Microarray, qPCR, and KCNJ5 Sequencing of Aldosterone-Producing Adenomas Reveal Differences in Genotype and Phenotype between Zona Glomerulosa- and Zona Fasciculata-Like Tumors


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Context: Aldosterone-producing adenomas (APA) are heterogeneous. The recent finding of somatic KCNJ5 mutations suggests a genetic explanation.

Objectives: The objectives of this study were the following: 1) to compare transcriptional profiles in APA and adjacent adrenal gland (AAG); 2) to test whether gene expression profile clusters with different cell histology; and 3) to measure the frequency of KCNJ5 mutations and determine the genotype-phenotype relationship.

Design/Setting: The design of the study included laboratory analyses of 46 unselected APA.

Patients: The patients in this study had primary hyperaldosteronism with unilateral APA.

Interventions: The objectives of this study were the following: 1) Illumina beadchip analysis of RNA from eight paired APA-AAG; 2) a blinded review of cell histology for 46 APA; 3) laser capture microdissection of zona glomerulosa (ZG) and zona fasciculata (ZF) cells; and 4) sequencing of KCNJ5 in 46 APA.

Main Outcome Measures: The main outcome measures of this study were the following: 1) a difference in gene expression profile and a correlation with histological markers of ZF; 2) a frequency of KCNJ5 mutations and phenotypic comparisons of wild type with mutant APA.

Results: The results of the study were the following: 1) a cluster analysis of microarray data separated APA from AAG. APA at opposite ends of the APA cluster had an approximately 800-fold difference in CYP17A1 mRNA expression, whereas histology showed 0% ZF-like cells in one vs. 100% in the other. A heat map ranking APA by CYP17A1 expression correctly predicted several genes (e.g. KCNK1, SLC24A3) to be enriched in laser capture microdissection samples of ZG; 2) known or novel mutations of KCNJ5 were found in 20 of 46 consecutive APA [43% (95% confidence interval [CI] (29, 58)%)]. The APA with KCNJ5 gene mutations were larger compared with tumors harboring the wild type, 1.63 [95% CI (1.37, 1.88)] cm vs. 1.14 [0.97, 1.30] cm (P = 0.0013), had predominantly ZF-like cells, and their CYP17A1 (log2-fold change) was higher than in wild type: −0.96 [95% CI (−0.07, −1.85)] vs. −2.54 [−1.61, −3.46], (P = 0.017).

Conclusions: KCNJ5 mutations are common in APA, particularly those arising from ZF. The long-recognized heterogeneity among APA may have a genetic basis. (J Clin Endocrinol Metab 97: E819–E829, 2012)
Primary hyperaldosteronism (PHA) is the most common form of secondary hypertension, being present in about 8–10% of patients with hypertension (1). The pathophysiology of PHA has been widely investigated in both the sporadic and familial forms of the disease (2–6). However, the causal genes and the cellular changes responsible for autonomous aldosterone production in PHA are still unclear. Sporadic PHA results from two major types of adrenal lesion: an aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia. APA are found in 30–40% of patients with PHA (7). The diagnosis of an APA is attractive because surgical therapy with unilateral adrenalectomy can cure the PHA. The name APA suggests a single etiology of cells producing aldosterone. However, the histological characteristics of most adrenal tumors classified as APA are heterogeneous, a mixture of cells from the different adrenal zones (8). Whether most APA arise from zona fasciculata (ZF) rather than the site of physiological aldosterone secretion, zona glomerulosa (ZG), as suggested by responses to ACTH and angiotensin II, remains unknown (9, 10).

