Effect of *KCNJ5* Mutations on Gene Expression in Aldosterone-Producing Adenomas and Adrenocortical Cells

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Context: Primary aldosteronism is a heterogeneous disease that includes both sporadic and familial forms. A point mutation in the *KCNJ5* gene is responsible for familial hyperaldosteronism type III. Somatic mutations in *KCNJ5* also occur in sporadic aldosterone producing adenomas (APA).

Objective: The objective of the study was to define the effect of the *KCNJ5* mutations on gene expression and aldosterone production using APA tissue and human adrenocortical cells.

Methods: A microarray analysis was used to compare the transcriptome profiles of female-derived APA samples with and without *KCNJ5* mutations and HAC15 adrenal cells overexpressing either mutated or wild-type *KCNJ5*. Real-time PCR validated a set of differentially expressed genes. Immunohistochemical staining localized the KCNJ5 expression in normal adrenals and APA.

Results: We report a 38% (18 of 47) prevalence of *KCNJ5* mutations in APA. KCNJ5 immunostaining was highest in the zona glomerulosa of NA and heterogeneous in APA tissue, and KCNJ5 mRNA was 4-fold higher in APA compared with normal adrenals ($P < 0.05$). APA with and without *KCNJ5* mutations displayed slightly different gene expression patterns, notably the aldosterone synthase gene (*CYP11B2*) was more highly expressed in APA with *KCNJ5* mutations. Overexpression of *KCNJ5* mutations in HAC15 increased aldosterone production and altered expression of 36 genes by greater than 2.5-fold ($P < 0.05$). Real-time PCR confirmed increases in *CYP11B2* and its transcriptional regulator, *NR4A2*.

Conclusions: *KCNJ5* mutations are prevalent in APA, and our data suggest that these mutations increase expression of *CYP11B2* and *NR4A2*, thus increasing aldosterone production. **(*J Clin Endocrinol Metab* 97: E1567–E1572, 2012)**

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Abbreviations: APA, Aldosterone-producing adenoma; FH, familial hyperaldosteronism; Hsp70, heat shock 70-kDa protein; PA, primary aldosteronism.

Primary aldosteronism (PA), which accounts for about 8% of hypertension cases, is characterized by hypertension, autonomous secretion of aldosterone and suppression of the renin-angiotensin system. Aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia are the most common causes of PA (1). So far, three forms of familial hyperaldosteronism have been described and are categorized as familial hyperaldosteronism (FH) types I, II, and III (2). FH-III is a very rare autosomal dominant form of PA, characterized by severe childhood onset of hypertension, hypokalemia, and high levels of hybrid steroids (3). Choi *et al.* (4) recently identified the cause of FH-III as a germline mutation in the *KCNJ5* gene, which encodes the inward rectifying K^+ channel Kir3.4. In the index family, the mutation p.T158A was responsible for loss of KCNJ5 ion selectivity, increased Na^+ conductance, and subsequent cell depolarization (4). Moreover, the authors reported *KCNJ5* somatic mutations (p.G151R, p.L168R) in sporadic APA. The current brief communication defines the transcriptome profiles of APA with and without *KCNJ5* mutations and demonstrates a link between mutated *KCNJ5* transcription and adrenal cell aldosterone synthase (*CYP11B2*) expression.

Materials and Methods

An expanded Materials and Methods section with statistical analyses is provided as a Supplemental File, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

Patient selection

Forty-seven human adrenals were collected from APA patients from different centers. PA patients were studied after procedures that have been described previously (5–7). All samples were used under institutional review board approval with written informed consent obtained from each patient.

Immunohistochemistry

Sections from formalin-fixed, paraffin-embedded specimens were incubated with anti-KCNJ5 antibody. EnVision reagent (Dako, Carpinteria, CA) coupled with peroxidase-labeled polymer was incubated as secondary antibody. The slides were visualized with 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2, counterstained with hematoxylin, and mounted.

Sequencing of KCNJ5

KCNJ5 cDNA was PCR amplified using intron-spanning primers (4) and sequenced using the following primers: forward, 5′-CGACCAAGAGTGGATTCCTT-3′, and reverse, 5′-AGGG TCTCCGCTCTCTTCTT-3′ (4).

RNA extraction and gene expression assays

RNA extraction and gene expression assays were performed as described previously (8).

Microarray analysis

RNA from 24 female APA samples were hybridized to an Illumina bead chip (Illumina, San Diego, CA). The arrays were scanned at high resolution on the iScan system (Illumina). Results were analyzed by GeneSpring GX (version 11.5) software (Silicon Genetics, Redwood City, CA).

