Aldosterone-Producing Adenomas

Different Expression of 11β-Hydroxylase and Aldosterone Synthase Between Aldosterone-Producing Microadenomas and Macroadenomas

Yoshikiyo Ono, Yasuhiro Nakamura, Takashi Maekawa, Saulo J.A. Felizola, Ryo Morimoto, Yoshitsugu Iwakura, Masataka Kudo, Kazumasa Seiji, Kei Takase, Yoichi Arai, Celso E. Gomez-Sanchez, Sadayoshi Ito, Hironobu Sasano, Fumitoshi Satoh

Abstract—Aldosterone-producing adenoma is a major subtype of primary aldosteronism. The number of cases of these adenomas, which are below the detection limit of computed tomography but diagnosed by adrenal venous sampling, has recently been increasing. However, the pathophysiology of these adenomas, especially those manifesting clinically overt hyperaldosteronism despite their small size, remains unknown. Therefore, we examined the correlation between tumor size and the status of intratumoral steroidogenic enzymes involved in aldosterone biosynthesis using immunohistochemistry. Forty patients with surgically proven aldosterone-producing adenomas were retrospectively studied. Multidetector computed tomography, adrenal venous sampling, and laparoscopic adrenalectomy were performed in all of the patients studied. The tumor area at the maximum diameter of the sections was precisely measured by ImageJ software. The status of the steroidogenic enzymes was immunohistochemically analyzed, and the findings were evaluated according to the H-score system, based on both the number of immunopositive cells and relative immunointensity. Adrenal masses were not detected by computed tomography in 20 patients. Blood pressure, plasma aldosterone concentration, urinary aldosterone excretion, and the number of antihypertensive agents also decreased significantly after the surgery in these patients, as well as in the patients with adenomas detectable by computed tomography. Maximum tumor area obtained in the specimens was significantly correlated with preoperative plasma aldosterone concentration, urinary aldosterone excretion, and the H score of 11β-hydroxylase and was inversely correlated with the H score of aldosterone synthase. These results demonstrated that small adenomas could produce sufficient aldosterone to cause clinically overt primary aldosteronism because of the significantly higher aldosterone synthase expression per tumor area.

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Key Words: adrenocortical adenoma • CYP11B1 • CYP11B2 • hyperaldosteronism • hypertension • immunohistochemistry

Primary aldosteronism (PA) is the most common form of secondary hypertension. The prevalence of PA is reported to be ≈5% to 10% in patients with hypertension and ≈20% in patients with resistant hypertension. Patients with PA are well known to have higher incidences of cardiovascular and cerebrovascular diseases than those with essential hypertension, even at the same blood pressure. In addition, we recently reported that PA should be detected early and treated to prevent the prevalence of chronic kidney disease. Therefore, it has become important to detect PA early in its clinical course.

More than 90% of PA cases involve bilateral adrenal hyperplasia and aldosterone-producing adenoma (APA); rare cases include unilateral adrenal hyperplasia, aldosterone-producing adrenocortical carcinoma, and familial forms. Patients with APA or unilateral adrenal hyperplasia can benefit from adrenalectomy, whereas those with bilateral hyperaldosteronism should be treated with mineralocorticoid receptor antagonists. Therefore, classifying the subtypes of PA is critical for developing clinical algorithms for patients. The Endocrine Society guidelines recommend adrenal computed tomographic (CT) scanning and adrenal venous sampling (AVS) before the surgical treatment of PA. AVS is currently considered the only reliable method for differentiating unilateral disease from bilateral hyperaldosteronism. Young et al reported that 110 of 194 patients were diagnosed with a unilateral source of hyperaldosteronism by AVS, whereas 31 (30%) of 102 patients with unilateral APA had a small tumor undetectable by CT; in addition, 8 of these 31 patients had...
CT-detectable nonfunctioning nodules in the contralateral adrenal gland. Furthermore, smaller APAs undetectable by CT, which can only be diagnosed by AVS, have been reported in other institutions.\textsuperscript{11-16} The prevalence of CT-undetectable APA among all APA patients is currently estimated to be 13% to 30%. In addition, hypertension was cured or markedly improved after adrenalectomy in almost all reported cases.\textsuperscript{13-16} Such small APAs undetectable by CT have been histopathologically analyzed, and the reasons why aldosterone hypersecretion from CT-undetectable small adenomas is sufficient to cause clinically overt PA have remained unknown. The main purpose of this study was to explore the reasons why the mean aldosterone secretion capacity of CT-undetectable small APA (microadenomas) could reach as much as that of CT-detectable large APA (macroadenomas) and the reasons why the clinical improvement after surgical treatment in both APA could be similar. Therefore, we evaluated the correlation between tumor size and the status of steroidogenic enzymes including 3β-hydroxysteroid dehydrogenase (HSD3B), 17α-hydroxylase (CYP17A1), 11β-hydroxylase (CYP11B1), and aldosterone synthase (CYP11B2), which are all related to aldosterone production, using immunohistochemistry to clarify the status of aldosterone biosynthesis in small APAs.

