Role for Germline Mutations and a Rare Coding Single Nucleotide Polymorphism Within the KCNJ5 Potassium Channel in a Large Cohort of Sporadic Cases of Primary Aldosteronism

Meena Murthy,* Shengxin Xu,* Gianmichele Massimo, Martin Wolley, Richard D. Gordon, Michael Stowasser, Kevin M. O’Shaughnessy

Abstract—Primary aldosteronism (autonomous aldosterone production with suppressed renin) plays an important pathophysiological role in what has been previously labeled as essential hypertension. Besides the recently described germline mutations in the KCNJ5 potassium channel associated with familial primary aldosteronism, somatic mutations in the same channel have been identified within aldosterone-producing adrenomas. In this study, we have resequenced the flanking and coding region of KCNJ5 in peripheral blood DNA from 251 white subjects with primary aldosteronism to look for rare variants that might be important for the pathophysiology of sporadic primary aldosteronism. We have identified 3 heterozygous missense mutations (R52H, E246K, and G247R) in the cohort and found that 12 (5% of the cohort) were carriers for the rare nonsynonymous single nucleotide polymorphism rs7102584 causing E282Q substitution of KCNJ5. By expressing the channels in Xenopus oocytes and human adrenal H295R cells, we have shown that the R52H, E246K, and E282Q substitutions are functional, but the G247R mutation is indistinguishable from wild type. Although the functional substitutions are remote from the selectivity filter, they affect the inward-rectification, the ability of the KCNJ5 channels to conduct Na+ currents and ATII-induced aldosterone release from the H295R cell line. Together these data suggest that germline variation in the KCNJ5 gene has a role to play in the common sporadic form as well as the much rarer syndromic forms of primary aldosteronism. (Hypertension. 2014;63:00-00.) • Online Data Supplement

Key Words: adrenal cortex ■ KCNJ5, potassium channel ■ mutation, missense ■ polymorphism, single nucleotide

Primary hyperaldosteronism (PA) is now recognized as a common, treatable, and potentially curable form of hypertension. In fact it may account for ≥40% of cases of what would previously have been labeled as essential hypertension.4,5 The excessive aldosterone production usually derives from either an aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia. The balance between these 2 pathologies varies; however, in most recently published series, bilateral adrenal hyperplasia is about twice as common as APA.3,4 Although the majority of PA is sporadic, there are monogenic familial forms of the condition (familial hyperaldosteronism types I, II, and III [FH-I, FH-II, and FH-III]). The molecular basis for FH-I, glucocorticoid-remediable aldosteronism, has been well understood for >2 decades and involves a recombination event that places the aldosterone synthase enzyme, CYP11B2, under the control of ACTH.5,6 In contrast, the molecular genetics for FHIII has been resolved recently with the discovery that mutations in the KCNJ5 potassium channel associated with familial primary aldosteronism, somatic mutations in the same channel have been identified within aldosterone-producing adrenomas. In this study, we have resequenced the flanking and coding region of KCNJ5 in peripheral blood DNA from 251 white subjects with primary aldosteronism to look for rare variants that might be important for the pathophysiology of sporadic primary aldosteronism. We have identified 3 heterozygous missense mutations (R52H, E246K, and G247R) in the cohort and found that 12 (5% of the cohort) were carriers for the rare nonsynonymous single nucleotide polymorphism rs7102584 causing E282Q substitution of KCNJ5. By expressing the channels in Xenopus oocytes and human adrenal H295R cells, we have shown that the R52H, E246K, and E282Q substitutions are functional, but the G247R mutation is indistinguishable from wild type. Although the functional substitutions are remote from the selectivity filter, they affect the inward-rectification, the ability of the KCNJ5 channels to conduct Na+ currents and ATII-induced aldosterone release from the H295R cell line. Together these data suggest that germline variation in the KCNJ5 gene has a role to play in the common sporadic form as well as the much rarer syndromic forms of primary aldosteronism.

