CLINICAL STUDY

Gene expression profiles in aldosterone-producing adenomas and adjacent adrenal glands

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Abstract

Background: Primary aldosteronism (PA) is the most common form of endocrine hypertension affecting ~8–10% of hypertensive subjects. Aldosterone production in PA occurs under low-renin conditions, and the mechanisms that maintain the production of aldosterone in PA remain unknown.

Objective: This study was designed to compare the transcript profiles between aldosterone-producing adenoma (APA) and their adjacent adrenal gland (AAG) from the same adrenal.

Methods: Total RNA was extracted from ten APA and ten AAG, and subsequently analyzed by microarray and real-time quantitative RT-PCR (qPCR). The microarray data were paired for each APA–AAG, and analyzed by GeneSpring GX 11 with paired t-test and fold change calculations for each transcript. Changes identified by microarray analysis were confirmed by qPCR.

Results: Microarray analysis indicated that 14 genes had significantly up-regulated expression in APA compared to AAG. Among the elevated genes were aldosterone synthase (CYP11B2) as well as novel transcription factors, calmodulin-binding proteins, and other genes that have not been previously studied in APA. Selective analysis of 11 steroidogenic enzymes using microarray demonstrated that only CYP11B2 showed a significantly higher transcript level in APA compared to AAG (P<0.001). In contrast, AKR1C3 (17β-hydroxysteroid dehydrogenase type 5), CYP17 (17α-hydroxylase/17,20 lyase), and CYB5 (cytochrome b5) showed significantly lower transcript level in APA (P<0.05).

Conclusion: The transcriptome analysis of APA compared with AAG showed several novel genes that are associated with APA phenotype. This gene list provides new candidates for the elucidation of the molecular mechanisms leading to PA.
Materials and methods

Subjects and tissues

Human adrenal glands, which contained APA tissue, were obtained as part of standard pathological evaluation in collaboration with Tohoku University Hospital (Sendai, Japan) and University of Texas Southwestern Medical Center (Dallas, TX, USA). Tumor and AAG tissues were macroscopically dissected and were then snap frozen and stored at \(-80^\circ\)C. All samples were used under University Ethics Committee approval, and written informed consent was obtained from each patient. Thirteen adrenal glands were collected from patient with APA, two APA samples, which showed less than twofold changes of CYP11B2 compared with the AAG tissue, were excluded from our study, and one showed low quality of RNA was also excluded. Herein, we used ten APA–AAG pairs for microarray analysis.

RNA isolation and cDNA generation

Total RNA was isolated from frozen tissue using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instruction. The purity and quantity were assessed by a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

For cDNA generation, 2 \(\mu\)g total RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer recommendations and incubated at 25 \(^\circ\)C for 10 min, 37 \(^\circ\)C for 2 h, and 85 \(^\circ\)C for 5 s. The synthesized cDNA was subjected to 1:10 dilution for qPCR.

Microarray analysis

Total RNA isolated from APA and AAG tissues was treated with a first- and second-strand RT step, followed by a single in vitro transcription amplification that incorporated biotin-labeled nucleotides. Then, the labeled RNA was hybridized to a bead chip containing more than 48 000 probes representing over 25 000 human genes (Illumina, San Diego, CA, USA).

The arrays were scanned at high resolution on the iScan system (Illumina). Results were analyzed using GeneSpring GX (version 11) software (Silicon Genetics, Redwood City, CA, USA) to identify transcriptome differences between APA and AAG.