\textbf{Methods}

\textbf{Patients}

From May 2010 to October 2012, we experienced 100 APA cases that consisted of 20 CT-undetectable cases and 80 CT-detectable cases. We then selected 1 of every 4 cases continually among CT-detectable cases to be able to compare the same number of the cases. Therefore, we could study 40 patients with APAs in this study. The study protocols were approved by the Ethics Committee of Tohoku University School of Medicine, and all patients provided informed consent before participation. All patients were diagnosed with PA on the basis of our previously published protocols.\textsuperscript{16} Blood pressure was measured with Omron Hem 907 (Omron Healthcare Co. Ltd, Kyoto, Japan) after \( \geq 15 \) -minute rest in a sedentary position, and the average of 3 consecutive measurements was recorded.\textsuperscript{17} Patients were treated with calcium channel blockers and \( \alpha \)-blockers during the workup of PA. Patients with a plasma aldosterone concentration (PAC)/plasma renin activity ratio (ng·d\textsuperscript{-1}·mL\textsuperscript{-1}·h\textsuperscript{-1}) \( > 20 \) after challenge with captopril 50 mg were diagnosed with PA. No patients examined had concurrent cortisol-producing adenoma, which was confirmed by an overnight dexamethasone 1 mg suppression test.

\textbf{Measurements}

CT scanning was performed with a 64-channel multidetector row CT (Aquilion, Toshiba, Tokyo, Japan), which can analyze adrenal glands in contiguous 1.0-mm-thick slices at 1.0-mm intervals. A nonionic iodinated contrast agent was administered intravenously in a routine manner.\textsuperscript{18} All the cases were evaluated by 3 radiologists well trained in adrenal imaging, and discordant interpretations were resolved by consensus.

All the cases underwent AVS following the protocol, which is shown in the online-only Data Supplement. All patients underwent laparoscopic unilateral adrenalectomy on the basis of AVS findings and subsequent pathological examination to confirm the existence of APA in the resected adrenal gland.

\textbf{Quantitative Histopathology and Immunohistochemistry}

Forty adrenal gland specimens were retrieved from the surgical pathology files of Tohoku University Hospital. The specimens had been fixed in 10% formalin for 48 hours at room temperature, sectioned at 2-mm intervals, and embedded in paraffin. Adrenocortical adenomas were morphologically defined as well-circumscribed solitary adrenocortical masses of different sizes. Because adrenal tumors are not always round and are often elliptical, the maximum diameter and area of each tumor were determined on hematoxylin and cosin-stained tissue slides by ImageJ version 1.47 (National Institutes of Health, Bethesda, MD). The adrenal tumors were photographed by a CCD camera attached to a light microscope.

Immunohistochemical staining was performed with antibodies against CYP11B1 (1:200, rat monoclonal), CYP11B2 (1:750, mouse monoclonal), HSD3B (rabbit polyclonal),\textsuperscript{19} and CYP17A1 (rabbit polyclonal),\textsuperscript{20} and the antibodies against CYP11B1 and CYP11B2 were recently developed in the laboratory of Dr Gomez Sanchez (Department of Endocrinology, University of Mississippi Medical Center, Jackson, MS).\textsuperscript{21} Detailed technical aspects of immunohistochemical staining are summarized in the online-only Data Supplement.

