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See Editorial Commentary, pp XX–XX

Abstract—Primary aldosteronism is the most frequent cause of endocrine hypertension. Three forms of familial hyperaldosteronism (FH) have been described, named FH-I to -III. Recently, a mutation of *KCNJ5* has been shown to be associated with FH-III, whereas the cause of FH-II is still unknown. In this study we searched for mutations in *KCNJ5* in 46 patients from 21 families with FH, in which FH-I was excluded. We identified a new germline G151E mutation in 2 primary aldosteronism-affected subjects from an Italian family and 3 somatic mutations in aldosterone-producing adenomas, T158A described previously as a germline mutation associated with FH-III, and G151R and L168R both described as somatic mutations in aldosterone-producing adenoma. The phenotype of the family with the G151E mutation was remarkably milder compared with the previously described American family, in terms of both clinical and biochemical parameters. Furthermore, patients with somatic *KCNJ5* mutations displayed a phenotype indistinguishable from that of sporadic primary aldosteronism. The functional characterization of the effects of the G151E mutation in vitro showed a profound alteration of the channel function, with loss of K⁺ selectivity, Na⁺ influx, and membrane depolarization. These alterations have been postulated to be responsible for voltage gate Ca²⁺ channel activation, increase in cytosolic calcium, and stimulation of aldosterone production and adrenal cell proliferation. In conclusion, we describe herein a new mutation in the *KCNJ5* potassium channel associated with FH-III, responsible for marked alterations of channel function but associated with a mild clinical and hormonal phenotype. (*Hypertension*. 2012;59:00-00.) • Online Data Supplement

Key Words: familial hyperaldosteronism ■ endocrine hypertension ■ primary aldosteronism ■ aldosterone ■ *KCNJ5*

Primary aldosteronism (PA) is the most frequent cause of secondary hypertension, and its prevalence increases with blood pressure severity.¹ PA diagnosis is of paramount importance for the patient, not only because specific therapy is available, mineralocorticoid receptor antagonists for bilateral forms and adrenalectomy for unilateral forms,² but also because aldosterone causes detrimental effects on the cardiovascular system that are at least partly independent of blood pressure levels.^{3,4} Current guidelines of the Endocrine Society recommend the confirmation or exclusion of PA in all groups of patients with an increased risk of the disease,¹ which include hypertensive patients with a family history of early onset hypertension or cerebrovascular events at a young

age and all hypertensive first-degree relatives of patients with PA. These indications recognize the importance of testing for PA in patients at risk for familial hyperaldosteronism (FH). Three forms of FH have been described to date, named FH-I to -III.^{5–8} FH-I, also known as glucocorticoid-remediable aldosteronism (GRA), is attributed to a chimeric *CYP11B1/CYP11B2* gene encoding a hybrid enzyme able to synthesize aldosterone under corticotropin control.⁵ Most affected individuals develop severe hypertension in early life, and GRA families are prone to cerebrovascular events, although different degrees of severity and even normotension have been described.⁹ FH-I/GRA diagnosis is made by long-range PCR testing for the chimeric gene.

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Table 1. Clinical and Hormonal Parameters of the Population Studied

Parameters	Torino Families	Paris Families	Munich+Würzburg Families
Patients/families, n/n	27/12	10/5	9/4
Sex, male/female	15/12	4/6	7/2
SBP/DBP, mm Hg	156±19/97±13	150±20/97±12	182±17/107±9
Age at diagnosis of PA, y	50±13	38±11	50±11
sK ⁺ , meq·L ⁻¹	3.8±0.6	3.5±0.7	3.1±0.6
sAldosterone, ng·dL ⁻¹	28.5 (22.3–41.6)	28.8 (25.2–32.2)	16.8 (12–18)
PRA, ng·mL ⁻¹ ·h ⁻¹	0.2 (0.1–0.5)	0.1 (0.1–0.3)	...
DAR, pg·mL ⁻¹	...	5.5 (1.5–11.7)	2.5 (1.8–6.3)
Subtype, APA/BAH/UND	2/16/9	5/4/1	4/3/2

SBP/DBP indicates systolic/diastolic blood pressure; sK⁺, serum potassium; PRA, plasma renin activity; DAR, direct active renin; APA, aldosterone-producing adenoma; BAH, bilateral adrenal hyperplasia; UND, primary aldosteronism with undetermined subtype; DST, dexamethasone-suppression testing. To convert aldosterone to nmol/L multiply by 0.0277; to convert PRA to ng·L⁻¹·s⁻¹ multiply by 0.2778; to convert DAR to pmol/L multiply by 0.0237. Data are expressed as mean±SD for normally distributed parameters and as median (25th to 75th percentile) for the other parameters.