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Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.113.02234
important in at least a subset of patients with sporadic PA. Hence, we have resequenced the coding region of KCNJ5 in a large cohort of patients with presumed sporadic PA and identified 3 missense mutations in the coding region. Despite being outside the selectivity filter, 2 of these mutations are functional when expressed in both Xenopus oocytes and the adrenal cell line H295R. We also noted carriers for a previously identified nonsynonymous SNP for KCNJ5, rs7102584 in our cohort. This SNP causes a charge-changing amino acid substitution (E282Q) in the pore region on the cytoplasmic side of the channel that is remote from the selectivity filter. Nevertheless, this substitution significantly alters channel function in vitro. Together these data suggest that germline missense mutations outside the selectivity filter of the KCNJ5 and a rare SNP variant of KCNJ5 channel are also important in the pathophysiology of PA.

Methods

KCNJ5 Resequencing

The subjects in this PA cohort were recruited (by M.S or R.D.G.) between 1999 and 2006 as referrals at either the Greenslopes or the Princess Alexandra Hospitals in Brisbane, Australia. Both are specialist endocrine hypertension referral centers and take patients from across Australian. Patients were identified based on baseline hypokalemia, a basal upright renin of <4 mU/L and an aldosterone:renin ratio of >150. The genetic study was approved by the Ethics Committee of University of Queensland, and informed consent for DNA collection and the genetic study was obtained from all participants. Genomic DNA was extracted using standard methods. The entire coding sequence (exons 2–3) and flanking regions of KCNJ5 were polymerase chain reaction amplified from this gDNA (see Table S1 in the online-only Data Supplement for details). The polymerase chain reaction products were purified and sequenced using an ABI 3730 DNA sequencer platform (PE Applied Biosystems).

Expression in Xenopus Oocytes

Xenopus laevis oocytes were harvested and defolliculated and cRNA synthesized as detailed previously.13–15 Briefly, cRNA (50 ng of KCNJ3+KCNJ5 wild type or the mutant forms) was injected in a total volume of 50 nL per oocyte as detailed elsewhere.15 Coinjection with 1 ng of KCNJ5 blocker (10 nmol/L).

Results

Expression in H295R Cells

Human adrenocortical carcinoma cells (NCI-H295R) were plated out and maintained as detailed previously.18 The wild-type (WT) human channel (containing the common variant of rs7102584, i.e., E282) was sourced in the commercial Coat-A-Count 125I-aldosterone radioimmunoassay from OriGene (Rockville, MD http://www.origene.com) and site-mutated as described previously.15 All plasmid constructs were confirmed by Sanger sequencing on a Beckman CEQ8000 platform. The plasmid constructs were transiently expressed in H295R cells by electroporation using the Nucleofector device and reagents from the Nucleofector kit R according to manufacturer instructions (http://www.Lonza.com). The transfection efficiency (typically 80% to 90%) was assessed using the green EGFP fluorescence signal measured by fluorescence-activated cell sorting (FACS Calibur machine, http://www.bdbiosciences.com). The percentage of transfected cells (GFP-positive cells) was then used to normalize aldosterone release from each well. Electroporated cells were plated onto 24-well plates for 24 hours, and then the medium was changed and they were grown on either in the presence or absence of ATIII (10 nmol/L) for a further 24 hours. The supernatants were then collected for aldosterone assay using 200 μL aliquots of the culture supernatant and the commercial Coat-A-Count 125I-aldosterone radioimmunoassay (http://www.healthcare.siemens.com/laboratory-diagnostics).

Cell Viability

Cell viability was measured using an MTT kit (http://www.milli-pore.com) according to manufacturer instructions. In brief, H295R cells (300,000 cells/well electroporated with different KCNJ5 mutant forms as above) were seeded into 96 well-plates and incubated at 37°C. The medium was changed after 24 hours and grown on for a further 24 hours before adding the MTT reagent and measuring the optical density at 540 nmol/L.

Channel Modeling

Ribbon diagrams to show the location of the KCNJ5 mutations were generated using the Discovery Studio Visualizer program (http://www.accelrys.com) as described previously.

Statistical Analysis

All data are presented as mean±SEM. Comparisons between groups was by ANOVA with post hoc testing using GraphPad/Prism 5 for Windows software. Statistical significance was set at P<0.05.