In each case, 500 parenchymal cells were evaluated in each region, and the ratio of positive cells in the tumors was subsequently obtained. Immunoreactivity was assessed semiquantitatively according to the McCarty H score, in which the percentage of stained cells is multiplied by a number from 0 to 3, reflecting the intensity of their immunopositivity.\textsuperscript{22} The relative immunointensity of specific immunoreactivity was characterized as not present (0), weak but detectable above control (1+), distinct (2+), or very strong (3+). In addition, in all APA cases examined, 3 independent observers (Y.O., Y.N., T.M.) evaluated the H scores, the averages of which were obtained in the blind fashion. The individual clinical data were not informed when they evaluated the H scores. When there was discordance or differences in their evaluation, the immunostained slides were simultaneously re-evaluated using multtheaded light microscopy until the consensus was reached. Therefore, the evaluation using H score of immunoreactivity of the enzymes is considered the best method currently available to allow the estimation of steroidogenic enzyme activity in the objective fashion in clinical materials of the patients.

\textbf{Statistical Analysis}

All data are presented as mean±SEM and 25th to 75th percentile ranges. Differences in measured parameters between groups were evaluated using the Mann–Whitney \( U \) test and \( \chi^2 \) test. Univariate correlations were determined by calculating Spearman rank correlation coefficients. The level of significance was set at \( P \leq 0.05 \). All analyses were performed using JMP version 10 (SAS Institute Inc, Cary, NC).

\textbf{Results}

\textbf{Clinicopathological Examination}

Histological examination indicated the range of the maximum tumor diameter and area as 2 to 28 mm and 3 to 439 mm\textsuperscript{2} (median, 60.6 mm\textsuperscript{2}). Because of the noncircular shapes of the tumors, the precise area determined by ImageJ (113.8±18.5 mm\textsuperscript{2}) differed significantly from the circular area calculated on the basis of the maximum diameter.
(148.4±24.0 mm²; P<0.001). Therefore, we tentatively classified 40 APA cases into the following 2 groups: the smaller and larger groups determined at 60 mm², which represents the median of the area of APAs that we could obtain using ImageJ. The average diameter and area of adrenal tumors were significantly smaller in the smaller group than in the larger group (P<0.001). There were no significant differences between these 2 groups with respect to the histological features (eg, clear/compact cell type predominance).

The preoperative clinical and endocrinologic characteristics of the patients are summarized in the Table. The prevalence of hypokalemia (P<0.001), PAC (P<0.001), and urinary aldosterone excretion (P<0.05) were significantly lower in the smaller group than in the larger group. However, the ratio of male patients, age, hypertension duration, and serum potassium level were significantly greater in the smaller than in the larger group (all P<0.05). The tumor area ratio of the larger/smaller group reached to >9x, but the capacity of aldosterone secretion per tumor area of the smaller group was estimated to be much higher than that of the larger group because the plasma aldosterone concentration ratio and an aldosterone urine excretion ratio in the larger group were 2.5x and 2x higher than those in the smaller group, respectively (Table).

This estimation could account for one of the reasons why the clinical improvements after surgical treatments in both groups were similar (shown in Figure 2). In addition, after unilateral adrenalectomy, the systolic/diastolic blood pressures significantly decreased by 22.2±4.3/18.3±3.6 and 25.3±5.0/15.6±2.5 mm Hg in the smaller and larger groups, respectively (mean±SEM; all P<0.05). Blood pressure reductions remained stable for 21 year of postoperative follow-up. Neither systolic blood pressure reductions nor diastolic blood pressure reductions differed significantly between the 2 groups during the 1-year postoperative follow-up period (Figure 2A and 2B). The number of antihypertensive agents, PAC, and urinary aldosterone excretion all decreased significantly in both groups, and serum potassium level was significantly elevated (Figure 2C–2F).