FH-II is a nonglucocorticoid-remediable form of PA, phenotypically indistinguishable from sporadic PA. The molecular basis of FH-II is still unknown, although a linkage with the chromosomal region 7p22 has been shown in some, but not all, FH-II families.^{6,10,11} FH-II diagnosis is made when PA diagnosis is confirmed in 2 members of the same family. Recently, the genetic cause of FH-III has been unraveled: this form is attributed to a mutation in the *KCNJ5* gene encoding the potassium channel Kir 3.4 (potassium inwardly rectifying channel, subfamily 1, member 5).¹² The mutation occurs near the selectivity filter for potassium resulting in increased sodium conductance and cell depolarization, which, in adrenal glomerulosa cells, produces opening of voltage-activated calcium channels with increased calcium signaling, followed by increased aldosterone production and cell proliferation.¹² Interestingly, somatic mutations in *KCNJ5* have also been shown in aldosterone-producing adenomas (APAs) from PA patients with severe hypertension and hypokalemia. So far, FH-III has been shown in a single family, affected by particularly severe hyperaldosteronism, resistant to aggressive pharmacotherapy and, therefore, requiring bilateral adrenalectomy. A characteristic feature of this family was the presence of particularly high levels of 18-hydroxycortisol and 18 oxo-cortisol and a paradoxical increase of aldosterone levels during dexamethasone-suppression testing (DST).⁷

The aim of our study was to search for the presence of mutations in *KCNJ5* in a sample of 21 European families with FH in which the presence of the chimeric gene responsible for FH-I/GRA was excluded.

Methods

An expanded Methods section is available in the online Data Supplement at <http://hyper.ahajournals.org>.

Diagnosis of PA and FH and Exclusion of FH-I/GRA

Torino

Diagnosis of PA and exclusion of FH-I/GRA were performed as described in detail elsewhere.^{13–15} Diagnosis of FH-II was made as

described in detail in the Primary Aldosteronism in Torino-Genetic Forms Study.¹⁶ A DST was performed only in patients carrying mutations in *KCNJ5*, as described previously.¹⁷

Paris

Diagnosis of PA was performed as described in detail elsewhere.^{18,19}

Munich and Würzburg

The diagnosis of PA was established as published previously.²⁰

Sequencing

DNA and RNA Isolation and RT-PCR

Genomic DNA was extracted from 46 patients with FH (27 from Torino, 10 from Paris, 5 from Munich, and 4 from Würzburg) and from 79 unaffected relatives (60 from Torino, 2 from Würzburg, 13 from Munich, and 4 from Paris). DNA or RNA was extracted from a total of 4 APAs (1 from Torino, 1 from Paris, and 2 from Würzburg).

KCNJ5 Sequencing

Sequencing primers were as described previously.¹²

Functional Study of the G151E Mutation

For functional expression in mammalian cells, specific cDNAs of human *KCNJ3*, *KCNJ5*, and mutant *KCNJ5*^{G151E} were purchased from Invitrogen/Geneart and subcloned into the pIRES-CD8 expression vector.²¹

Results

Identification of a Family Carrying a New Germline *KCNJ5* Mutation and 3 Subjects With Somatic Mutations

Twenty-seven PA patients (2 APA, 16 bilateral adrenal hyperplasia [BAH], and 9 undetermined) from 12 families identified in the Primary Aldosteronism in Torino-Genetic Forms Study¹⁶ were studied from Torino (60 unaffected relatives were available as controls). Ten PA patients (5 APA, 4 BAH, and 1 undetermined) from 5 families were studied from Paris (4 unaffected relatives were available as controls). Five PA patients (2 APA, 2 BAH, and 1 undetermined; 4 of 5 underwent adrenal vein sampling) from 2 families were studied from Munich (11 unaffected relatives were available as controls). Four PA patients (2 APA, 1 BAH, and 1 undetermined) from 2 families were studied from Würzburg (2 unaffected relatives were available as controls). Clinical