Results

KCNJ5 Resequencing

The entire coding region of KCNJ5 was resequenced in each of 251 patients with apparently sporadic florid PA. Although no mutations within the selectivity filter of KCNJ5 were detected, we identified 3 heterozygous missense mutations in KCNJ5 (Figure 1), one of which (E246K) was novel. The other 2 are present on the dbSNP database (R52H, rs144062083; G247R, rs2001706812) but are not validated. Neither appears in the 1000 genomes cohort, and R52H appears just once (1/22/1075) in the 1000 genomes cohort, and R52H appears just once (1/4296) in the National Heart and Lung Institute ESP6500, and G247R once (1/661) in the Clin-Seq-SNP cohorts, respectively. Another 12 subjects in the cohort were identified as carriers for the rare nonsynonymous SNP (population frequency of 2% [22/1075] in the 1000 genomes cohort), rs7102584, which causes a charge-changing substitution E282Q in KCNJ5. The phenotype of the rs7102584 carriers is similar to noncarriers (Table S1), although the values of their aldosterone:renin ratios clustered at the lower end of the distribution for the cohort (Figure S1). The subjects with the missense mutations were noticeably younger (Table S1).
Mutant KCNJ5 Channel Expression in Xenopus Oocytes

The mutant channels were expressed in oocytes where 2 of the missense mutations, and the coding SNP variant (E282Q) all significantly depolarized the oocytes in a high-Na\(^+\) bathing solution (Figure 2). In contrast, the G247R mutation did not alter the resting potential.

The steady-state current–voltage curves were generated with high-Na\(^+\) (92 mmol/L Na; 2 mmol/L K) with then high-K\(^+\) (45 mmol/L K; 0 mmol/L Na) in the bath solutions to look for evidence of altered Na\(^+\) permeability. The WT KCNJ5 shows typical inward-rectification in the high extracellular K medium; however, there are almost no measurable currents in the presence of high extracellular Na\(^+\) (Figure 3). Also as expected, the WT Ba-sensitive K\(^+\) current was almost completely blocked by the specific Kir channel inhibitor teretiapin-Q (Figure 3). The R52H channel in contrast showed no clear rectification, and the I-V curve was broadly unchanged by switching to a high extracellular Na\(^+\) solution confirming a substantial loss of K\(^+\) selectivity in this channel. The E246K mutation behaved similarly to R52H. However, the G247R mutation behaved like the WT channel with clear rectification, and almost no detectable current in the high-Na\(^+\) bathing solution (Figure S2). The E282Q channel behaved like the WT channel in the high extracellular K\(^+\); however, switching to the high-Na\(^+\) solution left the I-V essentially unchanged again, suggesting a substantial loss of K\(^+\) selectivity.

Impact of Mutants on Cell Viability of H295R Cells

To explore further a pathophysiological role for the KCNJ5 mutants, we transiently expressed the functionally active mutants and the E282Q SNP in H295R cells. After 48 hours of culture, all of the electrophysiologically active variants significantly reduced viability of the H295R cells (Figure S4). This reduced viability closely parallels that reported using the

![Figure 1. Sequence chromatograms for the 3 heterozygous missense mutations identified in the cohort.](image1)

![Figure 2. The resting membrane potential for Xenopus oocytes expressing either wild-type (WT) or one of the mutant KCNJ5 channels (mean±SEM; n=6). *Significantly different from WT, P<0.01.](image2)