### Table. Clinical Characteristics of the Smaller and Larger Aldosterone-Producing Adenoma Groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total</th>
<th>Smaller Group (&lt;60 mm²)</th>
<th>Larger Group (≥60 mm²)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male, female)*</td>
<td>28, 12</td>
<td>17, 3</td>
<td>11, 9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Age, y*</td>
<td>52.2±1.9 (43–61.5)</td>
<td>57.7±2.8 (51.2–66.0)</td>
<td>46.7±2.1 (38.3–53.0)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Duration of hypertension, y*</td>
<td>10.6±1.3 (5.0–13.5)</td>
<td>13.6±2.2 (5.5–20)</td>
<td>7.6±1.4 (4.0–11)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>No. of antihypertensive drugs</td>
<td>3.95±0.29 (3.0–5.0)</td>
<td>4.00±0.39 (2.3–5.0)</td>
<td>3.90±0.45 (3.0–4.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.4±0.5 (23.0–27.4)</td>
<td>25.9±0.7 (24.1–28.1)</td>
<td>24.8±0.8 (22.6–26.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>149.2±3.0 (134–161)</td>
<td>144.9±4.0 (130–161)</td>
<td>153.6±4.6 (139–165)</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>94.1±2.0 (85–102)</td>
<td>93.5±3.2 (84–102)</td>
<td>94.6±2.4 (88–102)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum creatinine, mg/dL</td>
<td>0.79±0.04 (0.7–0.9)</td>
<td>0.81±0.04 (0.7–0.9)</td>
<td>0.77±0.06 (0.6–1.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Sodium excretion, mEq/L</td>
<td>3.55±0.08 (3.1–4.0)</td>
<td>3.75±0.10 (3.5–4.0)</td>
<td>3.37±0.12 (2.9–3.8)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Prevalence of hypokalemia, %†</td>
<td>42.5</td>
<td>20</td>
<td>65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma aldosterone concentration, ng/dL†</td>
<td>31.9±3.8 (16.8–44.9)</td>
<td>18.5±1.9 (13.4–20.5)</td>
<td>45.4±6.2 (29.6–52.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum cortisol, µg/dL</td>
<td>7.55±0.53 (5.0–9.5)</td>
<td>7.38±0.60 (4.5–8.8)</td>
<td>7.7±0.97 (5.2–10)</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma renin activity, ng·mL⁻¹·h⁻¹</td>
<td>0.20±0.02 (0.1–0.3)</td>
<td>0.16±0.10 (0.10–0.28)</td>
<td>0.24±0.04 (0.1–0.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Aldosterone/renin activity, ng·dL⁻¹ per ng·mL⁻¹·h⁻¹</td>
<td>220.5±29.5 (71.5–283)</td>
<td>156.4±23.2 (64–199)</td>
<td>294.5±51.1 (81.4–522)</td>
<td>NS</td>
</tr>
<tr>
<td>Urinary aldosterone excretion, µg/d*</td>
<td>20.3±2.7 (10.5–25.0)</td>
<td>13.6±1.8 (8.5–15.7)</td>
<td>27.0±4.7 (16.0–30.3)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Urinary free cortisol excretion, µg/d</td>
<td>46.4±5.1 (25.4–54.3)</td>
<td>48.3±8.2 (24.3–58.6)</td>
<td>44.8±6.2 (27.2–49.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Corticosteroid challenge ARR, ng·dL⁻¹ per ng·mL⁻¹·h⁻¹</td>
<td>217.9±61.6 (57.2–261)</td>
<td>93.2±9.7 (64.9–120)</td>
<td>342.6±117.7 (43.2–356)</td>
<td>NS</td>
</tr>
<tr>
<td>Cortisol after DST, µg/dL</td>
<td>13.0±11.0 (8.1–18)</td>
<td>1.27±0.16 (0.8–1.8)</td>
<td>1.33±0.16 (0.8–1.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Lateralization index in AVS before cosyntropin loading*</td>
<td>18.19±4.0 (3.4–26.9)</td>
<td>11.1±3.0 (3.0–12.1)</td>
<td>26.60±7.3 (5.6–36.7)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Lateralization index in AVS after cosyntropin loading</td>
<td>9.47±1.05 (4.5–13.1)</td>
<td>7.95±0.97 (4.0–9.7)</td>
<td>11.46±1.83 (6.3–15.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Diameter of adrenal tumor, mm†</td>
<td>11.9±1.1 (6.3–17.3)</td>
<td>5.9±0.6 (4.0–7.8)</td>
<td>17.8±1.0 (15.0–20.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Area of adrenal tumor, mm²†</td>
<td>113.8±18.5 (16.6–189.4)</td>
<td>22.2±3.5 (9.8–31.0)</td>
<td>205.5±23.0 (132.3–257.0)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