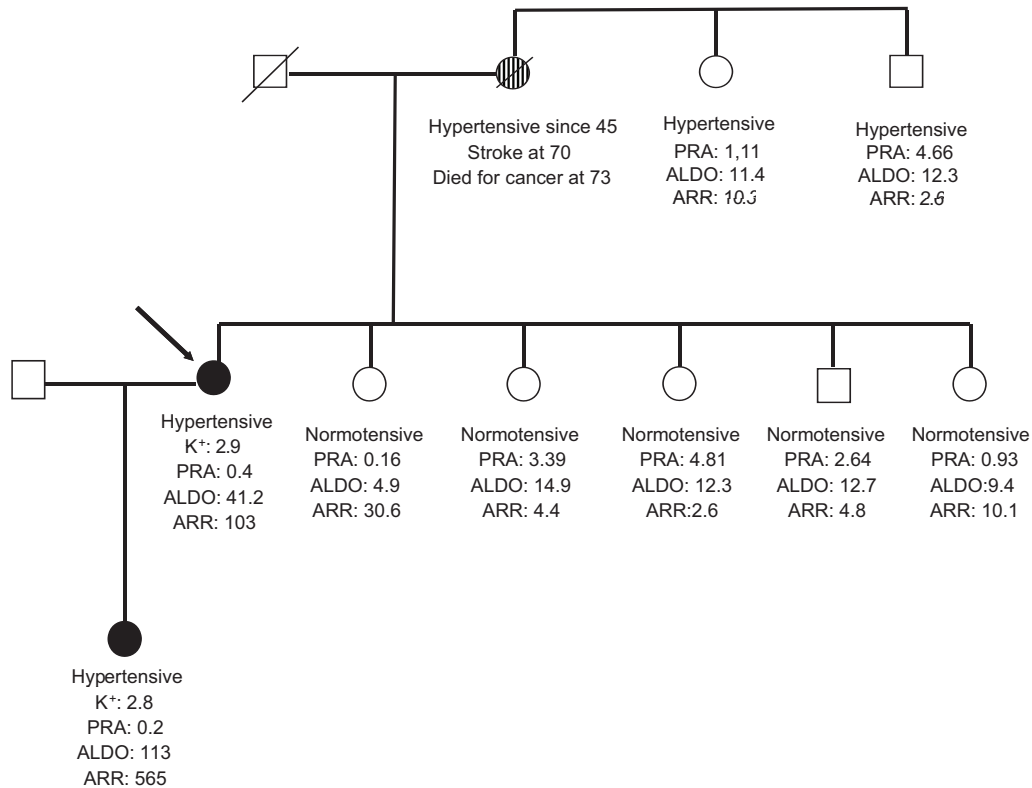


Figure 1. Family tree of the familial hyperaldosteronism (FH)-III family. Arrow indicates the index case. Black symbols indicate primary aldosteronism (PA) patients; circle with vertical lines indicate the mother of the index case, who was hypertensive since she was 45 years old and had a stroke at 70 years old. She died of cancer at 73 years of age. ALDO indicates aldosterone in $\text{ng} \cdot \text{dL}^{-1}$; PRA, plasma renin activity in $\text{ng} \cdot \text{mL}^{-1} \cdot \text{h}^{-1}$; ARR, ALDO/PRA ratio.

and biochemical parameters of PA patients are described in Table 1.

From a total of 21 families with FH we identified a novel heterozygous germline *KCNJ5* mutation (NM000890.3; c.452G>A [p.Gly151Glu]) in the 2 affected members of a single family (family 10 from Torino; Figure 1), which leads to the substitution of a glycine by glutamic acid. The mutation was absent in 7 investigated unaffected relatives (Figure 2A).

We also found 3 somatic APA mutations, 1 in a patient from Würzburg carrying the mutation c.451G>C/Gly151Arg and 1 in a patient from Paris carrying the mutation c.503T>G/p.Leu168Arg, both already described in APA patients,¹² and a third in a patient from Torino (family 7) carrying mutation

c.472A>G/Thr158Ala (Figure 2B). The latter mutation was described previously as a genomic mutation in the American family with FH-III.^{7,12} In all of the cases the mutations were absent in germline DNA from peripheral blood (Figure 2B).

Phenotype of the Family With FH-III

The index case is a white female, born in 1976. She had a history of polyuria in the first decade of life that disappeared during adolescence. She was found hypertensive in 1994 (18 years old); she was first seen in the Torino unit in 2003 when she was diagnosed with PA; she presented with high blood pressure (systolic/diastolic blood pressure: 190/115 mm Hg under doxazosin 4 mg + amlodipine 10 mg) and hypokalemia

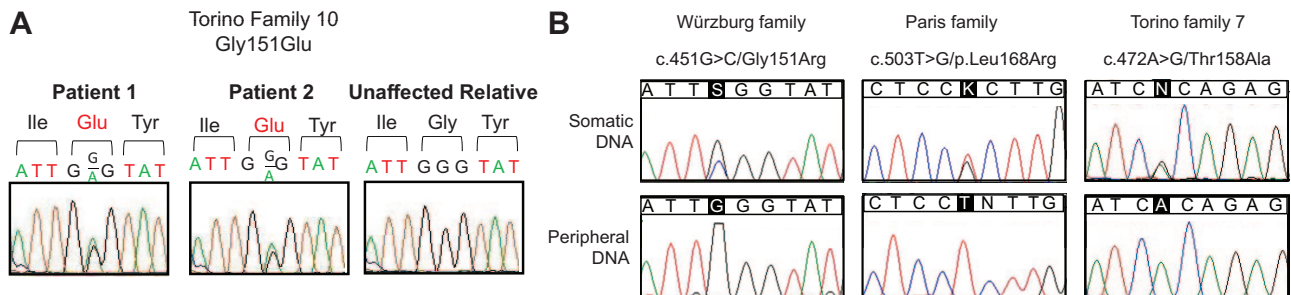


Figure 2. Mutations identified in patients with primary aldosteronism (PA). **A**, DNA sequencing showing the germline *KCNJ5* mutation G151E found in the 2 patients affected by PA in the familial hyperaldosteronism (FH)-III family and a normal sequence in 1 of the relatives. **B**, Chromatograms showing the 3 somatic *KCNJ5* mutations G151R, L168R, and T158A in aldosterone-producing adenomas (APAs) from the 3 PA patients from Würzburg, Paris, and Torino with apparent familial hyperaldosteronism described in the text and the paired normal sequences obtained on peripheral DNA.