Molecular cloning of KCNJ5

The cDNA encoding human $KCNJ5^{WT}$, $KCNJ5^{G151R}$, and $KCNJ5^{L168R}$ were purchased from Invitrogen/Geneart and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA).

Cell culture and experimentation

HAC15 human adrenocortical carcinoma cells were cultured as described previously (8, 9) and electroporated with the Amaxa electroporator (program X005; Amaxa Biosystems, Cologne, Germany). Culture medium was assayed for aldosterone by RIA.

Results

Tissue expression of KCNJ5

KCNJ5 mRNA levels were quantified by real-time PCR in human placenta (n = 4), testes (n = 3), ovarian follicles (n = 4), brain (n = 4), fetal adrenals (n = 4), adult adrenals (n = 30), and APA (n = 30). *KCNJ5* transcript levels were significantly higher in adrenocortical tissue compared with placenta, gonads, and brain ($P < 0.05$). Within the adrenal tissues, *KCNJ5* was 4-fold higher in APA compared with normal adrenals ($P < 0.05$) (Supplemental Fig. 1A). No significant difference in *KCNJ5* expression was observed between APA with or without *KCNJ5* mutations (data not shown). Immunohistochemical analysis revealed that KCNJ5 expression localizes in both the adrenal zona glomerulosa and the outer part of the fasciculata (Supplemental Fig. 1B); in APA, KCNJ5 expression was higher in the adenoma compared with the surrounding adrenal cortex (Supplemental Fig. 1C).

Prevalence of *KCNJ5* mutations in aldosterone-producing adenomas

Of the 47 APA tissue, the overall prevalence of *KCNJ5* mutations in APA was 38% (Supplemental Table 1). Among 18 APA with *KCNJ5* mutations, eight APA (17%) had p.G151R and 10 APA (21%) had p.L168R mutations. The remaining samples contained only wild-type *KCNJ5* sequences. Of the eight p.G151R mutations, two derived from the substitution c.451G>C and six from the substitution c.451G>A. Of note, *KCNJ5* mutations were more frequent in APA from female patients than males (71 *vs.* 29%, $P = 0.05$). Furthermore, patients with mutated *KCNJ5* displayed lower serum potassium levels compared with wild-type APA (Supplemental Table 2).

