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Analysis of mRNA Expression for Steroidogenic Enzymes in the Remaining Adrenal Cortices Attached to Adrenocortical Adenomas

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Abstract

We recently demonstrated that the adrenal cortices attached to aldosterone-producing adenoma (APA) contained microscopic subcapsular micronodules suggestive of active aldosterone production. In this study, we used in situ hybridization to investigate the mRNA expression of steroidogenic enzymes in the adrenal cortices attached to cortisol-producing adenoma (CPA) and clinically silent adenoma (nonfunctioning adenoma; NFA), in addition to APA. Microscopic subcapsular micronodules, which were several to hundreds of micrometers in size and spheroid in shape, were observed in the cortices attached to CPA and NFA, as well as APA, at high frequency. Most of the cortical nodules in zona fasciculata to zona reticularis showed a suppressed steroidogenesis in the cortices attached to adenoma, but some expressed intensely all necessary steroidogenic enzyme mRNAs for cortisol synthesis. It is thus necessary to keep in mind, on the occasion of subtotal adrenalectomy, that lesions with the potential to later develop into functional adrenocortical nodules may be present in other parts of the ipsilateral or contralateral adrenal cortices. (162 words)

Introduction

Hyperfunction of the adrenal cortex is associated with either hyperplasia, adenoma, or carcinoma. There are principally three forms of adrenocortical hyperfunction, namely primary aldosteronism (PA), Cushing's syndrome (CS) and congenital adrenal hyperplasia. PA and CS are thought to occur as quite distinct entities. Recently, case reports of adrenocortical adenoma overproducing both aldosterone and cortisol are becoming more frequent (1, 2), probably as the result of improvements in examination accuracy. Multiple or bilateral adrenocortical adenomas causing both PA and CS at the same time or at different periods have also been reported (3 - 5). These findings suggest that the same or a similar pathological condition may exist in the pathogenic background of adrenocortical adenomas, regardless of the type of hormone produced in excess.

In aldosterone-producing adenoma (APA), the remaining adrenal cortices often show hyperplasia of the zona glomerulosa (ZG), and one or more micro- or macronodules. Unlike the cortices adhering to APA, the remaining cortices in cortisol-producing

adenoma (CPA) invariably appear atrophic, but cortical nodules with a diffuse brown or yellow colour can also be seen occasionally, especially in cases of ACTH-independent bilateral or multiple adrenocortical adenomas (6, 7). Most of the cortical nodules in the cortices attached to functional adrenocortical adenomas have been thought to be incidental and nonfunctional, probably related to vascular sclerosis (8). However, histochemical and electron microscopic studies have shown that some ischemia-related cortical nodules had steroidogenic activity (9, 10). We recently demonstrated that the cortices attached to APA contained not only microscopic subcapsular micronodules suggesting active aldosterone production, as observed in BAH and UAH, but also cortical nodules suggesting the capability for producing cortisol (11). Hence, the possibility is suggested that buds with autonomous production of aldosterone or cortisol may exist in the cortices attached to APA.

Laparoscopically subtotal adrenalectomy with preservation of functional adrenal tissue has been attempted (12, 13). This approach would seem to be successful, although the postoperative long-term follow-up is often inadequate (5, 7, 14). Meanwhile, the recurrence of PA or CS following total or subtotal adrenalectomy has been also reported

(3, 15 – 17). Therefore, knowledge about what lesions are present in the remaining cortices is essential. In this study, we used in situ hybridization to examine the expression of mRNAs for steroidogenic enzymes in the remaining adrenal cortices attached to adrenocortical adenomas associated with PA and CS, as well as in clinically silent cases (NF).

Materials and Methods

Tissue Samples

Of the paraffin-embedded human adrenal tissues listed in the Division of Pathology, Nagasaki University Graduate School of Biomedical Sciences, and Red-Cross Nagasaki Atomic Bomb Hospital between 1988 and 2006, 19 cases of CP and 11 of NF were identified (Table 1). Seven cases of PA operated on between 2005 and 2006 were also selected (Table 1). As controls, we used five adrenal glands obtained from patients undergoing adrenalectomy together with pancreatectomy or nephrectomy for pancreatic

or renal cancer, who did not reveal any endocrine abnormalities. PA was diagnosed on the basis of an elevated plasma-aldosterone concentration and suppressed plasma-renin activity or concentration. The diagnosis of CS was based on symptoms such as obesity, moon face, buffalo hump, abdominal striae etc., elevated plasma-cortisol concentration with lack of diurnal rhythm, and suppressed plasma ACTH level. The exact location of the lesions in all cases of PA and CS was determined by adrenal venous sampling (AVS). Clinically silent adenoma (NFA), showing normal-range plasma aldosterone and cortisol concentrations, was incidentally detected by CT scan. However, cases 30, 32, 34, 36 and 37 showed low levels of plasma ACTH, loss of circadian rhythm, or isotope accumulation on an I^{131}-adosterol scintigram (Table 1). As shown in Table 2, all adrenal tumors examined were microscopically confirmed to be adrenocortical adenomas. All patients signed a form of informed consent prepared in accordance with the rules outlined by the Nagasaki University Ethics Committee.