Table 2. Clinical and Hormonal Parameters of Patients With KCNJ5 Mutations

Parameters	Torino Family 10 Index Case	Torino Family 10 Daughter	Torino Family 7 Index Case	Würzburg Family	Paris Family
Sex	Female	Female	Male	Male	Female
Age at diagnosis of hypertension, y	18	2	45	43	31
Age at diagnosis of PA, y	27	2	48	44	33
SBP/DBP, mm Hg	190/115	130/80*	155/95	190/110	132/88
sK ⁺ , mEq · L ⁻¹	2.9	2.8	3.5	3.9	3.4
Recumbent-upright sAldosterone, ng · dL ⁻¹	41.2/53.4	NA/113	26.5/23.5	16.8/NA	NA/21.9
Recumbent-upright PRA, ng · mL ⁻¹ · h ⁻¹	0.4/0.5	NA/0.1	0.3/0.3	1.6/NA†	1/NA†
Basal-postDST sAldosterone, ng · dL ⁻¹	76.2/75.4	100/104	NA	NA	NA
Basal-postDST SBP/DBP, mm Hg	135/85–133/82	132/91–131/94	NA	NA	NA
CT scanning	Normal adrenals	Normal adrenals	Right nodule 20 mm	Right nodule 16 mm‡	Left nodule 15 mm
u18OHF, µg · d ⁻¹	200	298	432	NA	NA
u18oxoF, µg · d ⁻¹	5.2	9.8	7.6	NA	NA

SBP/DBP indicates systolic/diastolic blood pressure; sK⁺, serum potassium; PRA, plasma renin activity; u18OHF, urinary 18-hydroxycortisol; u18oxoF, urinary 18-oxo-cortisol; NA, not available; DST, dexamethasone-suppression testing. To convert aldosterone to nmol/L multiply by 0.0277; to convert PRA to ng · L⁻¹ · s⁻¹ multiply by 0.2778; to convert 18OHF to µM/d multiply by 0.00265; to convert 18oxoF to µM/d multiply by 0.00266.

*The 99th percentile of blood pressure for age and height is 113/69 mm Hg.

†For these patients, direct active renin (pg · mL⁻¹) instead of PRA was measured.

‡For this patient, magnetic resonance instead of CT scanning was performed.

(serum potassium: 2.9 under potassium supplementation). Other clinical and hormonal parameters are summarized in Table 2. Long-PCR test for FH-I/GRA was negative, and hybrid steroid levels were within the normal range (Table 2) for patients with sporadic PA.¹⁶ CT scanning of the adrenal gland was normal. She refused to undergo adrenal vein sampling. At the last visit in July 2011, blood pressure was 130/85 mm Hg under candesartan 16 mg, nifedipine 60 mg, and spironolactone 25 mg, and serum potassium level was normal (4.0 meq/L, without supplements). Interestingly, the corrected QT was slightly prolonged (456 ms), despite the ECG being performed after correction of potassium levels.

A daughter was born in 2006 and referred to hospital for polyuria and polydipsia at 2 years of age. She was found hypertensive, hypokalemic, and severely hyperaldosteronemic (Table 2). She also displayed hypotonic urine (gravity: 1002 mg/dL) and hypercalciuria (urinary Ca²⁺/creatinine ratio: 0.92). 18-Hydroxycortisol level was in the range of PA patients, whereas 18-oxo-cortisol was slightly higher than in sporadic PA patients (Table 2) but much lower than FH-I/GRA patients and the reported FH-III family.^{7,8} Adrenal glands were normal at CT scanning. At her last visit at the end of 2010, she had normal blood pressure and potassium levels under canrenone (0.5 mg/kg per day)+amiloride (0.25 mg/kg per day). At variance with the American family with FH-III, both affected patients did not display an increase in blood pressure levels or a paradoxical increase in aldosterone levels during DST (Table 2).

Phenotype of the 3 Subjects With Somatic Mutations in KCNJ5

The patient from Torino (family 7) carrying the T158A somatic mutation is a male born in 1954. Diagnosis of hypertension was made in 1998 (45 years old); at this time

potassium was normal (4.4 meq/L), PRA was 0.3 ng/mL per hour, and aldosterone was 7.5 ng/dL. Blood pressure was 150/95 mm Hg when untreated and 135/85 mm Hg under fosinopril 20 mg. In 2002 (48 years old) he was referred to our unit for unprovoked mild hypokalemia, and diagnosis of PA attributed to a solitary APA was made (Table 2) by ARR, saline infusion, CT scanning, adrenal vein sampling, and postsurgery evaluation. Hybrid steroid levels were high but still in the range of patients with APA and much lower than FH-I/GRA and FH-III patients.

The patients from Paris and Würzburg carrying the L168R and G151R mutations, respectively, displayed phenotypes similar to those described previously by Choi et al¹² (Table 2). Clinical and hormonal parameters after adrenalectomy are described in Table S1 (available in the online Data Supplement).

