 2–5 MR. Pipettes were filled with an internal solution containing: 140 mM KCl, 5 mM HEPES, 1 mM EGTA, 1 mM CaCl₂ and 4 mM MgCl₂. Whole-cell voltage clamp recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) and Clampex 10.1 software (Molecular Devices, Sunnyvale, CA, USA). Signals were digitized at 10 kHz with a Digidata 1440A (Molecular Devices, Sunnyvale, CA, USA) and filtered at 4 kHz. Currents were elicited by delivering a second long pulse to –100 mV to +60 mV in 20 mV increments from a 0 mV holding. This protocol was repeated 3 times for each cell. Leak currents were recorded from empty vector controls and subtracted from each recording. A linear fit between averaged least negative and positive currents from each cell was used to estimate the reversal potential. All recordings were performed at room temperature. Recordings were analyzed using Clampfit 10.1 (Molecular Devices, Sunnyvale, CA, USA) and Microsoft Excel (Redmond, WA, USA).

Structural Biology Model Optimization and Evaluation

The model was initially subjected to energy minimization using the Gromacs-implemented force-fields to remove the geometrical strain (22, 23). It was subsequently solvated with Simple Point Charge (SPC) water using the truncated octahedron box extending to 7 Å from the model. Molecular dynamics were performed after that at 300K, 1 atm with 2 fs second step size, and for a total of ten nanoseconds, using the NVT ensemble in a canonical environment; NVT stands for Number of atoms, Volume and Temperature that remain constant throughout the simulation. The results of the molecular dynamics simulation were collected into a database by Gromacs, which can be subjected to further analysis (24). The resultant model was initially evaluated within the Gromacs package, version 4.5.5 (22, 23) by a residue packing quality function, which depends on the number of buried non-polar side chain groups and on hydrogen bonding. Moreover, the suite PROCHECK (25) was employed to further evaluate the quality of the produced model. Verify3D (26) was used to evaluate whether the model is similar to any known protein structures. The resultant models were visualized using PyMOL (27).

Results

Genetic studies

Sequencing of the *KCNJ5* gene in our patients revealed two novel germline heterozygous mutations. The first mutation (patient 1) was a single, heterozygous guanine to adenine (G → A) transition at nucleotide position 775 (c.775G>A) of the *KCNJ5* gene, resulting in valine (V) to methionine (M) substitution at amino acid position 259 (p.Val259Met) (Figure 1A). The second mutation (patient 2) was a single, heterozygous thymine to adenine (T → A) transversion at nucleotide position 1042 (c.1042T>A), which leads to tyrosine (Y) to asparagine (N) substitution at amino acid 348 (p. Tyr348Asn) (Figure 1B). These two mutations were not detected in 296 chromosomes, thereby excluding the possibility that they are common polymorphisms. It is important to note that both amino acids, V259 and Y348, are highly conserved across several species (Figure 2). *In silico* analysis of the amino acid changes detected revealed that both p.Val259Met (PolyPhen-2, SIFT) and p. Tyr348Asn (Poly-Phen 2, SIFT, Mutation Taster) mutations were predicted to be ‘damaging’. The *CYP11B1/CYP11B2* chimeric gene was not detected in our patients.

Electrophysiology

The properties of normal and mutant Kir3.4 channels were examined by co-expressing them with Kir3.1 in HEK 293T cells. In whole-cell voltage-clamp recordings, the currents were measured at voltages ranging between -100 mV and +60 mV. An inwardly rectifying current with a reversal potential of -27.7 ± 7.8 mV in the wild-type channels was observed. Similar to the previously reported mutations T158A, G151R, L168R and I157S (12, 13), the mutation Y348N showed greater rectification and a significantly less negative reversal potential of -1.3 ± 11.8 mV than the wild-type. The mutation V259M did not appear more rectified than the wild-type residue, as the reversal potential was not significantly different (-18.7 ± 10.5 mV) (Figure 3).