Microarray comparison between APA and adjacent adrenal glands

APA has been the principal model used to study changes in gene expression in sporadic PHA. Genome-wide expression (microarray) analysis and quantitative RT-PCR have become commonplace in the examination of gene expression. Numerous genes, such as aldosterone synthase, have been identified in previous microarray analysis as differentially expressed in APA and the adrenal cortex (7, 11, 12). However, these studies used as reference tissue the adrenal of nephrectomy patients. This approach diminishes the power for detecting differences between APA and nontumorous adrenal and may also reduce the possibility of detecting heterogeneity among APA. We therefore compared gene expression in APA with adjacent adrenal glands (AAG) from the same patient. Initial analysis indeed suggested two phenotypes of APA, each with a different gene profile; selected genes were further investigated by quantitative RT-PCR (qPCR) in a larger cohort.

KCNJ5 sequencing and post hoc analysis of phenotype-genotype

A possible molecular basis for the two types of APA arose from the discovery by Choi et al. (13) of two somatic mutations in the selectivity filter of the K+ channel KCNJ5 in eight of 22 APA; transfection of the mutants into human embryonic kidney-293T cells increased Na+ conductance and would be likely, through depolarization, to activate aldosterone secretion and/or cell division. The study by Choi et al. (13) also showed that a neighboring mutation in the filter region (T158A) caused a rare Mendelian form of primary aldosteronism, characterized by massive bilateral adrenal hyperplasia. The hyperplastic cells had ZF characteristics (i.e. clear cells with high cytoplasm to nucleus ratio). The accompanying editorial questioned whether KCNJ5 somatic mutations were a feature of atypical APA because the APA in the series by Choi et al. were rather large (mean diameter 2.8 cm) (14). Therefore, we aimed to measure the frequency of KCNJ5 mutations in unselected APA and determine whether the presence or absence of mutation correlated with the two phenotypes we found on gene expression and histological examination.

Materials and Methods

Subjects

Adrenal tissues were obtained from 46 patients with APA and 19 non-APA patients (pheochromocytoma, n = 9; Cushing’s syndrome, n = 9; adrenal cell cancer, n = 1) who underwent adrenalectomy at Addenbrooke’s Hospital (Cambridge, UK). We obtained informed consent from each patient and local ethical approval. Diagnosis of PHA and tissue collection is described in Supplemental Methods, published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org.

Extraction of total RNA

Approximately 0.125 cm³ of tissue was used for RNA extraction. Total RNA was isolated from AAG and APA using Trizol with a PureLink kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. On-column deoxyribonuclease treatment was performed on all RNA samples.

Microarray analysis

RNA samples from eight PHA patients, each with a paired APA and AAG were used for the microarray analysis. The eight APA were selected to span a spectrum of clinical parameters, including age, blood pressure response to adrenalectomy, and the size of APA (Table 1). Each RNA sample (total RNA, 1 μg) was converted to cDNA by reverse transcription and subjected to labeling and hybridization using the whole-genome cDNA-mediated annealing, selection, extension, and ligation (DASL) assay (Illumina HumanRef-8 version 3 Expression BeadChip; Illumina, San Diego, CA). Three DASL microarray slides containing 24,526 probes were obtained and analyzed by an Illumina certified provider (CSPro), the Cambridge Genomic Services, Department of Pathology, University of Cambridge (details at http://www.illumina.com/technology/whole_genome_dasl_assay.ilmn). Data were visualized and extracted with the GenomeStudio data analysis software (version 2009.2; Illumina) with Illumina’s default analysis settings. Two different methods were used to normalize global gene expression and identify genes with altered expression in APA (Supplementary Methods). Two heat maps were constructed. In one, the normalized expression of each gene was analyzed separately in APA and AAG and unsupervised cluster analysis performed. The second heat map analyzed fold change (FC) between each APA and its AAG, plotted to look for inverse
TABLE 1. Clinical features of patients before and after adrenalectomy whose APA and AAG were used for microarray analysis