In Situ Hybridization

cDNA fragments of StAR, CYP11A1, HSD3B2, CYP11B, CYP17A1, CYP21A2, SULT2A1 and MC2R, which were encoding human steroidogenic acute regulatory protein, cholesterol side-chain cleavage enzyme, 3β-hydroxysteroid dehydrogenase, 11β-hydroxylase and/or aldosterone synthase, 17α-hydroxylase, 21-hydroxylase, dehydroepiandrosterone sulfotransferase and receptor for ACTH, respectively, were obtained by reverse transcriptase-polymerase chain reaction and subcloned into pT-NOT vector (11, 18). Antisense strand cRNAs were synthesized using Digoxigenin (DIG)-UTP (Roche Diagnostics, Tokyo, Japan) with T3 or T7 RNA polymerase (Takara, Otsu, Japan). The probe for CYP11B used in this study could not discriminate between 11β-hydroxylase and aldosterone synthase, since the nucleotide sequences of CYP11B1 and CYP11B2 encoding 11β-hydroxylase and aldosterone synthase are 95 % identical (11, 19).

In situ hybridization was performed as described previously (11, 18). Briefly, 3 μm-thick sections were hybridized with DIG-labeled cRNA probes at 42^0C for 16 hr, and finally washed in 0.2 x SCC at 50^0C for 20 min. Hybridization signals were immunologically detected with alkaline phosphatase-conjugated anti-DIG Fab

fragments (diluted 1:500; Roche Diagnostics). The specificity of the signals was examined using three negative-control procedures; (a) hybridization with sense cRNA probe, (b) pretreatment of tissue sections with RNase A, and (c) displacement by addition of excess unlabeled antisense probe.

Real-Time PCR

To discriminate between the expression of CYP11B1 and CYP11B2 mRNAs, we performed real-time PCR. Frozen adrenal glands attached to adrenocortical adenomas were cut into 7 μm-thick and consecutively 300 μm-thick sections in a cryostat. The former were used to examine the expression of HSD3B2 and CYP17A1 mRNAs by in situ hybridization, to determine the presence of subcapsular micronodules, and the latter were stored at -80^{0}C until RNA extraction. Under stereomicroscope, the tissues containing subcapsular micronodules were enucleated with a 400-μm needle (inside diameter) from frozen 300 μm-thick sections of adrenal glands. Total RNA was collected using GenElute Mammalian Total RNA kit (Sigma-Aldrich, Inc. MO).

Approximately 300 ng of total RNA was extracted from 10 enucleated tissues. The RT reaction proceeded at 42^0C for 60 min with 200 ng total RNA in a reaction mixture containing 50 ng random primer (Life Technologies, Grand Island, NY), 0.5 mM deoxy-NTPs, 10 mM dithiothreitol, 1 X reaction buffer, 50 U ribonuclease inhibitor (Takara, Otsu, Japan), and 100 U RT enzyme (Life Technologies) in a final volume of 20 µl. The samples were heated at 95^0C for 10 min to inactivate RT activity and denature the RNA-cDNA hybrids. LightCycler Quick System 330 (Roche Diagnostic Co., Tokyo, Japan) was used for real-time PCR. Sequence-specific primers were designed, as previously described (20), and assigned the following GenBank accession numbers: CYP11B1 (X55764: 1435 – 1460 and 1534 - 1513), CYP11B2 (X54741: 2637 – 2658 and 2724 - 2699). Expression levels were standardized by 18S rRNA (M10098: 124 -148 and 256 – 232). All products were checked by electrophoresis using 3 % agarose gels and ethidium bromide staining with UV visualization to ensure the specificity of the PCR products and the absence of non-specific bands. Relative quantitation of gene expression was performed using the relative standard curve method. All real-time PCRs were done in triplicate. The sequence of PCR products was analyzed

using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan), in accordance with the manufacturer's recommendations. The results were shown as mean ± SEM.

Results

Expression of Steroidogenic Enzyme mRNAs in Control Adrenal Cortices

Expression levels were heterogeneous within each adrenal cortex and differed among cases, but the site of expression of each mRNA was constant. StAR mRNA was found throughout the whole adrenal cortex, but not in the medulla (Fig. 1, A and B). CYP11A1 and CYP17A1 mRNAs were expressed mainly in the zona fasciculata (ZF), followed by the zona reticularis (ZR) (Fig. 1, C and F). HSD3B2 mRNA was expressed in ZG and ZF, but only sporadically in ZR (Fig. 1, D). CYP11B and CYP21A2 mRNAs were intensely expressed in ZG and ZF, followed by ZR (Fig. 1 E and G). SULT2A1 mRNA expression was present mainly in ZR and extended to ZF (Fig. 1, H). MC2R mRNA was

expressed faintly in ZF and ZR (Fig. 1, I). In three cases, there were one or two microscopic subcapsular micronodules expressing StAR, CYP11A1, HSD3B2, CYP11B and CYP21A2, but not CYP17A1 (data not shown). These microscopic subcapsular micronodules were several to hundreds of micrometers in size and spheroid in shape. Cortical nodules in ZF to ZR expressing intensely for CYP17A1 mRNA were found in three cases, among which one expressed SULT2A1 mRNA (data not shown). There was no evidence of specific hybridization signals in the negative control (Fig. 1, J – L).

Expression of Steroidogenic Enzyme mRNAs in the Adrenal Cortices Attached to APA

The remaining cortices were of normal size and there was no suppression of expression for steroidogenic enzyme mRNAs (Fig. 2, A - F). SULT2A1 and MC2R mRNAs were expressed in ZF and ZR (Fig. 2, G and H) as in the control cortices. As expected, six cases (85.7 %) showed microscopic subcapsular micronodules suggesting aldosterone production, composed of spironolactone body-containing cells (Table 2; Fig. 3, A – F). Cortical nodules with expression of StAR, CYP11A1, HSD3B2, CYP17A1,

CYP11B and CYP21A2 mRNAs, suggesting the capability for producing cortisol, were present in all cases (Table 2; Fig. 3, G – K). The majority of these nodules also co-expressed SULT2A1 and MC2R mRNAs (Table 2; Fig. 3, L).