![Figure 3. The steady-state current–voltage curves for Xenopus oocytes expressing wild-type or mutant KCNJ5 channels in high-Na\(^+\) and high-K\(^+\) solutions.](image3)
Figure 3. The current–voltage (I-V) plots for clamped Xenopus oocytes expressing one of the KCNJ5 variants as indicated were generated in the presence of high extracellular K⁺ (left) or high extracellular Na⁺ (right). Plots in the absence and presence of 10 nmol/L tertiapin-Q are shown, respectively, by ● and ○ (mean±SEM; n=6–8).
We have identified 3 heterozygous missense mutations (1 is novel) in the KCNJ5 channel within our PA cohort. They all occur in regions of the channel whose sequence is highly conserved in evolution from the fly to the human suggesting functional importance (Figure S5). Importantly, we have shown that of the mutations (R52H and E246K) affect the behavior of KCNJ5 in the Xenopus oocyte expression system and aldosterone release from the human H295R cell line. We have also noted carriers for the infrequent KCNJ5 SNP rs7102584 in some 5% of our cohort, (12/251). There are no previous data to suggest whether this SNP is functional or not; therefore, our demonstration that the Q282 channel variant encoded by this SNP does affect KCNJ5 behavior in vitro is intriguing and novel. Taken together, these findings represent the first report of sequence variations outside of the selectivity filter affecting KCNJ5 channel behavior and being associated with sporadic PA.

Concerning the 2 functional missense mutations (R52H and E246K), we have not to date identified other cases of PA within their respective kinships (Figure S6). Hence, at the present time, we cannot rule out that they may be unrecognized pedigrees with a familial form of hyperaldosteronism (FHI). In the R52H kinship, the allele is transmitted to 2 sons who show no current features of PA. It is well known that the CYP11B2 mutations causing another familial form of PA, FHI, are highly variable in their penetrance. Hence, we are currently unable to say whether our carrier frequency is significantly higher than the population the cohort is taken from. In terms of the carrier phenotype, the clustering of aldosterone:renin ratio values in the carriers (Figure S1) suggests that this SNP may be associated with less florid PA. This is certainly suggested by its behavior in H295R cells where it produces a smaller increase in ATII-induced aldosterone release compared with the missense mutations (Figure 4). However, ongoing work using the Cambridge Bioresource (with >12,500 participants) to study the aldosterone:renin ratio prospectively in a larger cohort of carriers versus noncarriers should be able to answer these questions.

The exonic SNP we have identified in 5% of our cohort, rs7102584, is of more interest because it is nonsynonymous (E282Q) and within a highly conserved motif. It has a reported population frequency of 2% in the 1000 genomes project; therefore, it seems that the carrier frequency may be higher in our cohort. However, because our cohort was selected on the basis of a florid phenotype, there may be a selection bias. We also do not have an accurate figure for its frequency in the white Australasian population. Hence, we are currently unable to say whether our carrier frequency is significantly higher than the population the cohort is taken from. In terms of the carrier phenotype, the clustering of aldosterone:renin ratio values in the carriers (Figure S1) suggests that this SNP may be associated with less florid PA. This is certainly suggested by its behavior in H295R cells where it produces a smaller increase in ATII-induced aldosterone release compared with the missense mutations (Figure 4). However, ongoing work using the Cambridge Bioresource (with >12,500 participants) to study the aldosterone:renin ratio prospectively in a larger cohort of carriers versus noncarriers should be able to answer these questions.

The human H295R cell line is a well-established model for the human zona glomerulosa cell, and it expresses the KCNJ5 channel. Expression of a typical selectivity mutant KCNJ5 channel in the H295R cell has been reported to depolarize the cell and enhance ATII-induced aldosterone synthesis and release. We saw the same increase in ATII-induced aldosterone with another selectivity mutant, delI157, and in both the E282Q SNP and the 2 missense mutations that had altered channel behavior in the oocytes (Figure 4). Expressing the KCNJ5 channels in the oocyte gave a similar picture. The missense mutations and the E282Q channel behaved like typical selectivity mutants and depolarized the oocytes in high-Na+ as we reported previously with the typical delI157 selectivity filter mutant.

All of the germline mutations associated with FH-III and the somatic mutations within aldosteronomas reported to date have been localized within the selectivity filter of KCNJ5. This is a small but important domain in the protein that is expressed extracellularly and is highly conserved across the
inward-rectifying K channels.24 The effect of amino acid substitution within it is well understood, and the PA-associated mutations in KCNJ5 typically cause a marked reduction in K+ selectivity.7,15 Hence the channels carry a significant inward Na+ current, which is thought to depolarize the zona glomerulosa cells triggering increased aldosterone synthesis and release. It is striking then that 2 of the KCNJ5 missense mutations we identified in our cohort and the SNP variant Q282delI157.15 Both in this earlier report and here (Figure 1), the discontinuous I-V plot typical of a Kir channel is converted to a linear ohmic resistor in these mutants.