Functional Effects of the G151E Mutation In Vitro

KCNJ5 is an inwardly rectifying potassium channel that can associate with other protein, for example, KCNJ3, to form heteromeric channels.¹² The G151E mutation profoundly changed the ion selectivity of the KCNJ5 potassium channel. HEK cells cotransfected with KCNJ3 and wild-type KCNJ5 were hyperpolarized under control conditions (-52 ± 6 mV [$n=8$] versus -42 ± 4 mV [$n=10$] in empty vector transfected cells) and exhibited a strongly inward rectifying K⁺ conductance. This inward current was strongly augmented by increases in extracellular K⁺ (Figure S1A and S1C). Ba²⁺ (5 mmol/L) strongly inhibited the inward whole cell current of transfected cells by 68%. In cells cotransfected with KCNJ3 and KCNJ5^{G151E}, the membrane under control conditions was strongly depolarized to -18 ± 1 mV ($n=9$). The whole cell current showed mild inward rectification, but it was unchanged when extracellular Na⁺ was exchanged by

K⁺ (Figure S1B and S1C). The depolarized membrane voltage and the unchanged current size during replacement of Na⁺ by K⁺ indicate that the mutated channel complex is no longer K⁺ selective but similarly permeable for Na⁺ and K⁺. Interestingly, Ba²⁺ had almost no effect on the mutant channel complex; the current was only reduced by 5%. However, when the Na⁺ was replaced by the larger cation NMDG⁺, the whole cell inward current was almost abolished and the membrane hyperpolarized in cells expressing the mutant channel complex. Thus, the electrophysiological data clearly demonstrate a loss of K⁺ selectivity when the wild-type KCNJ5 subunit is replaced by KCNJ5^{G151E}. Instead of a K⁺ selective pore, the mutant channel allows Na⁺ and K⁺ to pass through the membrane. Taken together, the properties of KCNJ5^{G151E} are very similar to those reported recently for other mutations of KCNJ5.¹²

Discussion

In this study we investigated the presence of *KCNJ5* mutations in 46 patients from 21 families considered previously as being affected by FH-II. Until 2011, only 1 form of FH had a recognized genetic cause, FH-I/GRA, in which a chimeric gene arising from a recombination between *CYP11B1* and *CYP11B2* is responsible for aldosterone production under corticotropin control.⁵ Families with 2 PA affected members but without the hybrid gene were considered as affected by FH-II.^{6,10,11,16} In 2008, Geller et al⁷ reported an American family with a peculiar phenotype characterized by extremely severe PA and hypertension, resistant to multiple drug therapy, thus requiring bilateral adrenalectomy. This condition, named FH-III,²² was also characterized by a massive production of the hybrid steroids 18-hydroxycortisol and 18 oxo-cortisol and a paradoxical increase of aldosterone and blood pressure levels after DST.⁷ Recently, Choi et al,¹² demonstrated that a germline T158A mutation in *KCNJ5* was responsible for the disease and that another 2 somatic mutations in the same gene (G151R and L168R) were frequent in sporadic APA.¹²

In this study we demonstrate the presence of a newly described germline mutation in *KCNJ5*, responsible for FH. Interestingly, the phenotype of the 2 affected members was much milder compared with the American family. In particular, blood pressure levels and hypokalemia were easily corrected with medical therapy, which included MR antagonists and amiloride. The adrenals appeared normal by CT scanning, and hybrid steroids were produced at a much lower rate, in the range of other patients with sporadic PA. Furthermore, blood pressure and aldosterone levels were not paradoxically increased during DST. Recently, a *KCNJ5* mutation (G387R) has been associated to long QT syndrome.²³ This mutation was associated with a decreased activity of the channel because of a reduced expression of the protein at the plasma membrane. Interestingly, our index case with G151E mutation also displayed a mildly prolonged QT.

In *KCNJ5*, G151 lies in the selectivity filter at a position conserved among most K⁺ channels.¹² In vitro studies of this mutation demonstrated a striking loss of K⁺ selectivity. The electrophysiological consequences of the G151E mutation investigated in the present study are thereby very much

reminiscent of the mutations described recently by Choi et al.¹² The G151E mutant channel was similarly permeable for Na⁺ and K⁺, resulting in depolarization of the plasma membrane and a continuous Na⁺ influx. In adrenocortical cells, such a depolarization would activate voltage-gated Ca²⁺ channels and disturb the K⁺ sensory function of glomerulosa cells. Even more dramatic are probably the cellular consequences of the Na⁺ influx: the rise of the cytosolic Na⁺ concentration leads to cell swelling and impairs elimination of Ca²⁺ via Na⁺/Ca²⁺ exchangers.²⁴ At very much depolarized membrane voltages and high intracellular Na⁺, the driving forces could be inverted and Na⁺/Ca²⁺ exchangers might even work in reverse mode, that is, import of Ca²⁺ instead of extrusion. Therefore, the novel *KCNJ5* mutation G151E is potentially capable of modifying the cell biology of adrenocortical cells profoundly by disturbing membrane voltage and Ca²⁺ homeostasis explaining autonomous hormone production and disturbed cell cycle control.