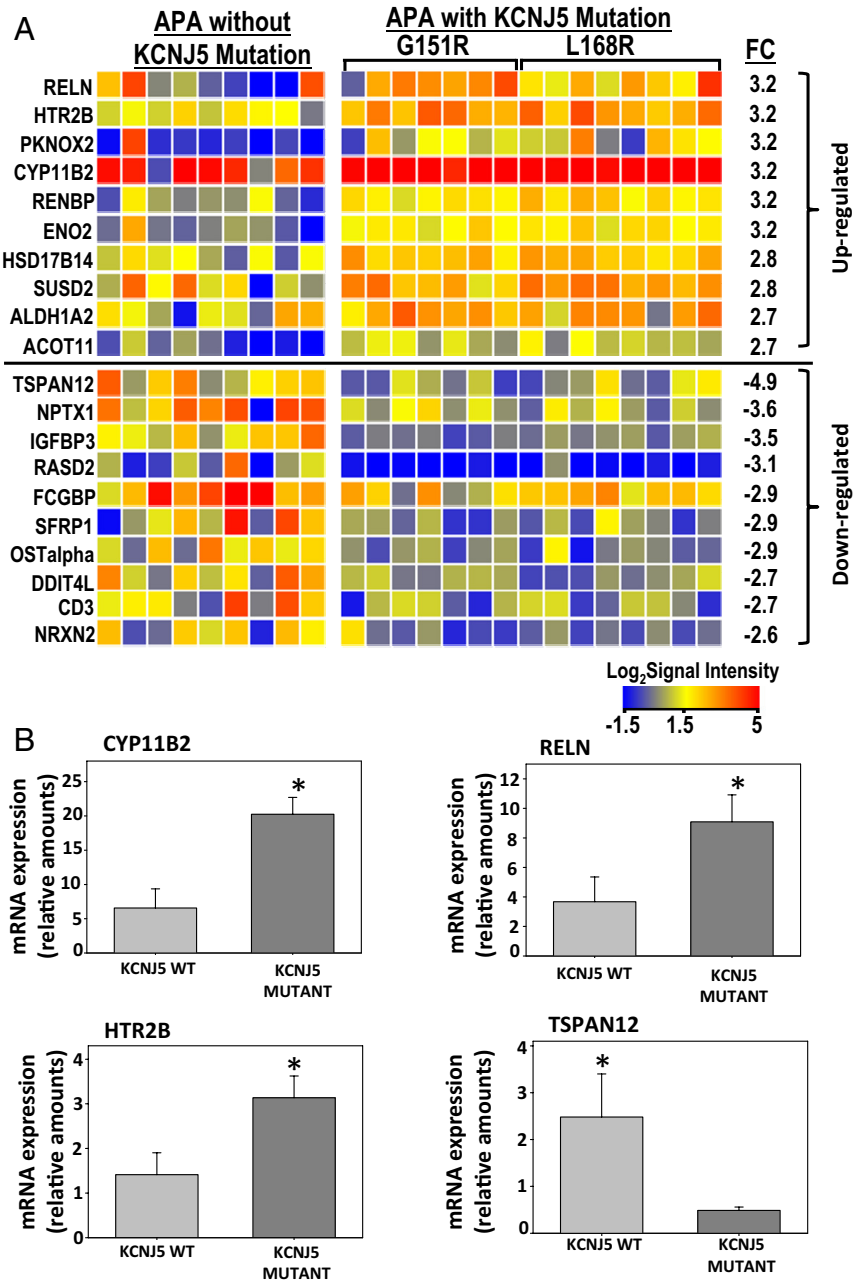

FIG. 1. A, Heat map representation of the 10 genes with the highest differential expression in female APA with or without *KCNJ5* mutations. Genes were selected based on a significance of $P < 0.05$ and a differential expression of at least 2.5-fold. Heat map data are presented as $\log_2$ of the signal intensity value. Absolute fold change (FC) is also provided. B, Validation of microarray using real-time PCR. Four genes were selected to confirm microarray analysis by using real-time PCR on a larger subset of RNA samples from women with APA (13 *KCNJ5*^{WT} and 20 *KCNJ5* mutant APA). Comparison of APA with and without *KCNJ5* mutations demonstrated a significant up-regulation of *CYP11B2* (3.1-fold change), *RELN* (2.5-fold change), and *HTR2B* (2.2-fold change) but a significant down-regulation of *TSPAN12* (−5.1-fold change). Data are presented as normalized (cyclophylin) transcript fold change with *each bar* representing the mean ± SE. *, $P < 0.05$ *vs.* APA with wild-type *KCNJ5*.

Transcriptome analysis of APA with and without KCNJ5 mutations

Oligonucleotide microarrays were used to perform transcriptome analysis of 24 APA from female patients, 15 with mutations in *KCNJ5* (eight p.L168R and seven p.G151R) and nine without mutations. APA with mutations in *KCNJ5* exhibited 24 differentially expressed genes compared with APA with wild-type *KCNJ5* (defined as 2.5-fold increase or decrease in mRNA levels; Fig. 1 and Supplemental Tables 3 and 4). Transcripts with the greatest differences in expression are shown in a heat map presentation in Fig. 1A. Interestingly, *CYP11B2* was one of the genes displaying differential expression. Our microarray analysis was validated by real-time PCR on a larger subset of samples, which showed 3-fold higher *CYP11B2* transcripts in tumors with the *KCNJ5* mutation compared with tumors without the mutations ($P < 0.05$, Fig. 1B).

Expression of KCNJ5 mutations in HAC15 cells

To better define the effects of the *KCNJ5* mutations on adrenal cell function, we overexpressed KCNJ5 in HAC15 adrenal cell model by transfection with pcDNA3.1/KCNJ5^{WT}, pcDNA3.1/KCNJ5^{G151R}, pcDNA3.1/KCNJ5^{L168R}, or empty vector. Gene expression was analyzed by oligonucleotide microarrays. A total of 36 up-regulated genes (including *CYP11B2)* and three down-regulated genes were identified with significantly altered expression ($P < 0.05$) in HAC15 cells expressing KCNJ5 mutations compared with wild-type KCNJ5 (Fig. 2A and Supplemental Tables 5 and 6). *HSPA6* (heat shock 70 kDa protein) and *NR4A2* (nuclear receptor subfamily 4, group A, member 2) were the two most up-regulated genes, with fold changes of 41 and 21, respectively. In addition, *NR4A3* (nuclear receptor subfamily 4, group A, member 3) was up-regulated (12-fold). Real-time PCR validated results on a broader sample set (Fig. 2B). Moreover, the overexpression of KCNJ5 mutations resulted in increased aldosterone production (1.9 ± 0.2 fold in 48 h), when compared with KCNJ5^{WT}-transfected cells.