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Age at adrenalectomy (yr)</th>
<th>Sex</th>
<th>SBP (mm Hg) Before</th>
<th>SBP (mm Hg) After</th>
<th>DBP (mm Hg) Before</th>
<th>DBP (mm Hg) After</th>
<th>Serum K+ (mmol/liter) Before</th>
<th>Serum K+ (mmol/liter) After</th>
<th>AVS ratio (aldo to cort) Left</th>
<th>AVS ratio (aldo to cort) Right</th>
<th>Plasma aldosterone (pmol/liter) Before</th>
<th>Plasma aldosterone (pmol/liter) After</th>
<th>Plasma rennin (mU/liter) Before</th>
<th>Plasma rennin (mU/liter) After</th>
<th>KCNJ5 mutation</th>
<th>APA diameter (mm)</th>
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<tr>
<td>ADR018</td>
<td>49</td>
<td>M</td>
<td>153</td>
<td>117</td>
<td>97</td>
<td>78</td>
<td>2.8</td>
<td>3.4</td>
<td>3.82</td>
<td>15.23</td>
<td>1126</td>
<td>465</td>
<td>2</td>
<td>40</td>
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<td>20</td>
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<tr>
<td>ADR032</td>
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<td>133</td>
<td>115</td>
<td>81</td>
<td>3.8</td>
<td>4.5</td>
<td>21.12</td>
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<td>108</td>
<td>33</td>
<td>127</td>
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<td>18</td>
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<tr>
<td>ADR040</td>
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<td>112</td>
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<td>72</td>
<td>3.2</td>
<td>4.2</td>
<td>0.48</td>
<td>1.68</td>
<td>1011</td>
<td>347</td>
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<td>22</td>
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<td>54</td>
<td>M</td>
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<td>3.3</td>
<td>4.2</td>
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<td>16.98</td>
<td>347</td>
<td>179</td>
<td>&lt;2</td>
<td>18</td>
<td>G151R</td>
<td>12</td>
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<tr>
<td>ADR049</td>
<td>66</td>
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<td>260</td>
<td>190</td>
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<td>9.68</td>
<td>5.77</td>
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<td>298</td>
<td>2</td>
<td>24</td>
<td>L168R</td>
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</tr>
<tr>
<td>ADR059</td>
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<td>F</td>
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<td>9</td>
<td>G151R</td>
<td>10</td>
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<tr>
<td>ADR063</td>
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<td>F</td>
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<td>3.8</td>
<td>3.9</td>
<td>46.72</td>
<td>1.72</td>
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<td>&lt;2</td>
<td>31</td>
<td>L168R</td>
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<td>F</td>
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<td>4.1</td>
<td>9.45</td>
<td>6.31</td>
<td>1016</td>
<td>158</td>
<td>3</td>
<td>9</td>
<td>None</td>
<td>9</td>
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</tbody>
</table>

All patients had a reduced plasma aldosterone and an increased renin after adrenalectomy. (This table is a detailed subset of Supplemental Table 1.) SBP, Systolic blood pressure; DBP, diastolic blood pressure; AVS, adrenal venous sampling; aldo, aldosterone; cort, cortisone; M, male; F, female.

correlations with CYP17A1 expression as a predictor of ZG expression.

Quantitative RT-PCR

qPCR was used for validation of microarray results and for investigating ZF/ZG genes. RNA samples of an additional 14 patients and the eight patients used for microarray analysis were reversely transcribed into cDNA (Promega, Southampton, UK). Details of cDNA synthesis and qPCR are described in Supplemental Methods. For validation of the microarray by qPCR, 16 genes significantly up- or down-regulated in APA were studied, together with a further 23 genes selected because of either a putative functional connection to aldosterone secretion or a correlation between their FC and that of CYP17A1 (Supplemental Fig. 1). The results from the qPCR also permitted a correlation analysis between the FC for each gene and that for the genes of ZF’s hallmark enzymes, CYP17A1 and CYP11B1.