Expression of Steroidogenic Enzyme mRNAs in the Adrenal Cortices Attached to CPA

The remaining cortices were atrophic, and consisted of mainly clear-type cells, in which the expression for steroidogenic enzymes was suppressed in general (Fig. 4). SULT2A1 mRNA was scattered throughout ZR and ZF cells. MC2R mRNA expression was also suppressed, but was present in a few cells of ZF and ZR (data not shown). Microscopic subcapsular micronodules (Table 2; Fig. 4) were noted in 15 cases (78.9 %). Most of the cortical nodules in ZF to ZR revealed a decreased expression for steroidogenic enzymes. However, 16 of 19 cases (84.2 %) contained cortical nodules expressing the mRNAs of steroidogenic enzymes necessary for production of cortisol (Table 2; Fig. 5, A – F). The composition of cells in some nodules showed pleomorphic nuclei and either eosinophilic or fine lipid-rich cytoplasm, as observed in primary

pigmented nodular adrenocortical disease (PPNAD). Of 16 cases, 9 cases had nodules that co-expressed SULT2A1 mRNA (Fig. 5, F) but not MC2R mRNA (Table 2). Only in case 22 did the nodule express both SULT2A1 and MC2R mRNAs (Table 2). Four cases of adrenal cortical hyperplasia with an unencapsulated extension into the periadrenal fat were composed of clear-type cells expressing HSD3B2 but not CYP17A1, and small compact-type cells expressing CYP17A1 but not HSD3B2 (Fig. 5, G and H). In 3 cases, pigmented cortical nodules were found, in which StAR, CYP11A1, CYP17A1 and SULT2A1 mRNAs were expressed, but not HSD3B2, CYP11B, CYP21A2 or MC2R mRNAs (data not shown).

Expression of Steroidogenic Enzyme mRNAs in the Adrenal Cortices Attached to NFA

Atrophic cortices were found in 7 of 11 cases. The expression of steroidogenic enzyme mRNAs was comparatively conserved. On the other hand, the suppressed expression of SULT2A1 and MC2R mRNAs was evident in the majority of cases. Microscopic subcapsular micronodules were found in 8 (72.7 %) of 11 cases (Table 2).

Cortical nodules expressing the mRNAs of steroidogenic enzymes necessary for production of cortisol were noted in 8 cases (72.7 %), but, among these, SULT2A1 or MC2R co-expression was confirmed in only 4 cases (Table 2).

Real-Time PCR

The tissues containing subcapsular micronodules expressing HSD3B2 but not CYP17A1 were examined (Fig. 6, A – C). As controls, APA tissues, and the capsular domain tissues without subcapsular micronodules were used. CYP11B1 and CYP11B2 mRNAs were detectable in all tissues examined. Fig. 6D shows amplification curves for CYP11B1, CYP11B2 and 18S rRNA. Agarose gels produced by electrophoresis indicated the single bands for CYP11B1, CYP11B2 and 18S rRNA PCR products (Fig. 6E), and the sequencing analysis demonstrated that the PCR products corresponded to CYP11B1 and CYP11B2 genes, respectively (data not shown). The ratio of CYP11B1/18S rRNA in the tissues containing subcapsular micronodules (2.44×10^{-3}) was slightly elevated, when compared with that in APA tissues ($n = 4$; 1.56 ± 0.09 x

10^{-3}) and the capsular domain tissues without subcapsular micronodules (n = 3; 1.88 ± 0.09 x 10^{-3}) (Fig. 7). On the other hand, the ratio of CYP11B2/18S rRNA in the tissues containing micronodules (1.81 x 10^{-4}) was situated between the levels of APA tissues (n = 4; 3.84 ± 0.49 x 10^{-4}) and the capsular domain tissues without subcapsular micronodules (n = 3; 0.36 ± 0.01 x 10^{-4}) (Fig. 7).

Discussion

In this study, we examined the mRNA expression of steroidogenic enzymes in adrenal cortices attached to adrenocortical adenomas associated with PA and CA, as well as clinically silent cases, by in situ hybridization. The results in the control adrenal glands were similar to those of previous reports (11, 21 - 23). Additionally, the distribution of mRNA expression corresponded well with the zonal functions of the adrenal cortex (24), although detection of mRNA expression was not necessarily equivalent to the actual steroidogenic activity of tissues examined (25). The expression signals obtained in this study were specific and not false-positive, as demonstrated by

three negative-control studies.

The remaining cortices in CPA were atrophic, reflecting the undetectable or very low levels of plasma ACTH resulting from the excessive neoplastic production of cortisol. The circulating ACTH concentrations also correlated well with the expression levels of SULT2A1 and MC2R mRNAs. In the atrophic cortices, the expression of SULT2A1 and MC2R mRNAs was suppressed, as described previously (23), while the cortices attached to APA were not atrophic and exhibited no suppression of either SULT2A1 or MC2R mRNAs. Atrophy was also observed in 7 of 11 cases assumed to be NFA. The past few years have seen an increase in the number of reports of adrenocortical adenoma associated with preclinical Cushing's syndrome identified in cases previously regarded as NFA (26, 27). Cases 30, 32, 34, 36 and 37 showed low levels of plasma ACTH, loss of circadian rhythm or a significant isotope accumulation on an I^{131}-aldosterol scintigram, providing the opportunity to correlate function with anatomic abnormalities (28). Hence, the possibility that at least these cases might actually be preclinical Cushing's syndrome could not be ruled out, although the examinations necessary for this diagnosis were not adequate.