Discussion

Although great strides have been made in our understanding of the pathophysiology of PA, the molecular

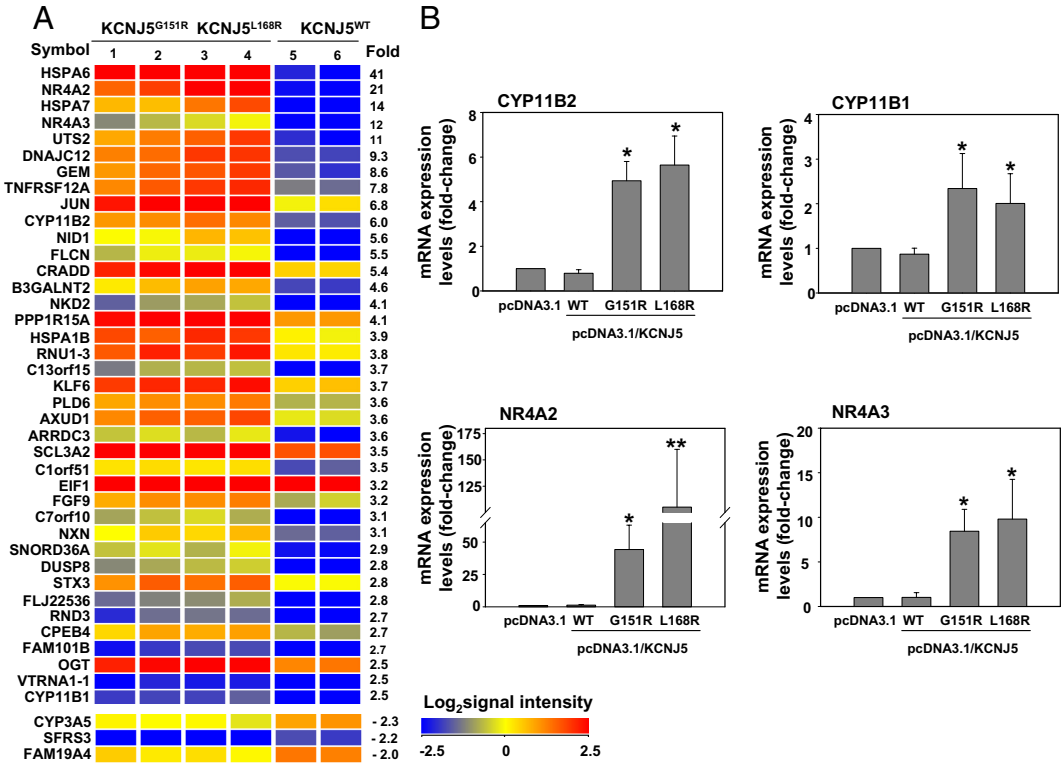

FIG. 2. A, Heat map representation of differentially expressed genes in HAC15 adrenal cells overexpressing either KCNJ5 mutations (p.G151R and p.L168R) or KCNJ5^{WT}. Genes were selected based on a significance of $P < 0.05$ and a differential expression of at least 2.0-fold. Heat map data are presented as $\log_2$ of the intensity value. Absolute fold change is also provided. Samples are HAC15 cells transfected with pcDNA3.1/KCNJ5^{G151R} (1 and 2); pcDNA3.1/KCNJ5^{L168R} (3 and 4); and pcDNA3.1/KCNJ5^{WT} (5 and 6). B, Validation of up-regulated genes using real-time PCR. *CYP11B2* was up-regulated 6.3 ± 0.6-fold and 7.2 ± 1.4-fold in HAC15 cells overexpressing KCNJ5^{G151R} or KCNJ5^{L168R}, respectively, compared with HAC15 cells overexpressing KCNJ5^{WT}. *NR4A2* was markedly up-regulated 98 ± 28-fold and 38 ± 6-fold in HAC15 cells overexpressing KCNJ5^{L168R} or KCNJ5^{G151R}, respectively, *vs.* KCNJ5^{WT}, with a relative fold change L168R/G151R of 2.6 ($P < 0.05$). No differences were observed between the effects of the G151R and L168R mutations on the gene expression levels of *CYP11B1*, *CYP11B2*, and *NR4A3* in HAC15 cells. No statistically significant difference was observed between HAC15 cells overexpressing KCNJ5^{WT} and pcDNA3.1 mock-transfected cells for any of the four selected genes. Each bar represents the mean ± SD of relative fold change of gene expression in five independent experiments. Each assay was performed in triplicate, and *PPAI* (cyclophylin) was used as endogenous control. *, $P < 0.05$ compared with WT; **, $P < 0.05$ compared with WT and G151R.

mechanisms causing the deregulated adrenal cell growth and aldosterone production remain poorly defined. Recently mutations in *KCNJ5* gene were implicated in the pathogenesis of both FH-III and sporadic APA (4, 10–12). The functional relevance of these mutations in the pathophysiology of APA and the regulation of aldosterone production are still unknown. Herein we confirm recently published prevalence findings and demonstrate that mutations in the *KCNJ5* gene cause augmented aldosterone production and *CYP11B2* expression in adrenal cells.