Structural Biology

In the mutated model, Valine is replaced by Methionine (p.Val259Met), whereas Tyrosine is replaced by Asparagine (p. Tyr348Asn). The wild-type Valine residue interacts with Gly265, Ile262 and Asn263 via a network of well-balanced hydrogen bonds. However, the Val259Met mutated model has lost its interaction to Ile262 completely. The Ile262 residue remains in close proximity to M259, but is not capable of establishing any form of interaction due to the restricting geometry of the Methionine residue (Figure 4A). The –SCH₃ moiety of the Methionine amino acid blocks the way by standing in the middle of the reactive –NH group of the M259 amino acid and the Ile262 residue.

The structural role of the Tyr348 amino acid is also very important, given that it supports its neighbouring residues via a strong hydrogen bond interaction between its –OH moiety and the Asp228 residue (Figure 4B). The Tyr348Asn mutated model reveals that the Asparagine residue at the 348 position is shorter, while it also lacks a reactive group at its end. The bulkier Tyrosine residue establishes hydrogen bonds with Asp352, Asn354 and Asp 228, whereas the less bulky Asparagine residue only interacts with Asp352 and Asn354. More specifically, Asp352 interacts with both –NH groups of the Asparagine residue, and this

results in a rather tilted conformation of the latter amino acid, which drives it away from Asp228, thereby preventing hydrogen bonding with it.

Discussion

We identified two novel germline heterozygous mutations of the *KCNJ5* gene, p.Val259Met and p. Tyr348Asn, in two patients with apparent essential hypertension without PA and radiologically “normal” adrenal glands, who exhibited ACTH-dependent aldosterone hypersecretion and responded to treatment with a mineralocorticoid receptor antagonist. It is likely that loss of ion selectivity caused by these mutations in Kir3.4 channel increased cell membrane depolarization and Ca^{2+} entry in the mutation-bearing adrenocortical cells, thereby increasing aldosterone synthesis and release. These findings expand on the clinical spectrum of phenotypes associated with *KCNJ5* gene mutations, and implicate mutations in the *KCNJ5* gene in the pathogenesis of hypertension characterized by an increased aldosterone response to ACTH stimulation.

Our electrophysiological studies revealed that the p.Tyr348Asn mutation showed less negative reversal potential than the wild-type, suggestive of loss of ion selectivity, whereas the p.Val259Met had a milder effect, despite the fact that the hormonal profiles of the two patients carrying these mutations were similar. These findings concur with previous studies that demonstrated striking variations in clinical phenotypes and clinical outcome, which result from different mutations of the *KCNJ5* gene or different mutations of the same amino acid in *KCNJ5* gene (12, 15), and may have implications for the diagnosis and management of PA with and without adrenal hyperplasia. Although the mutations detected in our study were found outside the selectivity filter, our functional studies demonstrated that they altered channel behavior and depolarized the cell membrane, thereby promoting aldosterone synthesis and release. It is also worth noting that these mutations are conserved among different species and were not detected in 296 chromosomes of the same population.

Our structural biology studies revealed that despite the fact that both amino acid positions Val259 and Tyr348 are found in unstructured loops of the protein, they are very important to its overall 3-dimensional (3D) arrangement. They both occupy space that is in very close proximity to a network of beta-sheet formations in the core domain of the *KCNJ5* protein (Figure 5), arranged in an antiparallel fashion in groups that contain four sheets each. The two sets of beta sheets are almost parallel to each other and their role is to structurally support the core domain of the *KCNJ5* protein. In amino acid position 259, the substitution of Valine (V) for Methionine (M) results in a bulkier, locally more unstable molecular system. The extra S and CH_3 groups of the Methionine residue hinder the reactivity of the –NH group of the same amino acid and block its interaction with Ile262. Therefore, the Met259 mutant is only capable of establishing weaker interactions than its wild-type Val259 counterpart. In contradistinction, the replacement of Tyrosine (Y) by Asparagine (N) at amino acid position 348 essentially represents a physicochemical change, since a polar amino acid with an uncharged side chain has occupied the place of the original wild-type Tyrosine residue. The interaction between Asparagine (N) 348 and Asparagine (N) 228 is no longer maintained by the much shorter Asparagine residue. This results in a very significant

loss of structural rigidity, given that the Y348 – N228 hydrogen bond plays a pivotal role in stabilizing the area adjacent to either sides of the 348 position (Figure 4B).