We demonstrated that some of the subcapsular micronodules intensely expressed the mRNAs of steroidogenic enzymes necessary for aldosterone production. Regardless of the size of the adenoma, subcapsular micronodules expressing StAR, CYP11A1, HSD3B2, CYP11B and CYP21A2, but not CYP17A1, were present in the cortices attached to APA at high frequency, as described previously (11). Unexpectedly, similar subcapsular micronodules were equally likely to be observed even in the controls and the cortices attached to CPA and NFA, although there were fewer subcapsular micronodules per case. As these micronodules were observed even in the controls showing no endocrine abnormalities, the possibility existed that the numbers of aldosterone-producing micronodules might be insufficient to provoke symptoms, or that the micronodules themselves might be unrelated to aldosterone production. However, subcapsular micronodules with the same morphology and expression pattern have been shown to be the main foci of aldosterone synthesis in some UAH cases (11). Additionally, the presence of spironolactone bodies in APA cases is proof that such microscopic subcapsular micronodules had enhanced steroidogenic activity, because spironolactone, a competitive antagonist of aldosterone receptors (29), directly

interferes with the overproduction of aldosterone through inhibition of 11β-hydroxylation and 18-hydroxylation in human adrenal cortical tissue (30). These observations support the possibility that some of the subcapsular micronodules might be an initial focus for the later development into hyperplasia or adenoma, causing PA (11, 31). By means of in situ hybridization, we could not confirm that excess aldosterone was actually synthesized in these micronodules, since the probe used for CYP11B could not distinguish 11β-hydroxylase from aldosterone synthase, being the enzyme for the final step of aldosterone synthesis. Enberg U et al. (31) have reported the expression of CYP11B2 mRNA in ZG by in situ hybridization using a radiolabeled oligonucleotide probe, and the autoradiogram exhibited seemed to indicate the presence of subcapsular micronodules expressing CYP11B2 mRNA. Hence, we performed real-time PCR to confirm the expression of CYP11B2 mRNA in the subcapsular micronodules. The level of CYP11B2 mRNA in the tissues containing micronodules was about 5.5-fold higher than that in the capsular domain tissues without micronodules, although the level of CYP11B2 mRNA was about 2-fold lower than that in APA tissues. These results indicate that, if not all, at least some subcapsular micronodules themselves expressed

levels of CYP11B2 mRNA sufficient for aldosterone production.

In older individuals and in association with hypertension, there is an increased frequency of cortical nodules in ZF to ZR (8). Vascular sclerosing is a frequent finding in PA and CS. Cortical nodules are also frequently found in the cortices attached to APA, CPA and NFA (8). In this study, most of the nodules in ZF to ZR showed a decreased expression for steroidogenic enzymes. However, some were expressing all the necessary steroidogenic enzymes for cortisol production. We also demonstrated that adrenal cortical hyperplasia, with extension of cortical cells into the periadrenal fat, was composed of clear-type cells expressing HSD3B2 but not CYP17A1 and compact-type cells expressing CYP17A1 but not HSD3B2. Such differential expression of HSD3B2 and CYP17A1 has been reported only in ACTH-independent macronodular adrenocortical hyperplasia (AIMAH) (32). Our results were consistent with those of previous reports showing the possibility that some nodules in the adrenal cortices of aging rats might be functional (9, 10). It is generally thought that cortical nodules occur as result of compensatory local tissue hyperplasia following ischemic damage (33). As is known, ACTH is an important factor to stimulate the growth of the adrenal cortex

through MC2R. Hence, we examined MC2R mRNA expression in the cortical nodules. MC2R mRNA was observed in the majority of APA cases, but absent in most of the CPA and NFA cases. Therefore, the nodules in the cortices of CPA or NFA were ACTH-independent, although those in the cortices attached to APA were considered to be still under ACTH regulation. At present, these nodules are generally thought to be mere distortions of the architecture of the cortex rather than clonal neoplastic growths, such as adenomas and carcinomas (33). Certainly, the relapse rate after adrenalectomy is extremely low. One possible explanation is that the function of most nodules indicated in this study may be episodic or transient. Another possibility is that a long time may be required for the nodules to develop sufficiently to cause endocrine disruption. Thirdly, the participation of factors other than ACTH may be required for symptoms to be produced. However, the possibility that some nodules in the cortices attached to adenoma may develop into autonomous lesions causing CS at a future point cannot be completely excluded, because relapse after even total adrenalectomy has been reported (3, 16), although rarely.

Laparoscopic unilateral adrenalectomy is now the accepted method of surgery for

unilateral benign adrenal tumor, but the choice between total and subtotal adrenalectomy remains controversial (5, 7, 14, 17). Ishidoya S et al. (17) experienced cases in which hypertension with endocrinological abnormality remained after subtotal adrenalectomy, and, hence, warned about the risk of subtotal adrenalectomy. It is thus necessary to keep in mind that lesions with the potential to later develop into functional adrenocortical nodules may be present in the remaining adrenal cortices, if subtotal adrenalectomy to remove only the adrenal mass is chosen.