All data are shown in the following order: mean±SEM (25th–75th percentile), except for sex and prevalence of hypokalemia. Hypokalemia is defined as serum potassium concentration <3.5 mmol/L. ARR indicates plasma aldosterone concentration per plasma renin activity; AVS, adrenal venous sampling; DST, 1 mg dexamethasone suppression test; and lateralization index, aldosterone/cortisol ratio.

*P<0.05, †P<0.001, both are significantly different between smaller group and larger group.

Immunohistochemistry of Steroidogenic Enzymes

Representative immunohistochemistry results of HSD3B, CYP17A1, CYP11B1, and CYP11B2 in the smaller APA group (case 1) and larger APA group (case 2) are shown in Figure 3. HSD3B immunoreactivity was markedly and diffusely present in all APA cases examined (Figure 3B and 3G). In contrast, CYP17A1-positive tumor cells were heterogeneously distributed, and their relative immunoreactivity was weak (Figure 3C and 3H). The immunoreactivity of CYP11B1 was weaker in the smaller APA group than in the larger APA group (Figure 3D and 3I). In contrast, the immunoreactivity of CYP11B2 was comparatively stronger in the smaller APA group (Figure 3J and 3K).
APA group than in the larger APA group (Figure 3E and 3J). The immunoreactivity of both CYP11B1 and CYP17A1 was mainly detected in the same tumor cells. We evaluated these steroidogenic immunoreactivities according to the H-score system (Figure 4). The H score of HSD3B was not significantly different between the 2 groups (Figure 4A). The H score of CYP17A1 immunoreactivity in adenoma cells tended to be higher in the larger APA group, but the difference did not reach statistical significance (Figure 4B).

In the smaller APA group, the H score of CYP11B1 was significantly lower ($P<0.005$) and the H score of CYP11B2 was significantly higher ($P<0.001$) than the corresponding scores in the larger APA group, respectively (Figure 4C and 4D).

Furthermore, we examined the correlation between the H scores and tumor area (Figure 5). Although the H scores of HSD3B and CYP17A1 were not significantly correlated with tumor area (Figure 5A and 5B), the H score of CYP11B1 was significantly correlated with tumor area (Figure 5C; $P<0.005$), and the H score of CYP11B2 was inversely significantly correlated with tumor area (Figure 5D; $P<0.005$). Both preoperative PAC and urinary aldosterone excretion were significantly correlated with the values of H score of CYP11B2 multiplied by tumor area (Figure S1A and S1B in the online-only Data Supplement) in our present study. Therefore, in the larger group, the higher levels of PAC and urinary aldosterone excretion were mainly postulated to be a result of the much larger volume of tumor. In addition, the values of PAC and urinary aldosterone excretion were also significantly correlated with those of H score of CYP11B1 multiplied by tumor area (Figure S1C and S1D). It is therefore reasonably postulated that the higher H score of CYP11B1 could synergistically contribute to the higher aldosterone secretion in the larger group.