In our study, the two novel germline *KCNJ5* gene mutations were detected in patients with apparent essential hypertension, who did not have PA but exhibited an enhanced aldosterone response to ultra-low dose ACTH (0.03 µg) stimulation (5). It is likely that these mutations sensitized the mutation-bearing adrenocortical cells to ACTH stimulation, leading to aldosterone hypersecretion. Aldosterone is physiologically regulated by AngII, [K⁺] and ACTH. AngII binds to its type 1 receptor and stimulates a variety of signaling cascades, leading to the entry of extracellular Ca²⁺ into the cytoplasm of cells and consequent cell membrane depolarization (4). Membrane depolarization by either elevation of extracellular K⁺ or closure of K⁺ channels by AngII activates voltage-gated Ca²⁺ channels, and increases intracellular Ca²⁺ concentrations. The latter provide signals for increased expression of proteins required for aldosterone biosynthesis and release, including the steroidogenic acute regulatory (StAR) protein and aldosterone synthase (CYP11B2) (28–30).

Given that ACTH is a potent stimulus of aldosterone secretion (15), we hypothesized that repetitive acute and/or chronic physical and/or psychological stress might induce ACTH-mediated aldosterone hypersecretion and/or prime the adrenal cortex to secrete aldosterone in response to ACTH (5). It is, thus, likely that under conditions of stress, the action of ACTH is potentiated by the already increased intracellular Ca²⁺ concentrations due to the presence of sensitizing *KCNJ5* mutations, leading to aldosterone hypersecretion.

In summary, we present two novel, germline, heterozygous mutations of the *KCNJ5* gene in two hypertensive patients without PA and with “normal” adrenal glands, who exhibited ACTH-dependent aldosterone hypersecretion and responded to treatment with a mineralocorticoid receptor antagonist. Both mutations caused loss of ion selectivity in Kir3.4 channels and increased cell membrane depolarization. These findings expand on the clinical spectrum of phenotypes associated with *KCNJ5* mutations, and implicate mutations in the *KCNJ5* gene in the pathogenesis of hypertension associated with increased aldosterone response to ACTH stimulation.

Acknowledgments

A positive control DNA sample for the presence of the *CYP11B1/CYP11B2* hybrid gene was kindly provided by Dr Shengxin Xu from Prof RD Gordon.

This work was supported in part by the National and Kapodistrian University of Athens Medical School, Athens, Greece, and the intramural program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA.

References

1. Douma S, Petidis K, Doumas M, et al. Prevalence of primary hyperaldosteronism in resistant hypertension: a retrospective observational study. *Lancet*. 2008; 371:1921–6. [PubMed: 18539224]
2. Gouli A, Kaltsas G, Tzonou A, et al. High prevalence of autonomous aldosterone secretion among patients with essential hypertension. *European journal of clinical investigation*. 2011; 41:1227–36. [PubMed: 21534948]
3. Rossi GP, Pessina AC, Heagerty AM. Primary aldosteronism: an update on screening, diagnosis and treatment. *Journal of hypertension*. 2008; 26:613–21. [PubMed: 18327065]