Although the selectivity filter is the narrowest part of the channel and bears the important K signature (TXGYG), it is not the only determinant of selectivity in Kir channels.25,26 For example, channels mediating the pacemaker current (i_p) in the heart have this canonical signature but show almost no selectivity for K+ over Na+,27 and mutations in both the transmembrane helix (M2) and the cytoplasmic domains of Kir channels have been reported to affect selectivity.25,26 Although the mechanisms for these substitutions are still poorly understood, they are thought to involve electrostatic effects, which are relevant to the E282Q and E246K mutations both lose inward-rectification and selectivity because the 2 features often correlate.25 However, better structural data on the cytoplasmic side of the GIRK4 pore are needed to resolve how these substitutions might alter the structure of the pore because currently there is only crystallographic data from the closely related GIRK2 channel.29

Overall, our findings further highlight the importance of KCNJ5 potassium channel in the pathophysiology of PA and specifically for its common sporadic form. The missense mutations and rare SNP variant we have reported in this study emphasize that the molecular genetics of PA is an unfinished story. Further work is needed to establish whether other SNP variants in KCNJ5 also have a role to play in PA and what impact germline variation in KCNJ5 might have on subjects with APs carrying somatic mutations in the same gene.

Perspective
PA is the cause of hypertension for a significant minority of patients. Its importance lies in the diagnosis offering the prospect of a curable form of hypertension. The molecular basis for rare syndromic forms of PA and the somatic mutations that may be driving sporadic adenoma formation is evolving rapidly. The role of KCNJ5 for the common sporadic form of PA is less well understood. In a cohort of 251 patients with sporadic PA, we identified 2 cases with functional heterozygous missense mutations in KCNJ5 (R52H and E236K). These mutations are distinct from the mutations previously identified in the selectivity filter of KCNJ5 in syndromic forms of PA or as somatic mutations in sporadic APs. Another 12 patients were carriers for a rare functional SNP variant of the KCNJ5 gene, rs7102584, that produces an E282Q amino acid substitution in the channel. We have shown that despite their location in the channel the functional behavior of these variants is similar to the selectivity mutants previously reported. These data suggest that mutant germline KCNJ5 channels are present in some apparently sporadic cases of PA. The clinical implications of this are unclear. It may be that they have a distinct phenotype or that PA patients with an adenoma and a germline mutation are less likely to have a long-term cure from unilateral adrenalectomy. Also the frequency of the functional SNP, rs7102584, could provide a germline basis for a significant minority of sporadic cases (≈5%). If it turns out that these germline mutations do affect the way we manage sporadic PA patients, then it will be necessary to routinely screen for them as part of their clinical workup.

Sources of Funding
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Disclosures
None.

References


Novelty and Significance

**What Is New?**

- Germline mutations in the K channel KCNJ5 are present in rare syndromic forms of primary aldosteronism (PA), and somatic mutations in the same gene are found in ~40% of aldosterone-secreting adenomas. We have found rare germline mutations in KCNJ5 in patients who do not seem to have the syndromic form of PA, whereas others are carriers for a rare coding single nucleotide polymorphism in KCNJ5.
- In short, we can say that sporadic cases of PA may occasionally have germline mutations in KCNJ5 or be carriers for a rare single nucleotide polymorphism variant of KCNJ5 (E282Q).
- These variants are functional and the mutations are novel because they involve a different part of the channel to the ones previously described in PA.

**What Is Relevant?**

- PA is present in ≤10% of patients who might previously have been labeled as having essential hypertension. Its pathophysiology is still unclear but at least in a substantial minority of patients it is driven by abnormal function of the KCNJ5 channel in the adrenal gland activating aldosterone secretion.