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Figure legends

Fig. 1. Expression of steroidogenic enzymes in the control. In the controls, StAR (B) mRNA was found throughout adrenal cortex. The expression of CYP11A1 (C) and CYP17A1 (F) mRNAs is found in ZF and ZR. HSD3B2 (D) mRNA is expressed in ZG and ZF, and sporadically expressed in ZR. CYP11B (E) and CYP21A2 (G) mRNAs are intensely expressed in ZG and ZF, followed by ZR. SULT2A1 (H) mRNA expression is present mainly in ZR and ZF. MC2R mRNA is expressed faintly in ZF and ZR (I). (J) – (L) show the negative controls; hybridization with StAR sense probe (J), pretreatment of tissue sections with RNase A before hybridization with StAR antisense probe (K), and displacement by addition of excess unlabeled StAR antisense probe (L). (A) stained with hematoxylin-eosin are consecutive sections of (B). med, medulla.

Fig. 2. Expression of steroidogenic enzymes in the adrenal cortices attached to APA. The adrenal cortices attached to APA cells express steroidogenic enzymes including HSD3B2 (B), SULT2A1 (C) and MC2R (D) at the same level as the controls. (A)

stained with hematoxylin-eosin are consecutive sections of (B).

Fig. 3. The expression of steroidogenic enzymes in the adrenal cortices attached to APA. Microscopic subcapsular micronodule with an intense expression of StAR (B), HSD3B2 (C), CYP11B (D) and CYP21A2 (F), but not CYP17A1 (E: round dotted lines), can be seen. The majority of subcapsular micronodules in the adrenal cortices attached to APA are composed of spironolactone-body containing cells (A: arrow in square box). The cortical nodule in ZF to ZR expresses StAR (H), CYP11A1, HSD3B2 (I), CYP11B (J), CYP17A1 (K) and CYP21A2 mRNAs, suggesting the capability for cortisol production, and expression of MC2R (L) mRNA. (A) and (G) stained with hematoxylin-eosin are consecutive sections of (B) and (H), respectively.

Fig. 4. Expression of steroidogenic enzymes in the adrenal cortex attached to CPA. In the adrenal cortices attached to CPA, the expression for steroidogenic enzymes is suppressed, but is comparatively conserved. In microscopic subcapsular micronodule, the expression of StAR (B), HSD3B2 (C), CYP11B (D) and CYP21A2 (F), but not

CYP17A1 (E: round dotted lines), can be seen. (A) stained with hematoxylin-eosin is consecutive sections of (B).

Fig. 5. The expression of steroidogenic enzymes in the adrenal cortices attached to CPA. (A) to (F) indicate five cortical nodules (a – e) in the attached adrenal cortex. (a) and (c) show the expression of HSD3B2 (B), CYP11B (C) and CYP21A2 (E) mRNAs, but absent or sporadic expression of CYP17A1 (D) mRNA, suggesting the capability for aldosterone production. (b) and (d) show decreased expression of steroidogenic enzymes. (e) expresses all steroidogenic enzymes including SULT2A1 (F) mRNA, suggesting the capability for cortisol synthesis. The adrenal cortical hyperplasia with unencapsulated extension into the periadrenal fat is composed of cells expressing HSD3B2 (G) but not CYP17A1 (H) and the cells expressing CYP17A1 but not HSD3B2 (G and H: triangles). (A) stained with hematoxylin-eosin are consecutive sections of (B). med, medulla.

Fig. 6. Analysis of CYP11B1 and CYP11B2 mRNA expression by real-time PCR. The

tissues containing a subcapsular micronodules expressing HSD3B2 (A) but not CYP17A1 (B, round dotted lines) are isolated. (C) is the section stained with toluidine blue after enucleation of the tissue containing subcapsular micronodule. (D) shows amplification curves for CYP11B1, CYP11B2 and 18S rRNA. (E) indicates the ethidium bromide staining of the 3 % agarose gels. Cap, the capsular domain tissues without subcapsular micronodules; M, the tissues containing subcapsular micronodule.

Fig. 7. Ratios of CYP11B1/18S rRNA and CYP11B2/18S rRNA mRNAs. The results are shown as the ratio of CYP11B1 and CYP11B2 to 18S rRNA. A dotted line shows the mean value in APA tissues and the capsular domain tissues without micronodule, respectively. Student's *t* test was used to compare APA tissues with the capsular domain tissues without subcapsular micronodules. Cap, the capsular domain tissues without subcapsular micronodules; M, the tissues containing subcapsular micronodule.