4. Rossi GP, Seccia TM, Pessina AC. A diagnostic algorithm--the holy grail of primary aldosteronism. *Nature reviews Endocrinology*. 2011; 7:697–9.
5. Markou A, Sertedaki A, Kaltsas G, et al. Stress-induced Aldosterone Hyper-Secretion in a Substantial Subset of Patients With Essential Hypertension. *The Journal of clinical endocrinology and metabolism*. 2015; 100:2857–64. [PubMed: 25974737]
6. Funder JW, Carey RM, Fardella C, et al. Case detection, diagnosis, and treatment of patients with primary aldosteronism: an endocrine society clinical practice guideline. *The Journal of clinical endocrinology and metabolism*. 2008; 93:3266–81. [PubMed: 18552288]
7. Connell JM, MacKenzie SM, Freel EM, et al. A lifetime of aldosterone excess: long-term consequences of altered regulation of aldosterone production for cardiovascular function. *Endocrine reviews*. 2008; 29:133–54. [PubMed: 18292466]
8. Mulatero P, Tizzani D, Viola A, et al. Prevalence and characteristics of familial hyperaldosteronism: the PATOGEN study (Primary Aldosteronism in TORino-GENetic forms). *Hypertension*. 2011; 58:797–803. [PubMed: 21876069]
9. Lifton RP, Dluhy RG, Powers M, et al. Hereditary hypertension caused by chimaeric gene duplications and ectopic expression of aldosterone synthase. *Nature genetics*. 1992; 2:66–74. [PubMed: 1303253]
10. Halperin F, Dluhy RG. Glucocorticoid-remediable aldosteronism. *Endocrinology and metabolism clinics of North America*. 2011; 40:333–41. viii. [PubMed: 21565670]
11. Choi M, Scholl UI, Yue P, et al. K⁺ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science*. 2011; 331:768–72. [PubMed: 21311022]
12. Scholl UI, Nelson-Williams C, Yue P, et al. Hypertension with or without adrenal hyperplasia due to different inherited mutations in the potassium channel KCNJ5. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109:2533–8. [PubMed: 22308486]
13. Charmandari E, Sertedaki A, Kino T, et al. A novel point mutation in the KCNJ5 gene causing primary hyperaldosteronism and early-onset autosomal dominant hypertension. *The Journal of clinical endocrinology and metabolism*. 2012; 97:E1532–9. [PubMed: 22628607]
14. Xekouki P, Hatch MM, Lin L, et al. KCNJ5 mutations in the National Institutes of Health cohort of patients with primary hyperaldosteronism: an infrequent genetic cause of Conn's syndrome. *Endocrine-related cancer*. 2012; 19:255–60. [PubMed: 22323562]
15. Mulatero P, Monticone S, Rainey WE, et al. Role of KCNJ5 in familial and sporadic primary aldosteronism. *Nature reviews Endocrinology*. 2013; 9:104–12.
16. Mulatero P, Tauber P, Zennaro MC, et al. KCNJ5 mutations in European families with nonglucocorticoid remediable familial hyperaldosteronism. *Hypertension*. 2012; 59:235–40. [PubMed: 22203740]
17. Geller DS, Zhang J, Wisgerhof MV, et al. A novel form of human mendelian hypertension featuring nonglucocorticoid-remediable aldosteronism. *The Journal of clinical endocrinology and metabolism*. 2008; 93:3117–23. [PubMed: 18505761]
18. Mulatero P. A new form of hereditary primary aldosteronism: familial hyperaldosteronism type III. *The Journal of clinical endocrinology and metabolism*. 2008; 93:2972–4. [PubMed: 18685118]
19. Velarde-Miranda C, Gomez-Sanchez EP, Gomez-Sanchez CE. Regulation of aldosterone biosynthesis by the Kir3.4 (KCNJ5) potassium channel. *Clinical and experimental pharmacology & physiology*. 2013; 40:895–901. [PubMed: 23829355]
20. Rayfield EJ, Rose LI, Dluhy RG, et al. Aldosterone secretory and glucocorticoid excretory responses to alpha 1–24 ACTH (Cortrosyn) in sodium-depleted normal man. *The Journal of clinical endocrinology and metabolism*. 1973; 36:30–5. [PubMed: 4344308]
21. Jonsson JR, Klemm SA, Tunny TJ, et al. A new genetic test for familial hyperaldosteronism type I aids in the detection of curable hypertension. *Biochemical and biophysical research communications*. 1995; 207:565–71. [PubMed: 7864844]
22. Hess BKC, van der Spoel D, Lindahl E. GROMACS 4: Algorithms for Highly Efficient, Load-Balanced, and Scalable Molecular Simulation. *J Chem Theory Comput*. 2008; 4:435–47. [PubMed: 26620784]
23. Van Der Spoel D, Lindahl E, Hess B, et al. GROMACS: fast, flexible, and free. *Journal of computational chemistry*. 2005; 26:1701–18. [PubMed: 16211538]