The phenotypic heterogeneity in FH-III recalls that observed for FH-I/GRA, where the same genetic defect results in markedly different phenotypes between affected families^{5,16,25} and also within the same family.²⁶ In line with this observation is the phenotype of the patient from Torino who carried a somatic mutation in the APA that correspond with the mutation (T158A) reported as being responsible for the severe phenotype in the American family. This patient displayed a phenotype indistinguishable from a patient with sporadic APA displaying hypertension grades 1 to 2. The observation in this study that 3 patients with apparent FH, treated by unilateral adrenalectomy, had *KCNJ5* mutations present only in the APA raises questions about the classification of these 2 families as affected by FH and, therefore, diagnosis of FH in families with only 2 PA affected members should be performed with some caution. Two situations are theoretically possible: first, the 6 subjects of the 3 families were in fact affected by sporadic PA, and the *KCNJ5* mutation was somatic and thus present only in the APA; this could be possible because the relatively high frequency of PA in the hypertensive population could result in a casual association of PA in 2 subjects of the same family; second, >1 genetic alteration is necessary to obtain a PA phenotype, and the 3 families display a genetic background predisposing to PA that developed into an APA only in patients carrying the *KCNJ5* mutations and in BAH in the other subjects possibly affected by another genetic alteration. The difficulty in studying adrenal samples from bilateral forms of FH makes this conundrum unresolved at present.

Perspectives

The genetic cause of FH type III has been demonstrated recently to be attributed to a mutation on *KCNJ5*, contributing to the understanding of the pathophysiology of genetic and sporadic hyperaldosteronism and possibly to hypertension in general. We have demonstrated a new mutation in the gene encoding the Kir 3.4 potassium channel, causing an in vitro phenotype similar to that described by Choi et al¹² but with a much milder clinical phenotype. Therefore, other patients with *KCNJ5* mutations may have been incorrectly classified as affected by FH-II, and, thus, it would be interesting to

investigate the presence of mutations in *KCNJ5* in other series of FH-II families. Future research will better define the clinical spectrum of alterations determined by *KCNJ5* mutations and the role of this channel in the pathophysiology of hypertension and PA.

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Disclosures

None.

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Online Supplement

KCNJ5 Mutations in European Families with Non-Glucocorticoid Remediable Familial Hyperaldosteronism

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Expanded Methods

Diagnosis of PA and FH and exclusion of FH-I/GRA

Torino

Diagnosis of PA was performed as described in detail elsewhere (1). Briefly, patients were screened using the aldosterone (PAC)/plasma renin activity (PRA) ratio (ARR) and PA was confirmed using the intravenous saline load test. All patients with confirmed PA underwent CT scanning and adrenal vein sampling (AVS). AVS was considered successful if the adrenal vein/inferior vena cava cortisol gradient was at least 3 (2) (or at least 2 before 2008); lateralization was considered when the aldosterone/cortisol ratio (A/C) from one adrenal was at least 4 times that of the other adrenal gland. A definitive diagnosis of APA was made as defined elsewhere (1). Diagnosis of FH-I/GRA was excluded by long-range PCR as described previously (3). Diagnosis of FH-II was made when at least two members of the same family displayed confirmed PA and a negative long-range PCR test for FH-I/GRA as described in detail in the PATOGEN study (4). Hormone measurements were performed as described previously (1). A dexamethasone suppression test (DST) was performed only in patients carrying mutations in KCNJ5, as described previously (5).

Paris

Diagnosis of PA was performed as described in detail elsewhere (6). Patients with hypokalemic hypertension or resistant hypertension were screened for PA that was considered present if ARR was at least 64 pmol/mU (107 pmol/ng) in two separate measurements and aldosterone concentration exceeded 500 pmol/l (18 ng/dl) or urinary aldosterone excretion exceeded 63 nmol/day (23 mcg/day). Two patients underwent a saline infusion test and five patients underwent an adrenal venous sampling. Thin-slice CT scanning and AVS for subtype differentiation were performed and interpreted as described before (6,7). Renin was measured as direct active renin by a direct radioimmunoassay (ERIA renin active Pasteur CT, reference 79970, Paris, France). Plasma aldosterone was measured by radioimmunoassay.

Munich and Würzburg

The diagnosis of PA, was established as published previously (8). In brief, screening was performed by measurement of the aldosterone to renin ratio (ARR, cut-off 12.1 ng/L), followed by confirmatory testing using saline infusion suppression. Medication known to influence the renin aldosterone ratio was stopped whenever possible or adjusted according current guidelines. The diagnosis of unilateral aldosterone excess, mainly aldosterone producing adenoma, was based on adrenal vein sampling (AVS). In short, blood was drawn consecutively from both adrenal veins without ACTH stimulation. A selectivity index of ≥ 2.0 of the cortisol concentrations within the adrenal vein compared to a simultaneously drawn peripheral sample was applied. Unilateral disease was assumed if the lateralisation index of the aldosterone to cortisol ratio was ≥ 4.0 of the dominant versus the non-dominant side. In patients without AVS (<5% since 2004) unilateral disease was assumed if computed tomography or magnetic resonance imaging of the adrenal glands demonstrated a unilateral nodular enlargement and a contralateral normal appearing adrenal gland. Plasma aldosterone levels were measured using the radioimmunoassay "Aldosterone Coat-a-Count" (Siemens Germany), and plasma direct renin levels by radioimmunoassay Renin III generation (Cisbio, Codolet, France).