**Summary**

Patients with apparent sporadic PA occasionally harbor rare mutations in the KCNJ5 gene or are carriers for a rare coding single nucleotide polymorphism in KCNJ5. Both of these germline variations affect regions the KCNJ5 gene outside the selectivity filter where previous mutations in KCNJ5 were clustered.
Role for Germline Mutations and a Rare Coding Single Nucleotide Polymorphism Within the KCNJ5 Potassium Channel in a Large Cohort of Sporadic Cases of Primary Aldosteronism

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A role for germ-line mutations and a rare coding SNP within the KCNJ5 potassium channel in a large cohort of sporadic cases of primary aldosteronism.

Meena Murthy, †Shengxin Xu, Gianmichele Massimo, †Martin Wolley, †Richard D Gordon, †Michael Stowasser and Kevin M O'Shaughnessy.

*Clinical Pharmacology Unit, Department of Medicine, University of Cambridge, Addenbrooke’s Hospital, Cambridge, UK and †Endocrine Hypertension Research Centre, University of Queensland School of Medicine, Brisbane, Australia.*
Table S1. PCR* primers used to amplify the coding and flanking regions of hKCNJ5

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* PCR was performed on 25ng of gDNA in a final volume of 30 µL containing 1.5 mM MgCl2, 0.2 mM of each primer, 0.2 mM deoxynucleotide triphosphate and 1.0 unit of Taq DNA Polymerase (Life Technologies Corporation, Australia).

Table S2. Clinical phenotype of the PA cohort by mutation and rs7102584 genotype

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<th>Sex (F/M)</th>
<th>Age at diagnosis</th>
<th>Aldo (pmol/l)</th>
<th>Renin (mU/L)</th>
<th>ARR</th>
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<td>476±244</td>
<td>4.3±5.3</td>
<td>134±67</td>
<td>3/9</td>
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</table>

NB Aldosterone, renin and ARR are taken on the 1st day of a fludrocortisone suppression test

*Final diagnosis (APA, aldosterone-producing adenoma or BAH, bilateral adrenal hyperplasia) was based on the appearances of the adrenal CT scan and results of adrenal vein sampling (AVS). The AVS results for the index cases with missense mutations are shown below (the cut-off used for lateralisation is >4):

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<th>R cort</th>
<th>R Ratio</th>
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<td>3050</td>
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Figure S1. Plot of individual log ARR values for non-carriers and carriers for the rs7102584 SNP and each missense mutation.

Figure S2. Current-voltage (I-V) plots for clamped Xenopus oocytes expressing G247R KCNJ5 variant were generated in the presence of high extracellular K⁺ (left) or high extracellular Na⁺ (right). Plots on the left-hand figure in the absence and presence of 10 nmol/L tertiapine-Q are shown respectively by ● and ○ (mean ±sem, n=6-8).
Figure S3. The basal and ATII (10 nmol/l)-induced aldosterone release from H295R cells transiently transfected with either the control (WT), G247R or somatic mutant dell157 KCNJ5 channel as indicated (n=6). * P<0.01 versus control.

Figure S4. Cell viability of H295R cells 48 hours after transfection with one of the electrophysiologically active variants as indicted. Results are expressed as fold changes in the OD of the MTT assay product versus control. * P<0.05 versus WT or empty vector control.
### SUPPLEMENTAL MATERIAL

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Figure S5. Protein alignments for KCNJ5 homologues across species.
**SUPPLEMENTAL MATERIAL**

**E246K Pedigree**

- ARR: 290
- Renin: 1.3
- Aldo: 365

**R52H Pedigree**

- ARR: 28
- Renin: 10
- Aldo: 285

- ARR: 8
- Renin: 23
- Aldo: 178

Figure S6. Pedigrees for the E246K and R52H index cases. Affecteds (index cases) are infilled in black and where DNA is available for other members the border colour shows the genotype: missense mutation present in red and WT in green.
Figure S7. Picture showing a ribbon diagram for the KCNJ5 channel seen from below with the sites of the mutations and rs7102584 SNP indicated.
Figure S8. Picture showing a ribbon diagram for the KCNJ5 channel seen from the side with the sites of the mutations and rs7102584 SNP indicated and the location of the selectivity filter mutations in KCNJ5.