24. Wang JCCP, Kollman PA. How well does a restrained electrostatic potential (resp) model perform in calculating conformational energies of organic and biological molecules. *Comput Chem Mol Model*. 2000; 21:1049–74.
25. Laskowski RA, Rullmannn JA, MacArthur MW, et al. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *Journal of biomolecular NMR*. 1996; 8:477–86. [PubMed: 9008363]
26. Eisenberg D, Luthy R, Bowie JU. VERIFY3D: assessment of protein models with three-dimensional profiles. *Methods in enzymology*. 1997; 277:396–404. [PubMed: 9379925]
27. De Lano, WL. *The PyMOL User's Manual*. San Carlos, CA, USA: DeLano Scientific; 2002.
28. Kojima I, Kojima K, Rasmussen H. Role of calcium and cAMP in the action of adrenocorticotropin on aldosterone secretion. *The Journal of biological chemistry*. 1985; 260:4248–56. [PubMed: 2579947]
29. Spat A, Hunyady L. Control of aldosterone secretion: a model for convergence in cellular signaling pathways. *Physiological reviews*. 2004; 84:489–539. [PubMed: 15044681]
30. Foster RH. Reciprocal influences between the signalling pathways regulating proliferation and steroidogenesis in adrenal glomerulosa cells. *Journal of molecular endocrinology*. 2004; 32:893–902. [PubMed: 15171720]

Figure 1.

Sequencing chromatograms showing the two novel mutations detected in the *KCNJ5* gene.

(**A**) G to A substitution at nucleotide 775 (c.775G>A), altering codon 259 from Valine to Methionine p.Val259Met, in exon 2. (**B**) T to A substitution at nucleotide 1042 (c.1042T>A), altering codon 348 from Tyrosine to Asparagine, p.Tyr348Asn in exon 3.

A

		258			
243	QTKEGEFIPLNQTDINVF	FDTGDDRLFLVSP	LIISHEINQKSPFW	MSQAQLHQEEFEV	302 P48544 IRK5_HUMAN
243	QTKEGEFIPLNQTDINVF	FDTGDDRLFLVSP	LIISHEINEKSPFW	MSRAQLQEEFEV	302 P48545 IRK5_MOUSE
243	QTKEGEFIPLNQTDINVF	FDTGDDRLFLVSP	LIISHEINEKSPFW	MSRAQLQEEFEV	302 P48548 IRK5_RAT
243	QTKEGEFIPLNQTDINVF	FDTGDDRLFLVSP	LIISHEINEKSPFW	MSRAQLNQEFEV	302 O02670 IRK5_PIG
254	QTKEGEFIPLNQTDINVF	FDTGDDRLFLVSP	LIICHEINEKSPFW	MSRAQLTQEFEV	313 F1MYR9 F1MYR9_BOVIN
243	QTKEGEFIPLNQTDINVF	FDTGDDRLFLVSP	LIISHEINEKSPFW	MSQAQLHQEEFEV	302 F7EPF6 F7EPF6_MACMU
243	QTKEGEFIPLNQTDINVF	FDTGDDRLFLVSP	LIISHEINEKSPFW	MSRAQLDQEFEV	302 E2RPY4 E2RPY4_CANFA