APA histology

Hematoxylin and eosin-stained sections of all but one of the 46 APA were reexamined by two adrenal histopathologists blinded to the clinical and gene expression profile data. Semi-quantitative assessment of the APA was made based on known features of ZF cells when compared with ZG cells, in particular higher cytoplasm to nucleus ratio. Representative fields of the 22 APA characterized by qPCR were photographed.

Laser capture microdissection

To estimate whether APA characterized through gene expression profiling or histology as ZF-like or ZG-like might arise, respectively, from ZF and ZG cells, we used laser capture microdissection (LCM) of AAG, followed by qPCR, to compare expression of steroidogenic enzymes and several putative ZG genes in the nontumorous ZF and ZG cells from AAG (Supplemental Methods and Supplemental Fig. 2).

KCNJ5 sequencing and genotype-phenotype analyses

Sanger sequencing of the selectivity filter region of KCNJ5 was performed in 46 PHA patients using the primers and systems described in Supplemental Methods. Sequencing used cDNA for both APA and AAG, except in four cases in which the only available material was genomic DNA from APA and either AAG or blood. qPCR analysis of steroidogenic enzymes (CYP11B2, CYP11B1, and CYP17A1) was undertaken in the 42 from whom cDNA was available, including the 22 patients already characterized by microarray and/or qPCR (Supplemental Table 1). In these 22 patients, a multivariate analysis was performed to compare, between wild-type and mutant APA, the expression of putative ZG genes (those that showed significant inverse correlation with CYP17A1 and/or CYP11B1).

KCNJ5 immunohistochemistry

Sections of APA and AAG were stained with polyclonal anti-KCNJ5 (no. HPA017353; Sigma, St. Louis, MO) as detailed in Supplemental Methods.

Statistical analyses

All data are presented as mean ± SEM unless stated otherwise. Quantitative comparisons between groups were by Student’s t test or multivariate ANOVA; frequencies were compared by Fisher’s exact test; associations between continuous variables were investigated using multiple regression analyses. Statistical relationships not otherwise stated are to be assumed nonsignificant (P > 0.05).

Results

Patient information

The clinical features of the patients whose APA were studied by microarray are shown in Table 1. All patients responded to adrenalectomy with a reduction of plasma aldosterone and an increase of plasma renin.

Microarray analysis and validation

A gene list was compiled on the basis of both methods of normalization and statistical analysis (P ≤ 0.05). Using these criteria, 886 genes were identified. Unsupervised hierarchical clustering analysis (Cluster 3.0; Stanford University, Palo Alto, CA) of the 886 significant genes separated all APA from their paired AAG (Fig. 1A). Many of the known up-regulated genes, namely those differentially
expressed in previous microarray studies, are in the list, including our top genes, \textit{MYB}, \textit{HTR4}, \textit{PRRX1}, \textit{ALDH1A2}, and \textit{CYP11B2} [Supplemental Table 2, A and B (7, 15, 16)]. However, the FC of \textit{CYP11B2}, between APA and AAG, was relatively small compared with previous studies. Further validation of results was therefore performed by comparing the microarray and qPCR results for 39 genes in the eight pairs of APA-AAG. The qPCR results correlated well with microarray analysis (Supplemental Fig. 1). Of interest, exclusion of \textit{CYP11B2} increased the correlation between microarray and qPCR (from $R^2 = 0.54$ to $R^2 = 0.65$), supporting the probable underestimation of the FC for \textit{CYP11B2} in the microarray analysis. \textit{PPY} showed the highest change, an average of 13-fold among all the APA but was absent from one APA (18T) (Supplemental Table 2A). This gene along with 15

![Heat map representation of differentially expressed genes in aldosterone-producing adenomas.](https://academic.oup.com/jcem/article-abstract/97/5/E819/2536642)
other interesting genes were qPCR interrogated in a larger cohort of APA (n = 22; Table 2).