Table 1. Clinical Data

No.	Age	Clin	BP	Ald	F	Renin	17	ACTH	CR	Dex	Sca	Drug
1	37	PA	144/90	205	10.8	0.2	n	18.7	n	n	+	A
2	38	PA	200	232	n	4.0	0.4	n	n	n	+	A
3	42	PA	148/110	352	13.4	<2.3	7.1	n	n	n	+	A
4	48	PA	150/100	520	10.84	<2.0	0.3	32	n	n	+	A
5	54	PA	160/100	500	n	<0.2	n	n	n	n	+	A
6	51	PA	140/90	249	6.06	n	n	n	n	n	+	A
7	52	PA	200/110	168	8.18	3.3	0.3	n	+	n	+	A
8	27	CS	160/100	n	22.3	n	n	nd	-	-	+	B
9	51	CS	155/93	n	22.5	n	9.3	nd	-	-	+	B
10	48	CS	135/85	n	20.2	n	12.2	nd	-	-	+	B
11	67	CS	148/96	n	19.8	n	14.3	nd	-	-	+	B
12	47	CS	150/92	n	23.3	n	11.1	nd	-	-	+	A
13	52	CS	160/100	n	22.7	n	10.8	nd	-	-	+	B
14	48	CS	200	n	19.3	n	14.7	nd	-	-	+	A
15	87	CS	200/100	n	19.4	2.3	7.8	nd	-	-	+	A
16	29	CS	105/82	<10	22.7	3.9	15.8	3.3	-	-	+	-
17	48	CS	147/92	n	21.2	n	9.8	nd	-	-	+	B
18	54	CS	158/106	n	22.5	n	n	5.0	-	-	+	-
19	37	CS	180	n	25.1	n	11.6	nd	-	-	+	B
20	45	CS	160/96	44	22.3	78.2	n	nd	-	-	+	A
21	37	CS	155/90	32	21.5	20.6	0.7	nd	-	-	+	B
22	32	CS	148/108	n	23.3	n	15.7	nd	-	-	+	-
23	37	CS	145/110	n	24.2	n	15.0	nd	-	-	+	B
24	65	CS	200/100	30	15	2.4	13.7	nd	n	-	+	A
25	42	CS	150	*189	13.9	n	n	nd	-	-	+	-
26	38	CS	127/76	n	17.7	n	n	nd	-	-	+	-
27	67	NF	194/98	n	7.2	n	n	n	n	n	n	n
28	48	NF	128/88	10.0	14.1	0.2	n	14	+	n	n	-
29	74	NF	136/99	43	11.1	3.5	n	n	+	n	n	-
30	58	NF	127/76	n	17.7	n	n	nd	-	n	n	-
31	65	NF	125/87	2.2	5.9	6.5	n	n	n	n	n	-
32	54	NF	195/101	10	17.8	n	n	< 5.0	n	n	+	A
33	66	NF	160/90	120	6.6	n	n	20	+	n	n	B
34	40	NF	144/99	40	15.7	n	n	< 5.0	n	n	+	B
35	31	NF	110/80	n	6.5	n	n	n	n	n	n	-
36	56	NF	204/100	59	14.3	n	n	11	-	n	+	A
37	28	NF	115/66	53	18.2	0.9	6.2	nd	+	n	+	-

Clin, clinical diagnosis; PA, primary aldosteronism; CS, Cushing's syndrome; NF, clinical silent; BP, blood pressure (mmHg); Ald, plasma aldosterone (29.9 ~159 pg/ml, *35.7～240 pg/ml), F, plasma cortisol (6.2 ~ 19.4 μg/ml); Renin, plasma renin (2.5 ~ 21.4 pg/ml); 17, 17-OHCS (2.2 ~ 7.3 mg/day); ACTH, plasma ACTH (7 ~56 pg/ml), CR, circadian rhythm: Dex, dexamethasone suppression test; Sca, isotope accumulation on an I^{131}-aldosterol scintigram; Drug, ant-hypertensive agents, A (spironolactone), B (calcium antagonists); nd, not detection; n, not dermined

Table 2. Expression pattern in the adrenal cortices attached to APA, CAP and NFA

No.	Diag	Site	Size	Atro	(a)	(b)	(c)	(d)	(e)
1	APA	R	2.0	-	+	+	+	+	-
2	APA	L	1.5	-	+	+	+	+	-
3	APA	R	1.5	-	+	+	+	+	-
4	APA	L	0.9	-	+	+	+	+	+
5	APA	R	2.2	-	+	+	+	+	+
6	APA	L	1.2	-	-	+	+	+	+
7	APA	R	2.0	+	+	+	+	+	+
8	CPA	L	3.0	+	+	+	-	-	+
9	CPA	L	3.0	+	+	+	-	-	+
10	CPA	L	3.4	+	-	-	-	-	+
11	CPA	L	3.5	+	+	-	-	-	+
12	CPA	L	4.2	+	-	+	-	-	+
13	CPA	L	4.1	+	+	+	+	-	+
14	CPA	R	4.0	+	+	+	-	-	+
15	CPA	L	4.0	+	+	+	+	-	+
16	CPA	L	4.5	+	-	-	-	-	-
17	CPA	L	3.0	+	+	+	-	-	+
18	CPA	L	3.2	+	+	+	-	-	-
19	CPA	L	3.0	+	+	+	+	-	-
20	CPA	L	2.5	+	+	+	+	-	+
21	CPA	L	3.0	+	-	+	+	-	+
22	CPA	R	2.4	+	+	+	+	+	-
23	CPA	L	3.7	+	+	+	+	-	+
24	CPA	R	3.0	+	+	+	+	-	-
25	CPA	L	3.3	+	+	+	+	-	-
26	CPA	L	3.8	+	+	+	+	-	-
27	NFA	L	3.0	-	+	+	+	+	+
28	NFA	R	4.0	+	+	-	-	-	+
29	NFA	L	4.0	-	-	+	-	+	+
30	NFA	R	3.0	+	+	+	-	-	+
31	NFA	L	4.0	-	+	+	-	+	-
32	NFA	L	5.0	+	-	-	-	-	+
33	NFA	R	3.0	+	+	-	-	-	+
34	NFA	L	3.0	+	+	+	+	-	+
35	NFA	L	3.2	-	+	+	-	-	+
36	NFA	L	3.2	+	+	+	-	-	+
37	NFA	R	3.5	+	-	+	-	-	+

a, nodules suggesting the capability for producing aldosterone; b, nodules expressing an intense CYP17A1 but not SULT2A1; c, nodules co-expressing SULT2A1; d, nodules co-expressing MC2R; e, nodules with decreased expression for steroidogenic enzymes; Diag, pathological diagnosis; APA, aldosterone-producing adenoma; CPA, cortisol-producing adenoma; NFA, nonfunctioning adenoma; “Atrophy” indicates the pathological finding in the attached remaining adrenal cortex.