Real-time quantitative RT-PCR

Primers and probes, for selected genes, were designed based on published sequences, and gene amplification was performed using Taqman Gene Expression Assays (Applied Biosystems). The gene symbols and AB assay numbers are listed in Table 1. The primer and probe set for human CYP11B2 and CYP17 were designed using Primer Express 3.0 (Applied Biosystems) and purchased from IDT (Integrated DNA Technologies, Inc., Coralville, IA, USA) as described previously (19).

qPCR was performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems), with 5 \(\mu\)l cDNA, 10 \(\mu\)l TaqMan Fast Universal PCR Master Mix (2X; Applied Biosystems), 900 nM of each primer, and 400 nM probe in a total volume of 20 \(\mu\)l per well according to the manufacturer’s instruction. Negative controls contained water instead of cDNA. Quantitative normalization of cDNA in each tissue-derived sample was performed using expression of 18S rRNA as an internal control. The generated \(C_t\) value of each gene was normalized by its respective \(C_t\) value of 18S rRNA (\(\triangle C_t\)). Each APA gene was then further normalized to the \(\triangle C_t\) value of its corresponding AAG (\(\triangle \triangle C_t\)). The final fold expression changes were calculated using the equation \(2^{-\triangle \triangle C_t}\).

Statistical analysis

The fold change of qPCR data between APA and AAG was converted to logarithms, and then analyzed using paired t-test with the SigmaStat 3.0 software package (Chicago, IL, USA). The microarray data were analyzed using paired t-test with Benjamini and Hochberg False discovery rate (FDR) correction using GeneSpring GX11 software. \(P\) value <0.05 was considered to be significant.

Table 1 Comparison of results from qPCR and microarray for APA–AAG pairs. The \(\log_2\) fold changes were calculated for each APA–AAG pair and presented as mean ± S.E.M.

<table>
<thead>
<tr>
<th>Gene symbols</th>
<th>AB assay no.</th>
<th>qPCR (log(_2) fold change)</th>
<th>Microarray (log(_2) fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± S.E.M.</td>
<td>(P)</td>
</tr>
<tr>
<td>CYP11B2</td>
<td>Hs01113638_m1</td>
<td>6.01 ± 0.88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PFC4</td>
<td>Hs00353682_m1</td>
<td>3.30 ± 0.40</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PRRX1</td>
<td>Hs00246561_m1</td>
<td>1.69 ± 0.25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALDH1A2</td>
<td>Hs00180254_m1</td>
<td>1.09 ± 0.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CYB5</td>
<td>Hs00157217_m1</td>
<td>0.97 ± 0.41</td>
<td>0.042</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>Hs00366261_m1</td>
<td>1.77 ± 0.53</td>
<td>0.009</td>
</tr>
</tbody>
</table>

\(P\) value was determined by paired t-test for each transcript.

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Results

Patient information

Before the removal of adrenal glands, all APA patients included in this study were diagnosed using adrenal vein sampling (AVS), which demonstrated lateralization of excess aldosterone production. All patients responded to adrenalectomy with normalization of aldosterone/renin ratio (ARR) and with decreased blood pressure and/or normalization of hypokalemia (Table 2). All adenoma cases were histopathologically diagnosed as an adrenocortical adenoma.

Differential gene expression in APA

To evaluate global changes in gene expression associated with APA, RNA samples from eight adenomas were compared against the corresponding AAG tissue using microarray analysis. A gene list was compiled based on a minimum of a 2.5-fold increased level of expression that was found to be statistically significant ($P < 0.05$). Fourteen genes were identified using these criteria, and a heat map representation of these genes is shown in Fig. 1. Five genes in this list were chosen for qPCR validation, which confirmed the microarray data (Fig. 2, and Table 1). The major gene difference, which was observed in every APA–AAG sample pair, was CYP11B2 (Fig. 2). Microarray data indicated a mean of 9.7-fold increased expression in APA ($P < 0.001$), and the more quantitative method, qPCR, indicated a mean difference of 49-fold (Table 1). A comparison of microarray and qPCR was also performed for Purkinje cell protein 4 (PCP4), pre-B lymphocyte 3 (VPREB3), and paired related homebox 1 (PRRX1). In each case, qPCR generally agreed with microarray analysis (Table 1).