**Differences between APA at opposite ends of the cluster analysis**

One APA (18 T) was clustered closer to AAG than to the other APA (Fig. 1A). Further inspection of its gene expression profile revealed that 18 T was a clear outlier from the rest of the APA. On histology review, the tumor cells of 18 T were entirely clear type, with a high cytoplasm to nucleus ratio, resembling classic ZF morphology (Supplemental Fig. 3). A gene expression profile review also showed that whereas CYP17A1, a gene that encodes an enzyme absent from human ZG (17, 18), was down-regulated in most APA (Fig. 1B), CYP17A1 expression in 18 T was similar to that in AAG. Using CYP17A1 as a biomarker for ZF-like cells, two APA from each end of the CYP17A1 expression spectrum were compared histologically. 18 T, as previously mentioned, and 49 T, which had high expression of CYP17A1, had clear cells with a high cytoplasm to nucleus ratio, whereas 67 T and 40 T, which had low expression of CYP17A1, had compact cells with low cytoplasm to nucleus ratio (Supplemental Fig. 3). There was a similar contrast in CYP11B1 expression between these two pairs of APA (data not shown).

**Further investigation of CYP17A1 and CYP11B1 as biomarkers of ZF-like cells**

Correlation of CYP17A1 and CYP11B1 expression with ZF-like cell histology was further analyzed in the cohort of APA (n = 22) used for interrogating the microarray results. Although the levels of CYP17A1 and CYP11B1 mRNA were (as in the microarray) overall lower in APA compared with their paired AAG (the geometric mean FC were 0.2 ± 0.1 and 0.4 ± 0.1, respectively), there was a large spectrum of expression. We termed APA that had a low FC of CYP17A1 and low expression of CYP11B1 as ZG-like APA (nine of 22) and APA with high FC of CYP17A1 as ZF-like APA (nine of 22) (Supplemental Fig. 4; details in Supplemental Methods). On histopathology review, ZF-like APA appeared to contain a majority of clear cells, whereas ZG-like APA had a majority of compact cells (Supplemental Fig. 5). The remaining four APA that were not termed ZG-like or ZF-like due to not meeting the gene expression criteria appeared to contain a majority of cells with high cytoplasm to nucleus ratio.

**Investigation of ZF/ZG genes**

A secondary heat map was constructed of 3009 genes, which correlated with CYP17A1 (−0.66<r>0.66; P < 0.06); this is shown in Supplemental Fig. 6, which highlights 21 genes whose expression was subsequently compared by qPCR between the nine ZF- and ZG-like APA (Supplemental Table 3). To explore whether genes that had a trend toward higher expression in ZG-like APA than ZF-like APA were also more highly expressed in normal ZG than in normal ZF, we proceeded to LCM. Because this technique provides limited material from each adrenal, only seven genes together with the three steroidogenic

**Table 2. Confirmation of microarray result by qPCR**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Illumina probe ID</th>
<th>Normal expression Mean ± SEM APA expression Mean ± SEM</th>
<th>Fold change APA vs. normal</th>
<th>Adjusted P value</th>
<th>Fold change (geomean) APA/normal Mean ± SEM APA/normal</th>
<th>Two-tailed t test, P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYY</td>
<td>3140148</td>
<td>125 ± 50</td>
<td>1694 ± 519</td>
<td>13.5</td>
<td>0.00005</td>
<td>29.9 ± 190.4</td>
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<tr>
<td>GPR51</td>
<td>1710577</td>
<td>79 ± 23</td>
<td>572 ± 131</td>
<td>7.3</td>
<td>0.001</td>
<td>3.0 ± 1.3</td>
</tr>
<tr>
<td>PPARA</td>
<td>7610411</td>
<td>325 ± 77</td>
<td>2070 ± 351</td>
<td>6.4</td>
<td>0.0007</td>
<td>8.5 ± 24.0</td>
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<td>SLC9A2</td>
<td>3710563</td>
<td>209 ± 25</td>
<td>829 ± 53</td>
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<td>0.0002</td>
<td>3.3 ± 1.4</td>
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<tr>
<td>ESRRB</td>
<td>4810142</td>
<td>60 ± 16</td>
<td>195 ± 57</td>
<td>3.2</td>
<td>0.04</td>
<td>2.8 ± 15.0</td>
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<tr>
<td>KCNK12</td>
<td>1940193</td>
<td>1073 ± 566</td>
<td>3300 ± 973</td>
<td>3.0</td>
<td>0.0003</td>
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<tr>
<td>CYP11B2</td>
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<td>0.0000001</td>
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<tr>
<td>SLC24A3</td>
<td>150309</td>
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<td>0.02</td>
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<td>0.00001</td>
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<td>239 ± 172</td>
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</tr>
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</table>