Recent studies have reported the prevalence of mutated *KCNJ5* in sporadic APA from different centers to range between 14 and 65% (11, 12). We observed an overall 38% prevalence of *KCNJ5* mutations in APA, with a higher percentage in females. Furthermore, all five of our Japanese APA had *KCNJ5* mutations. This result might relate to the patient population or center differences in referral patterns, yet a recent study also showed a high (65%) prevalence of *KCNJ5* mutations in Japanese APA. Further studies on a larger cohort of samples are warranted to determine whether mutations APA are related to ethnicity. Serum K^+ levels were lower in patients with *KCNJ5* mutations, whereas plasma aldosterone, PRA, and blood pressure did not differ between groups. The reasons for the discrepancy between serum K+ and aldosterone are not clear but may relate to the impact of antihypertensive medications, dietary sodium, conditions/timing of sampling, or an assay method on aldosterone levels.

To better define molecular differences between tumors with and without *KCNJ5* mutations, we performed a transcriptome analysis on APA tumors. Array comparison was done using only female samples to avoid the presence of gender bias in gene discovery. APA with and without *KCNJ5* mutations had a slightly different gene expression patterns, with reelin (*RELN*) and 5-hydroxytryptamine (serotonin) receptor 2B (*HTR2B*) as the two most up-regulated genes. Real-time PCR on a larger subset of APA samples also validated these results. The expression of the

transcript encoding the late rate-limiting enzyme involved in aldosterone synthesis, *CYP11B2*, was also significantly higher in APA with *KCNJ5* mutations than in tumors without these mutations. We have previously established the pathways that regulate *CYP11B2* transcription, and increased intracellular calcium is a key step in the signaling mechanisms shared by both angiotensin II and potassium (13–17). Both KCNJ5 amino acid substitutions, p.G151R and p.L168R, seem to increase sodium influx, cell depolarization, and subsequent overproduction of aldosterone (4).

To better define the influence of *KCNJ5* mutations on *CYP11B2* expression, we overexpressed wild-type or mutant KCNJ5 in the HAC15 cells. Overexpression of KCNJ5 mutations increased aldosterone secretion, whereas wild-type *KCNJ5* did not. In addition, both p.G151R and p.L168R mutations increased *CYP11B2* transcription compared with wild-type KCNJ5, suggesting a direct link between *KCNJ5* mutations and activation of aldosterone production through increased *CYP11B2* transcript levels. Importantly, we also observed an increase in key regulators of *CYP11B2* transcription, *NR4A2* and *NR4A3*, the final effectors of the multiple signaling pathways activated by angiotensin II and potassium in adrenal cells (15, 16). However, no difference in transcript levels for *NR4A2* or *NR4A3* was observed between APA with or without *KCNJ5* mutations because these transcription factors are likely a common final event needed for increased transcription of *CYP11B2*, regardless of the primary molecular mechanism. In this respect, a cell model might be a better way to define genes that are regulated in the short term by mutated *KCNJ5*. Exposure of cells to various stresses, as probably occurs with the sodium and calcium influx in cells transfected with KCNJ5 mutants could explain the overexpression of the heat shock proteins *HSPA6*, *HSPA7*, and *HSPA1B* in HAC15 cells with mutant KCNJ5, especially, heat shock 70-kDa protein (Hsp70), which is involved in protection from stress-induced apoptosis (18).

In conclusion, we propose that KCNJ5 is primarily an adrenal glomerulosa-expressed protein, found at high levels in APA. Although the role of the wild-type KCNJ5 protein in the regulation of aldosterone biosynthesis remains unclear, our findings confirm that two recurring mutations in the *KCNJ5* gene are commonly found in APA tumors. Transcriptome and real-time PCR analyses demonstrate that APA with *KCNJ5* mutations exhibit enhanced *CYP11B2* expression. Finally, we found that overexpressing the KCNJ5 mutations but not wild-type KCNJ5 in adrenal cells increased aldosterone production by augmenting the transcription of *CYP11B2* and of its regulatory transcription factors. Together our findings support a model in which the recurring *KCNJ5* mutations p.G151R and p.L168R cause PA by activating the transcription of genes required for aldosterone production in adrenal cells.

Acknowledgments

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