Steroidogenic enzymes

Eleven genes involved in the production of all classes of adrenal steroids were analyzed by microarray (Fig. 3). In addition to a 9.7-fold increase in CYP11B2 expression, AKR1C3 (17β-hydroxysteroid dehydrogenase type 5), CYP17A1 (steroid 17α-hydroxylase/17,20 lyase), and Cytochrome b5 (CYB5) showed significantly lower expression in APA compared with AAG ($P < 0.05$; Fig. 3). These genes also showed significant differences between APA and AAG when analyzed by qPCR (Fig. 4, and Table 2). Additionally, microarray analysis did not indicate any significant differences for the expression of cholesterol side-chain cleavage (CYP11A1), StAR protein (StAR), 3β-hydroxysteroid dehydrogenase (HSD3B2), 21-hydroxylase (CYP21A2), 11β-hydroxylase (CYP11B1), cytochrome P450 oxidoreductase (POR), or steroid sulfotransferase type 2A1 (SULT2A1; Fig. 3).

Discussion

In this study, we investigated the molecular mechanisms leading to APA by comparing the whole transcriptome of APA with their AAG tissues. Using microarray analysis, 14 transcripts were significantly elevated in APA, and qPCR assay confirmed the microarray results for the genes of interest. Most of the genes have not...
previously been studied in adrenal carcinoma or adenoma tissues, and several are good candidates as regulators of aldosterone secretion (8, 10–12, 20–22). One such example is PCP4, which is capable of binding calmodulin and regulating the dissociation of calcium from calmodulin (23, 24). The calcium–calmodulin cascade plays an important role in the expression of CYP11B2 (11, 13, 25), and therefore PCP4 could participate in regulating aldosterone production. Another example is the gene that encoding PRRX1, a member of the paired family of homeobox proteins which is localized in the nucleus and functions as a transcription co-activator (26, 27).

One unique feature of the current study is its use of paired APA–AAG samples from the same patient to study gene expression differences. This comparison led to the identification of several genes not previously noted in APA array studies, in which the control adrenal tissues were from autopsy or patients who underwent adrenalectomy along with renal carcinoma. Compared with these adrenal tissues, AAG showed morphological remodeling, but without transcriptional phenotype difference (28). Moreover, APA production of aldosterone causes these patients to have low plasma renin activity and Ang II (1, 5). Thus, the adjacent adrenal tissue in this study is exposed to relatively lower Ang II levels, which was not controlled in previous studies (8, 10). So the transcriptome comparison with paired APA–AAG could have less variation because these tissues are exposed to the same hormonal milieu. Interestingly, a qPCR analysis showed that CYP11B2 expression between our AAG and normal adrenal gland (obtained from autopsy or renal cancer surgery) was not statistically different; and compared with the normal adrenal glands, the AAG showed smaller variation in CYP11B2 expression (data not shown). This is likely because most subjects, even those without PA, are on a relatively high sodium diet and a relatively inactive glomerulosa. This concept is supported by the use of recently developed CYP11B2 antibodies show that normal adrenal glands exhibit few cells in the glomerulosa with detectable expression (29).

Several studies suggested that APA has altered expression of several genes encoding various steroidsigenic enzymes when compared to normal adrenal tissue. This observation has led many to speculate that the abnormal production of aldosterone and ‘hybrid’ steroids such as 18-oxocortisol by APA tissue may result from the disordered expression of multiple steroidsigenic enzymes (8, 11). As expected, our microarray data demonstrated that CYP11B2 was expressed at much higher levels in APA compared with AAG. In contrast, most transcripts encoding other steroidsigenic enzymes were not different in APA, including StAR, which controls the acute rate-limiting first step in steroidsigenesis (30, 31). Nevertheless, we

Figure 1 Heat map representation from microarray analysis showing transcripts with elevated expression in APA versus AAG tissue. The list was prepared based on genes that were significantly increased by >2.5-fold (P < 0.05) in APA compared to AAG. RNA from ten APA–AAG pairs was used for microarray analysis on 20 arrays. The heat map color indicates the expression value of each gene in the corresponding sample based on the log2 of signal strength (see color bar). The mean fold induction of each APA–AAG pair with P value between all APA and AAG using paired t-test is also provided (n = 10). APA–AAG sample sets are paired by the indicated numbers. (** indicates a P value < 0.001, T = APA; N = AAG). Full colour version of this figure available via http://dx.doi.org/10.1530/EJE-10-1085.