The 16 interesting genes that were highlighted by the microarray (n = 8) were validated by qPCR. Fourteen of the genes remain significant after validation (P < 0.05). [Fold changes of genes are presented as geometric mean (geomean) ± SEM. Validation of gene using qPCR technique was performed in 22 APA-AAG pairs except for PYY, which was performed in 17 pairs due to lack of cDNA. The data in this table are a subset of Supplemental Fig. 1.]
genes were selected for analysis. The results are shown in Table 3. In addition, a correlation analysis was performed between FC in all the genes studied by qPCR, and those of CYP11B1 and/or CYP17A1, as the hallmark of ZF steroidogenic enzymes. Twelve genes showed significant negative correlation with CYP17A1 (KCNK1, KCNJ3, SLC24A3, KCNJ5, ASMT, GAD1, GNRHR, KCNH3, SLC30A10, INPP4B, ESRRB, and SLC35A1), and two genes showed significant negative correlation with CYP17A1 (PPY and GAD1).

**KCNJ5 sequencing**

The mutations were G151R (n = 11), L168R (n = 8), and a novel I157del (ss470828695) (Supplemental Figs. 7 and 8). Several differences were apparent between the demography and APA of the patients with somatic mutations compared with those with wild-type APA.

Clinically the patients with mutations were younger at the time of adrenalectomy (Fig. 2A), and their APA were about 50% larger in diameter [1.63 cm (95% CI [1.37, 1.88])] than the wild-type APA [1.14 cm (95% CI [0.97, 1.31]) (P = 0.0013)].

On gene expression profiling, CYP17A1 in mutant APA was more abundant than in wild-type APA: log₂ FC −0.96 [95% CI (−0.07, −1.85)] vs. −2.54 [−1.61, −3.46] (P = 0.017). In a multiple regression of log₂ CYP17A1 FC on clinical and biochemical variables, the significant predictors out of age, size, gender, and genotype were gender (β = 0.35, P = 0.041) and genotype (β = 0.46, P = 0.011). Values for individual mutations are shown in Supplemental Table 4. A quantitative comparison of putative ZG gene expression was performed between the mutant and wild-type APA, using the 13 genes whose FC had been found to correlate inversely with that of CYP11B1 and/or CYP17A1 expression. On multivariate analysis, the genes that correlated with CYP17A1 had lower expression in the mutant APA (P = 0.023).

On histological review, the percentage of ZG-like, compact cells in APA with KCNJ5 mutations [15.8 (95% CI [3.6, 27.9%])] was lower than wild-type [39.8 (29.1, 50.4%)] (F = 11.6, P = 0.009) (Fig. 2, B–D). The wild-type APA seem to divide into two groups, one with predominantly compact cells and one with predominantly clear cells (Fig. 2D).

All 19 non-APA tumors had wild-type sequence of KCNJ5.
**KCNJ5 immunohistochemistry (IHC)**

IHC showed expression of KCNJ5 in both ZG and ZF of AAG from PHA patients (n = 7; Fig. 3 and Supplemental Fig. 9). APA, regardless of KCNJ5 mutation, also showed expression of KCNJ5 (n = 4; Supplemental Fig. 9).