**Discussion**

To the best of our knowledge, this is the first study to evaluate the correlation between APA tumor area and CYP11B1
and CYP11B2 immunoreactivity using specific monoclonal antibodies. Immunoreactivity was analyzed in a quantitative fashion according to the H-score system to evaluate both the percentage and the intensity of positively stained cells, whereas tumor area was precisely measured by ImageJ. In both smaller and larger groups, laparoscopic adrenalectomy based on the results of AVS significantly improved blood pressure, PAC, urinary aldosterone excretion, and the number of anti-hypertensive drugs. These results also support the clinical use and validity of the AVS-based diagnosis and management of APA. In our present study, the H score of CYP11B1 was significantly higher in the larger group but that of CYP11B2 was significantly higher in the smaller group. However, the status of autonomous cortisol production in the tumors diagnosed by the dexamethasone suppression test or urinary free cortisol excretion was not significantly different between these 2 groups. Therefore, the different H-score status of intratumoral CYP11B1 and CYP11B2 in these 2 groups does not necessarily account for the difference in cortisol production but could explain the difference of aldosterone production between these 2 groups. Therefore, the different H-score status of intratumoral CYP11B1 and CYP11B2 in these 2 groups does not necessarily account for the difference in cortisol production but could explain the difference of aldosterone production between these 2 groups. These findings did indicate that much larger amounts of precursors of aldosterone such as corticosterone could be produced by CYP11B1 in the larger APA than in the smaller APA group, and such precursors could be converted to aldosterone by CYP11B2 in the hyperplastic zona glomerulosa of adjacent adrenal cortex, but this hypothesis required further studies.

The tumor area of APAs determined by quantitative histological analysis was significantly correlated with preoperative aldosterone levels and urinary aldosterone excretion. The total production of steroids including aldosterone was generally considered higher in the larger APA group than in the smaller APA group. However, tumor area was inversely correlated with the H score of CYP11B2, which is the rate-limiting step of aldosterone biosynthesis, and positively correlated with the H score of CYP11B1. These findings did demonstrate that the smaller tumors had higher CYP11B2 expression per area and cell. It is entirely true that CYP11B2 levels alone do not necessarily represent abundant aldosterone production, because several other factors (eg, the levels of steroidogenic enzymes upstream of CYP11B2) also play pivotal roles in overall aldosterone production.23,24 However, this marked expression of CYP11B2 per area and cell in the tumors may at least explain why small APAs below the detection limit of CT can result in clinically overt hyperaldosteronism. Of particular interest, both PAC and urinary aldosterone excretion were significantly correlated with the values of H scores of CYP11B2 multiplied by tumor area. These findings did indicate that both higher levels of PAC and urinary aldosterone excretion could synergistically contribute to the higher PAC and urinary aldosterone excretion...
detected in the larger group of APA patients. However, further investigations are also required to clarify the reasons why larger APA expressed the low H score of CYP11B2, despite the high H scores of HSD3B and CYP11B1.

CT is currently considered one of the best diagnostic tools for detecting adrenal tumors. However, it is also difficult to differentiate unilateral from bilateral hyperaldosteronism solely on the basis of CT findings, because CT and AVS findings are reported to be discordant in only 50% to 70% of cases.11,12,16 In addition, CT has a detection limit on the size of adrenal mass lesions. For example, Omura et al13 reported that CT could not detect adrenal tumors ≤6 mm in diameter. In the smaller APA group in our present study, there were 7 cases in which the tumor diameter evaluated on histological sections exceeded 6 mm (range, 7–10 mm), whereas CT did not detect these tumors. The maximum widths of the 95th percentile measurements of normal adrenal glands were 12.2 and 9.9 mm in the left and right sides, respectively.25 Therefore, if a small adrenocortical tumor is located and embedded in a relatively thick section, CT would be unable to detect it. These clinical difficulties in detecting smaller sized adrenocortical tumors could account for the older age and longer duration of hypertension in the smaller group than in the larger group.

Perspectives

Results of the present study did reveal that hypertension was markedly ameliorated after adrenalectomy in both smaller and larger groups. In addition, the relatively higher CYP11B2 expression per area in the smaller group could clinically cause the older age and longer duration of hypertension in the smaller group than in the larger group.