Figure 2 Quantification of up-regulated gene transcripts in APA–AAG pairs using qPCR. The vertical point scatter plot compares the expression of CYP11B2, PCP4, PRRX1, VPREB3, and ALDH1A2 between each APA–AAG pair. Each plot represents the fold change within each sample pair, normalized to 18S rRNA.
found decreased expression of the transcripts normally associated with adrenal androgen production including AKR1C3, CYP17A1, and CYB5 in APA. These results were consistent with the selectively increased aldosterone production from APA.

In the current study, microarray analysis suggested that the fold difference for CYP11B2 expression was smaller than we and others have previously observed when APA tissue was compared to adrenal glands from autopsy and renal cancer patients (8, 10, 32, 33). However, our qPCR analysis of the CYP11B2 transcript agrees with previous reports of a larger than tenfold difference between APA and normal adrenal tissue (8). This may be related to some cross-reactivity of the Illumina microarray CYP11B2 oligonucleotide with the highly similar 11β-hydroxylase (CYP11B1) transcripts. CYP11B2 is overexpressed in APA, which has been widely reported and also confirmed by our study (1, 10, 12). In vitro and in vivo studies have shown that CYP11B2 expression is mainly regulated by Ang II, whereas PA patients have low plasma renin activity, leaving the mechanism of autonomous CYP11B2 expression in APA unknown. Ye et al. (10) showed that the expression of certain G-protein-coupled receptors (GPCRs) were greatly up-regulated in APA when compared with normal adrenal glands. We found a small but significant increase in the expression of several genes encoding GPCRs in APA using our pairwise comparison, but the overall fold increase was <2.5 (data not shown). These results further suggest that some GPCRs may be involved in the molecular mechanisms of APA, at least in some tumors. As noted previously, the ectopic expression of GPCRs may occur in only a small subset of APA (10). Other studies suggest that the expression of transcription factors such as nerve growth factor-induced clone B (NGFIB), steroidogenic factor-1 (SF-1), and dosage-sensitive sex reversal, adrenal hypoplasia congenita critical region on the X chromosome gene 1 (DAX-1) (8), or signaling molecules such as calcium/calmodulin-dependent protein kinase I (CAMKI) might increase CYP11B2 expression in APA (12); however, these genes were not found to be differentially up-regulated in our study using pairwise comparison.

Microarray analysis probes tens of thousands of genes simultaneously; therefore, it has become a useful tool to identify genes involved in tumorigenesis (18, 33–36). Several lines of evidence support the validity of our microarray analysis. First, our microarray results are in general agreement with the data obtained from qPCR. Second, a high CYP11B2 expression and low expression of AKR1C3, CYP17, and CYB5 are found in APA tissue, which is consistent with previous studies (8, 11, 32). Our results also indicate a series of novel genes, which are highly expressed along with CYP11B2 in APA. These transcripts sort with CYP11B2 as markers for
APA versus AAG and could contribute to Ang II-independent CYP11B2 expression, aldosterone production, and growth in APA. Elucidating the action of these genes in the adrenal cortex and APA might identify important mechanisms of APA growth and function, which might then lead to new strategies to diagnose and treat PA.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by National Institutes of Health Grants DK-43140 (to W E Rainey), a Synergy Award from the Cardiovascular Discovery Institute, Medical College of Georgia (to W E Rainey and M A Edwards), a Clinical Scientist Award in Translational Research (to W E Rainey), an R01-HL-090991 (to W E Rainey), a Synergy Award from the Cardiovascular Discovery Institute, Medical College of Georgia (to W E Rainey and M A Edwards), an R01-DK-43140 (to W E Rainey), a Synergy Award from the Cardiovascular Discovery Institute, Medical College of Georgia (to W E Rainey and M A Edwards), and the Houston J and Florence A Dowell Center for the Development of New Approaches for the Treatment of Hypertension at UT Southwestern.

Acknowledgement

We would also like to acknowledge the editorial assistance of Dr Mary Bassett.

References


Received 18 November 2010
Accepted 1 January 2011