Fig. 1

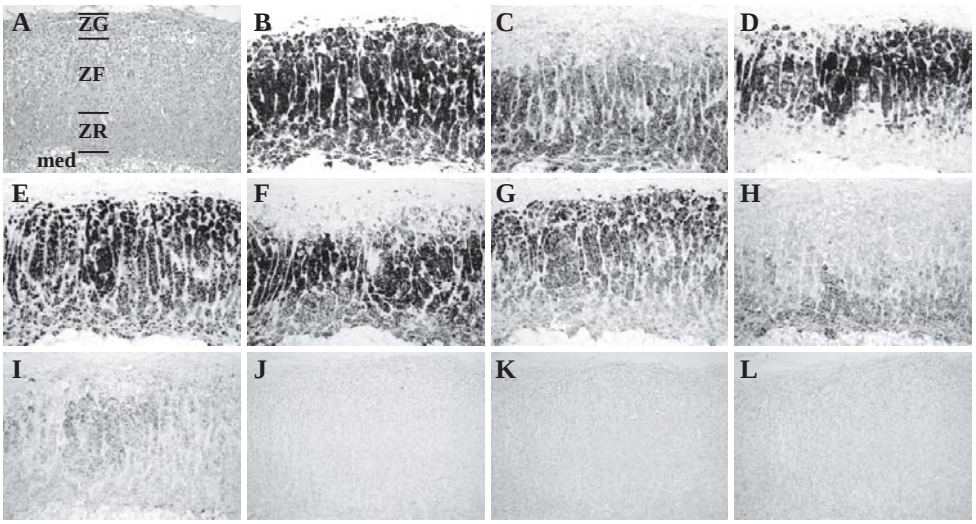


Fig. 2

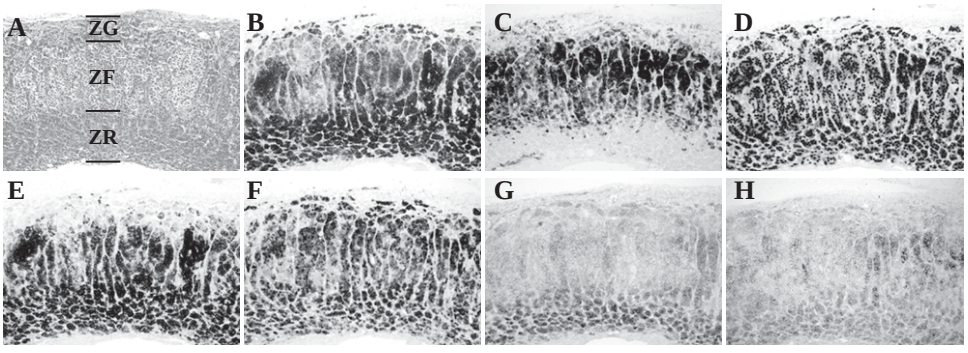


Fig. 3

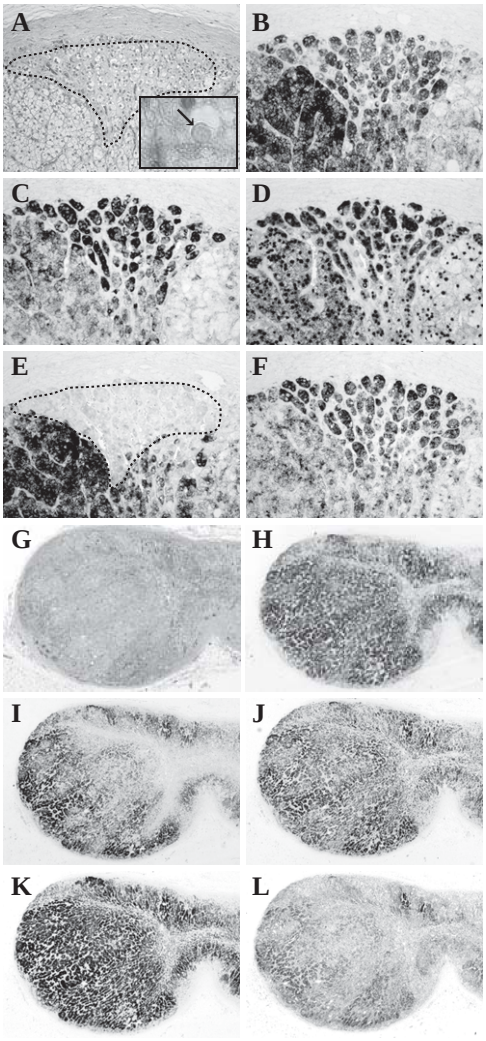
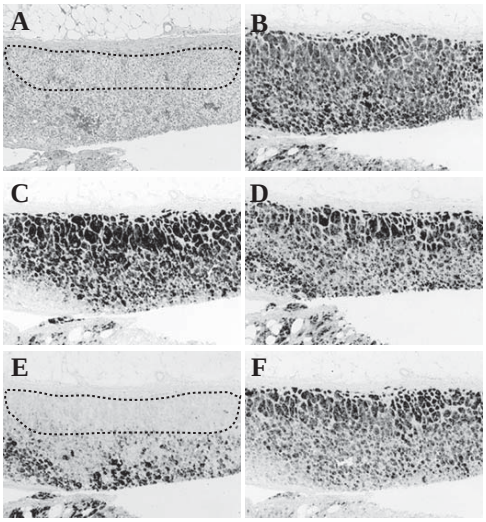