B

		348			
303	VILEGMVEATGTCQARSS	YMDTEVLWGH	RFTPVLTLE-KG	FYEVYNTFHD	TYETNTPS 361 P48544 IRK5_HUMAN
303	VILEGMVEATGTCQARSS	YMDTEVLWGH	RFTPVLTLE-KG	FYEVYNTFHD	TYETNTPS 361 P48545 IRK5_MOUSE
303	VILEGMVEATGTCQARSS	YMDTEVLWGH	RFTPVLTLE-KG	FYEVYNTFHD	TYETNTPS 361 P48548 IRK5_RAT
303	VILEGMVEATGTCQARSS	YMDTEVLWGH	RFTPVLTLE-KG	FYEVYNTFHD	TYETNTPS 361 O02670 IRK5_PIG3
14	VILEGMVEATGTCQARSS	YMDTEVLWGH	RFTPVLTLE-KG	FYEVYNTFHD	TYETNTPS 372 F1MYR9 F1MYR9_BOVIN
303	VILEGMVEATGTCQARSS	YMDTEVLWGH	RFTPVLTLE-KG	FYEVYNTFHD	TYETNTPS 361 F7EPF6 F7EPF6_MACMU
303	VILEGMVEATGTCQARSS	YMDTEVLWGH	RFTPVLTLE-KG	FYEVYNTFHD	TYETNTPS 361 E2RPY4 E2RPY4_CANFA

Figure 2.

Uniprot multiple alignment of the KCNJ5 protein sequence in different species, indicating conservation of the residues Val258 (A) and Tyr348 (B) affected by these mutations.

Figure 3.

Kir3.4 currents from HEK 293T cells expressing the *KCNJ5* wild-type (WT) channels and the two mutants *KCNJ5*^{V259M} and *KCNJ5*^{Y348N}. (A) Examples of current traces evoked by -100 to +60mV voltage steps (1s, 20mV increment; scale bars: 100pA/100ms). (B) *KCNJ5* mutations result in depolarization of current reversal potential. Reversal potentials are plotted for the wild-type (WT) Kir3.4(*KCNJ5*) channels and each Kir3.4 (*KCNJ5*) mutant. WT channels (black box) show a negative reversal potential. The mutant *KCNJ5*^{Y348N} (green box) shows significantly less negative reversal potentials compared with the WT channel ($P < 0.05$), suggesting a loss of ion selectivity. The mutant *KCNJ5*^{V259M} (orange box) does not affect the Kir3.4 current significantly. Numbers in parentheses represent the number of the cells recorded.

Figure 4.

Molecular interactions of the wild-type and the mutated model of KCNJ5 protein. **(A)** The p.Val259Met position, where only Valine is capable of hydrogen bonding to Ile262. **(B)** The p.Tyr348Asn position, where the less bulky Asparagine is not capable of hydrogen bonding to Asp228 as the wild-type Tyrosine does.

Figure 5. Superimposed snapshot of the wild-type (orange) and mutant (blue) model of the KCNJ5 protein. **(A)** The p.Val259Met mutation, where the $-CH_3$ moiety stands in the mid-space between the 259 residue position and the network of beta-sheets. **(B)** The p.Tyr348Asn mutation, where the Asparagine residue (N) is less bulky than the Tyrosine (Y) wild-type amino acid, thus preventing its hydrogen bonding interactions with amino acids in the nearby beta-sheets.

Table 1

Responses to ultra-low ACTH dose (0.03 µg) stimulation in the two patients

	0 min	15 min	30 min
Patient 1			
ACTH (pg/mL)	29		
Cortisol (µg/dL) [nmol/L]	11 [293]	26 [712]	25 [696]
Aldosterone (ng/dL) [pmol/L]	15.3 [425]	102 [2827]	57 [1584]
Renin (µU/mL)	27	26	24
ARR(ng/dL/ U/mL) [pmol/mIU]	0.58[16]	3.9 [109]	2.3 [66]
Patient 2			
ACTH (pg/mL)	27		
Cortisol (µg/dL) [nmol/L]	15 [401]	24 [658]	26 [719]
Aldosterone (ng/dL) [pmol/L]	12.9 [357]	53 [1480]	56 [1563]
Renin (µU/mL)	7	8	6
ARR (ng/dL/MU/mL) [pmol/mIU]	1.8 [51]	6.6 [353]	9.3 [261]