**Discussion**

Microarray and qPCR analysis showed a spectrum of expression that could be correlated, first, with a histological assessment between the two extremes of ZF-like and ZG-
like and, subsequently, with the presence or absence of somatic mutations in the selectivity-filter region of KCNJ5. This is of interest as an example of somatic genotype-phenotype relationship. This relationship offers a retrospective explanation for the well-recognized heterogeneity in histology reports of APA. Looking ahead and with caveats about whether ZF-like and ZG-like necessarily denote the cell of origin, we suggest that our findings may point the way to new candidates for aldosterone regulation, either its physiological secretion from ZG, or pathological induction in ZF.

Within this overall conclusion, there is considerable interest in finding that somatic KCNJ5 mutations are indeed frequent in APA. Far from their being a curiosity of atypical, large APA, they turn out to be as common, if not more common, in our series of 46 unselected APA as in the original cohort of Choi et al. (13) of 22 APA. Although the average size of our APA was smaller than in the original (mean diameter of 1.4 vs. 2.8 cm), we did find that those with mutations were closer in size to those of Choi et al. than our wild-type APA. Inspection of Fig. 2A suggests that KCNJ5 genotype partially differentiates a phenotype of mainly younger women with larger APA from one of mainly older men with smaller APA. On the other hand, our wild-type APA are clearly smaller than those of Choi et al., which interestingly approximate the 4-cm diameter of Conn’s original, but atypically large, adenoma. We have not looked for the loss of heterozygosity reported by Choi et al. Whether there are perhaps three rather than two broad types of APA may emerge with the reporting of further cohorts, and we have started to pool our data with those from different geographical areas (19).

In addition to the previously discovered G151R and L168R mutants, sequencing of KCNJ5 in all 46 APA revealed a novel three-base deletion mutation, I157del (ss470828695), which is also located within the selectivity filter of the K⁺ channel. Indeed, the I157del is immediately adjacent to the mutation found in the Mendelian form of severe aldosteronism, T158A, and we have found similar effects of these two mutations on KCNJ5 current when expressed in frog oocytes (Murthy M., and K. M. O. Shaughnessy, unpublished data).

Although most of our histological analysis was undertaken by blinded review of paraffin-embedded sections, we did undertake a prospective study of KCNJ5 immunohistochemistry in adrenals from PHA patients. This was motivated by our finding of ZF-like appearance and gene expression profile in the KCNJ5 mutant APA, whereas the original report suggested expression of KCNJ5 in the ZG of normal adrenal cortex. It is apparent, however, that there is both ZF and ZG expression of KCNJ5 (and this was confirmed, at least at the RNA level, by qPCR of RNA extracted separately, by LCM, from normal ZF and ZG cells; Table 3). It may be that the apparent difference between our findings and the original report relates to the latter’s use of adrenals from patients with pheochromocytoma. However, inspection of the published figures suggest that the discrepancy is more apparent than real (13). In further support of our hypothesis that APA with a somatic KCNJ5 mutation arise from ZF cells is the finding that patients with the Mendelian form of severe aldosteronism who inherited the T158A KCNJ5 mutation had massive bilateral adrenal hyperplasia composed of ZF-like cells with an atrophic ZG (20). As is also true of our own results, relating biochemistry to histology, we cannot exclude the possibility that ZF-like cytological features in a tumor is due to changes with ZG-derived tumor cells, e.g. greater metabolic activity, or induction of lipid uptake. On balance, however, the
similarities between the gene expression profiles of ZF-like APA cells and normal ZF-cells acquired by LCM (for example, expression of CYP17A1, SLC24A3, and KCNJ1) favors the simpler conclusion that ZF-like appearance of cells is most commonly due to a ZF origin.