Sequencing

DNA and RNA isolation and RT-PCR

Genomic DNA was extracted from 46 patients with FH (27 from Torino, 10 from Paris, 5 from Munich, 4 from Würzburg), and from 79 unaffected relatives (60 from Torino, 2 from Würzburg, 13 from Munich and 4 from Paris). DNA or RNA was extracted from a total of 4 APA (1 from Torino, 1 from Paris and 2 from Würzburg), Tumor DNA was extracted using QIAamp DNA midi kit (Qiagen); DNA from peripheral blood leukocytes was prepared using salt-extraction or using the Blood & Cell Culture DNA Kit (Qiagen). Total RNA was isolated from frozen tissue using Trizol

(Invitrogen, Carlsbad, Ca, USA) and then cleaned-up on silica columns using RNeasy Micro Kit (Qiagen). The integrity and quality of the RNA were systematically checked using an Agilent 2100 bioanalyzer with the RNA6000 Nano Assay (Agilent Technologies). After DNaseI treatment (Invitrogen), 500 ng of total RNA were retro-transcribed using Superscript II RT (Invitrogen) and random hexamers (Promega, Madison, WI, USA).

KCNJ5 sequencing

Coding sequence of genomic DNA was investigated by exome sequencing. The KCNJ5 coding sequence spanning amino acids 122 to 199 was amplified and sequenced as previously reported (9). PCR was performed on 100 ng of DNA (4 µl of RT product) in a final volume of 25 µl containing 0.75 mM MgCl₂ (without MgCl₂ for cDNA), 400 nM of each primer, 200 µM deoxynucleotide triphosphate and 1.25 U Platinum Taq DNA Polymerase (Invitrogen). Cycling conditions were as previously described with an annealing temperature of 58°C (56°C for cDNA). Direct sequencing of PCR products was performed using the ABI Prism Big Dye Terminator® v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Sequencing primers were as previously described (9).

Functional Study of the G151E Mutation

For functional expression in mammalian cells, specific cDNAs of human KCNJ3, KCNJ5 and mutant KCNJ5^{G151E} were purchased from Invitrogen/Geneart and subcloned into the pIRES-CD8 expression vector (10). For functional studies in HEK293 cells, wild type human KCNJ3 was cotransfected with wildtype KCNJ5 or with mutated KCNJ5^{G151E} using Lipofectamin. Patch clamp recordings were performed using an EPC-10 amplifier without leak subtraction (HEKA, Germany). The following solutions were used: control solution (10 mM HEPES pH 7.4, 140 mM NaCl, 5 mM KCl, 1.8 mM MgCl₂, 1.8 mM CaCl₂), 15 mM K⁺ solution (10 mM HEPES pH 7.4, 130 mM NaCl, 15 mM KCl, 1.8 mM MgCl₂, 1.8 mM CaCl₂), 50 mM K⁺ solution (10 mM HEPES pH 7.4, 95 mM NaCl, 50 mM KCl, 1.8 mM MgCl₂, 1.8 mM CaCl₂), Na⁺-free solution (10 mM HEPES pH 7.4, 5 mM KCl, 1.8 mM MgCl₂, 1.8 mM CaCl₂, 140 mM N-methyl-D-glucamine chloride (NMDG⁺)), pipette solution (5 mM HEPES pH 7.4, 140 mM KCl, 4 mM MgCl₂, 1 mM CaCl₂, 1 mM EGTA).