Disclosures

Y. Nakamura was partly supported by the Takeda Science Foundation. The other authors report no conflicts.

References


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**Novelty and Significance**

**What Is New?**

- The present study is the first to investigate the correlations between aldosterone-producing adenoma tumor area size and CYP11B1 and CYP11B2 immunoreactivity using specific monoclonal antibodies.

**What Is Relevant?**

- Significantly higher CYP11B2 expression in small aldosterone-producing adenomas could explain why small aldosterone-producing adenomas cause aldosteronism, although they are undetectable by computed tomography.

**Summary**

Small adenomas could produce sufficient aldosterone to cause clinically overt primary aldosteronism because of the significantly higher CYP11B2 expression per tumor area.
Different Expression of 11β-Hydroxylase and Aldosterone Synthase Between Aldosterone-Producing Microadenomas and Macroadenomas
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ONLINE DATA SUPPLEMENT

The different expression of CYP11B1 and CYP11B2 between aldosterone-producing microadenomas and macroadenomas

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Methods of adrenal venous sampling (AVS)

Bilateral adrenal veins were simultaneously catheterized in all patients. After a 60-min rest in the supine position, 2 venous catheters were introduced via the bilateral femoral veins. After baseline samples were simultaneously obtained from both adrenal veins, all the patients received an intravenous bolus injection of cosyntropin (200 µg). A second set of blood samples was collected from the same sites 15 min later. Continuous cosyntropin infusion (50 µg/h) was started 30 min after cosyntropin bolus injection. All blood samples in AVS were collected at 15-45 minutes after 200 µg of cosyntropin bolus infusion. The catheter placements and success of adrenal venous cannulation were confirmed as just before and after sampling using a very small amount of contrast medium. The selectivity index was defined as the level of cortisol in the adrenal vein divided by that in the inferior vena cava following cosyntropin administration. The smallest selectivity index among the patients was 11.5. Meanwhile, the lateralization index was used to determine the laterality of aldosterone excess. The lateralization index was defined as the aldosterone/cortisol ratio in the adrenal vein divided by that in the contralateral adrenal vein. The lateralization index after cosyntropin stimulation exceeded 4.0 in 33 patients and was between 2.6 and 4 in 7 patients (mean: 9.5, range: 2.6–36). The lateralization index under basal condition with 2.6 and 4 after cosyntropin stimulation was 9.4±4.0 (range: 1.1-31.7).

Methods of immunohistochemical staining

For immunohistochemical staining, 5-µm-thick sections were cut on a microtome and deparaffinized with xylene and ethanol. To detect CYP17A, sections were antigen-retrieved with an autoclave (5 min in citric acid buffer, pH 6.0). To detect HSD3B and CYP17A, sections were treated with a blocking reagent (Histofine, Nichirei, Tokyo, Japan) for 30 min at room temperature. Sections were incubated with either αHSD3B (1:2,500) or αCYP17A1 (1:500) overnight at 4°C. Immunoreactivity was visualized with 3,3′-diaminobenzidine (DAB; brown staining) with a peroxidase-based Histofine Simple Stain Kit (Nichirei, Tokyo, Japan) and counterstained with hematoxylin. Immunostaining for CYP11B1 and CYP11B2 was performed using the streptavidin-biotin amplification method using ImmPRESS reagent (Vector, Burlingame, CA, USA). Antigens were retrieved by heating the glue-coated slides in ethylenediaminetetraacetic acid (EDTA; pH 9.0) in an autoclave for 5 min. Blocking was performed for 1 hour using blocking buffer (normal horse serum 5% with sodium dodecyl sulfate 0.5%) at room temperature. Antigen-antibody complexes were visualized with DAB solution (1 mmol/L DAB, 50 mmol/L Tris-HCl buffer [pH 7.6], and 0.006% H2O2) and counterstained with hematoxylin.
**Figure S1.** The value of H-score of CYP11B2 multiplied by tumor area was significantly correlated with plasma aldosterone concentration (A) and urinary aldosterone excretion (B). The value of H-score of CYP11B1 multiplied by tumor area was significantly correlated with plasma aldosterone concentration (C) and urinary aldosterone excretion (D).