CYP17A1 encodes for the enzyme that converts pregnenolone or progesterone to 17α-hydroxypregnenolone or 17α-hydroxyprogesterone, respectively, precursors of glucocorticoids, androgens, and estrogens. CYP17A1 has been of recent interest in hypertension, in which genome-wide association studies identified the locus as one of the top hits (21–23). In contrast to humans, adrenals of rodents do not express the CYP17A1 gene and therefore produce corticosterone instead of cortisol. Although many microarray studies have reported down-regulation of CYP17A1 in APA, none of these studies commented on the large spectrum of CYP17A1 expression in APA (11, 15, 24, 25). However, in IHC studies, the distribution of CYP17A1 is variable in APA (17, 18). In one case, no CYP17A1 expression was found and the authors concluded, from the cytological features of the APA, in situ hybridization, and RT-PCR that 100% of the cells were of ZG-like phenotype (26). In contrast to CYP17A1, CYP11B2 expression failed to discriminate between the two phenotypes. Perhaps this is unsurprising because all APA synthesize aldosterone, but it will be of interest now to discover why depolarization of ZF-cells by KCNJ5 mutations appears to induce CYP11B2 expression.

Investigators have been aware of subtypes of APA for some time. Classically most APA have been regarded as unresponsive to angiotensin II stimulation but responsive to ACTH, in contrast to the picture in bilateral adrenal hyperplasia; however, in the 1990s, investigators found a minority of APA that were responsive to angiotensin II stimulation (10, 27–29). These APA that were responsive to angiotensin II stimulation consisted of a variable proportion of ZG-like cells, whereas the APA unresponsive to angiotensin II stimulation contained predominantly ZF-like cells (10, 27, 30). Although this notion is not universally accepted (31), in our study we too have found a higher expression of AGTR1, the gene that encodes for the angiotensin II receptor, in ZG-like APA (Supplemental Fig. 6). All responsiveness in an Australian cohort was limited to patients lacking a KCNJ5 mutation in the APA, which we now believe more likely to be of ZG phenotype (19).

Other G protein-coupled receptors may regulate aldosterone secretion, and the evidence again points to their importance varying between types of APA. For example, the increment of plasma aldosterone after administration of the dopamine-2 receptor antagonist, metoclopramide, correlated inversely with the percentage of ZF cells subsequently found in the APA (4). Similarly, our study found large increases in G protein-coupled receptors like the GnRH receptor, as previously reported (7, 32), but only in small numbers of KCNJ5 wild-type APA, which had the most extreme ZG-like phenotype (Supplemental Fig. 6).

The finding that the heterogeneity of APA is partially measurable as a spectrum between two extremes, defined by genotype, gene expression, and histological appearance, prompts the question whether there is any clinically useful prognostic information in establishing the genotype or phenotype of an individual APA. Probably at least half of patients undergoing adrenalectomy for APA still require some antihypertensive medication postoperatively, albeit at lower doses or with fewer drugs (1, 33, 34). There is no good agreement on clinical parameters that predict responses to surgery, but our own experience is that younger patients with larger tumors tend to fare better. It will be of interest to establish in larger numbers whether it is genotype or phenotype that is the more predictive. However, a potentially more important clinical implication of our findings is that small size should not be an argument against vigorous investigation and treatment. If the wild-type APA comprise more ZG-like than ZF-like cells, their smaller total size is predictable but is not a good guide to their cell number and aldosterone production. It might well be that failure to find or remove small APA at a young age explains in part why their common presentation is in older men with resistant hypertension (35).

Microarray analyses play an important role in identifying novel targets for understanding physiological and pathological processes and ultimately novel modes of therapy. The finding that, as long suspected, many APA appear to arise from ZF rather than ZG cells and that genotypically there are two partially distinct phenotypes suggests the need to separate physiological regulation of aldosterone secretion in ZG from pathological induction of CYP11B2 in ZF. After the next microarray, comparing APA of different genotypes with their ZF and ZG internal controls, it should be possible to define the pathways in ZF and ZG that may cause PHA and include targets for novel drug discovery.

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