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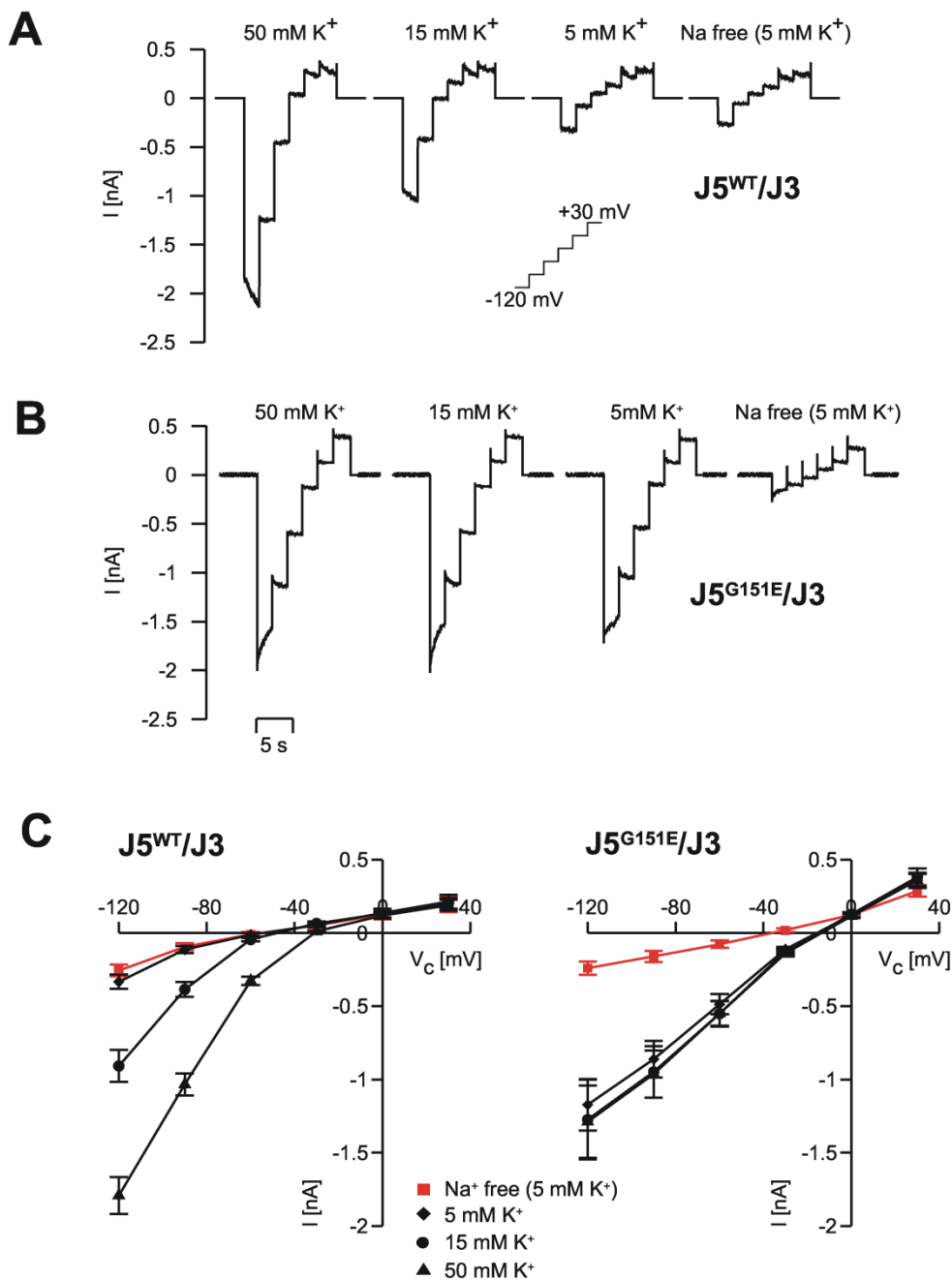
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Supplemental Table S1. Clinical and hormonal parameters of patients with somatic KCNJ5 mutations after adrenalectomy.

Parameters	TO Fam 7 Index Case	WU Fam	PA Fam
SBP/DBP (mmHg)	130/80	135/90	128/90
sK ⁺ (mEq L ⁻¹)	4.4	4.4	4.4
upright sAldosterone (ng dL ⁻¹)	7.5	3.5	9.6
upright PRA (ng mL ⁻¹ h ⁻¹)	0.3	-	-
DAR (pg mL ⁻¹)	-	18.8	19
hystology	single nodule 20 mm with normal surrounding cortex	21 mm nodule with surrounding micronodular hyperplasia	n.a.
number of drugs before/after surgery	1/1	3/0	1/0

SBP/DBP= systolic/diastolic blood pressure; sK⁺= serum potassium; PRA=plasma renin activity; DAR= direct active renin; n.a.= not available; to convert aldosterone to nmol/L multiply by 0.0277; to convert PRA to ng L⁻¹s⁻¹ multiply by 0.2778, to convert renin to pmol/L multiply by 0.0237.

Supplemental figure S1.



Legend to supplemental figure S1. Functional effects of the G151E mutation in HEK cell co-transfected with KCNJ5/KCNJ3 or KCNJ5^{G151E}/KCNJ3. (A) Typical patch clamp current traces of a KCNJ5/KCNJ3-transfected cell. A strong inward current was observed at 50 mM extracellular K^+ that was progressively decreased when extracellular K^+ was reduced. Replacement of Na^+ by the large cation NMDG⁺ had almost no effect on the whole cell current. (B) Typical patch clamp current traces of a KCNJ5^{G151E}/KCNJ3-transfected cell. The whole cell current was slightly inwardly rectifying and insensitive to reduction of K^+ from 50 to 5 mM. However, replacement of Na^+ by NMDG strongly reduced the whole cell current. (C) Summary of similar experiments. Please note that the I/V relationship of KCNJ5/KCNJ3 expressing cells ($n=8$) is strongly influenced by extracellular K^+ , but almost not modified, when Na^+ is replaced by NMDG⁺. In KCNJ5^{G151E}/KCNJ3 expressing cells ($n=9$), modification of extracellular K^+ has almost no effect. Na^+ replacement by

NMDG⁺, however, strongly reduces the current shifts the voltage at current = 0 to more hyperpolarized values.