Fig. 4



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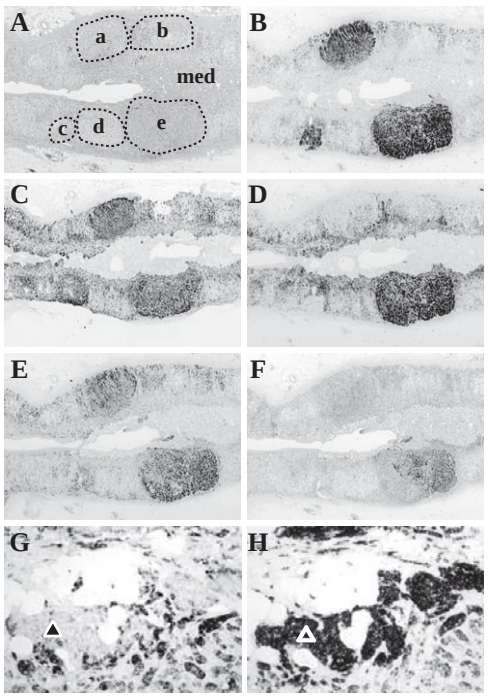


Fig. 6

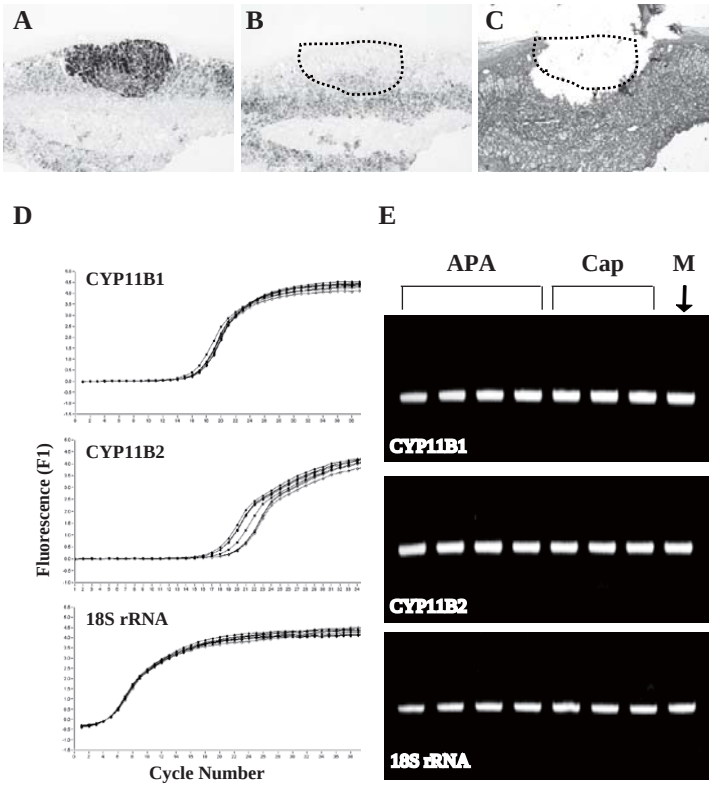
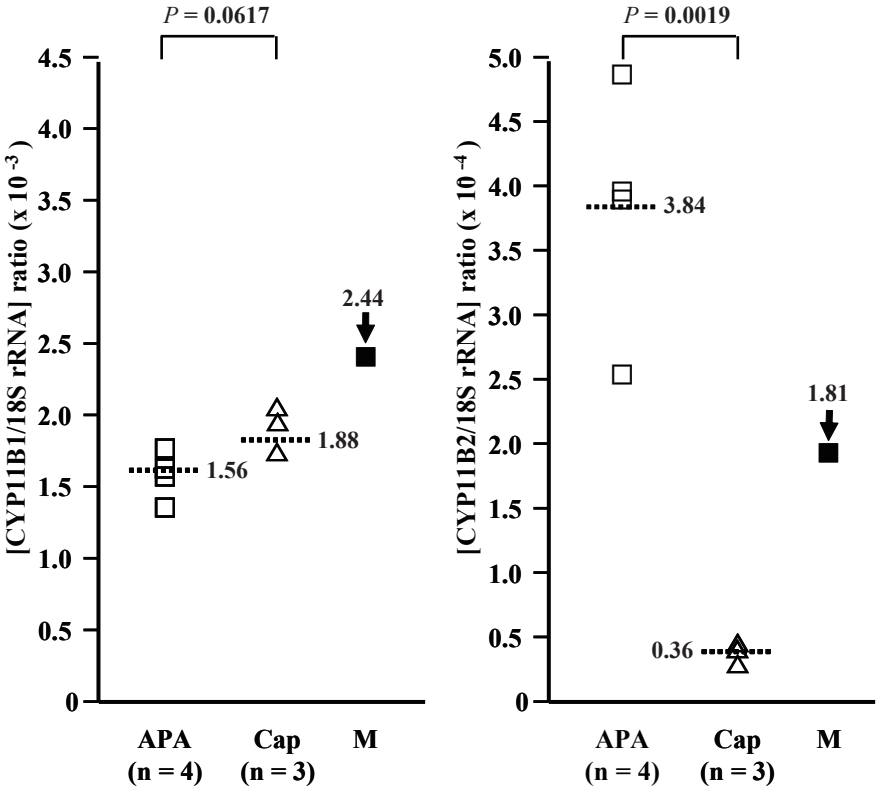


Fig. 7


P = 0.0617
[CYP11B1/18S rRNA] ratio (x 10 -3)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
2.44
1.88
1.56
APA
(n = 4)
Cap
(n = 3)
M
P = 0.0019
[CYP11B2/18S rRNA] ratio (x 10 -4)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
3.84
1.81
0.36
APA
(n = 4)
Cap
(n = 3)
M