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Inherited Adrenocorticotropin-Independent Macronodular Adrenal Hyperplasia with Abnormal Cortisol Secretion by Vasopressin and Catecholamines

Detection of the Aberrant Hormone Receptors on Adrenal Gland

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ACTH-independent macronodular adrenal hyperplasia (AIMAH) is a rare disorder and an unusual cause of Cushing's syndrome, of which familial transmission has rarely been reported. In this study, a mother and her son, the former affected with definite AIMAH and the latter with possible AIMAH, are described. Although the mother manifested overt Cushing's syndrome, her son remained with no stigmata of Cushing's syndrome except for bilateral adrenal tumor and mild hypertension, and a full suppression of plasma cortisol by low-dose dexamethasone was observed in him. Recently, aberrant expression of adrenal receptors for various ligands has been noted in AIMAH patients. In our cases, provocation tests in vivo suggested that AVP and catecholamines promoted cortisol production through V1a and/or V1b receptors and via β-adrenergic receptor, respectively. Reverse transcriptional-PCR analysis of the operated adrenal tissues of mother revealed the abnormal expression of mRNA of receptors for V1b, V2, and LH/hCG, none of which was observed in a normal control. Inherited AIMAH is very rare, and the son might be at the earliest developmental stage of AIMAH among the cases reported so far. An intervention could be tried to prevent the development of overt Cushing's syndrome by suppression of the possible endogenous ligands or by blockade of the receptors that may be aberrantly expressed in his adrenal glands.

Key Words: ACTH-independent macronodular adrenal hyperplasia; familial transmission; aberrant hormone receptors; vasopressin; β-adrenergic receptors.

Introduction

Adrenocorticotropin (ACTH)-independent macronodular bilateral adrenal hyperplasia (AIMAH) is a rare disorder and an unusual cause of Cushing's syndrome *(1,2)*. Most of ACTH-independent Cushing's syndrome result from unilateral adrenal adenoma or carcinoma, and bilateral macronodular adrenal hyperplasia has been thought to be associated with long-standing ACTH hypersecretion *(2)*. Among these bilateral adrenal disorders, AIMAH, with characteristic giant nodular hyperplasia of both adrenals, is now recognized to be a distinct etiology of Cushing's syndrome *(3)*. It has peculiar clinical features compared with other subtypes of this syndrome, such as milder Cushing's stigmata, more elderly onset, and higher frequency to be diagnosed incidentally as pre- or subclinical Cushing's syndrome. Histopathological and immunohistochemical characteristics of tissues from patients with AIMAH have been described; the adrenal glands contained multiple nodules composed of large clear cells and small compact cells, and immunoreactivity of P-450-17 alpha was predominant in the small compact cells, while that of 3 beta-hydroxysteroid dehydrogenase was observed exclusively in the large clear cells *(4,5)*. In addition, unique endocrinological features have recently been reported from several groups indicating that abnormal or ectopic expression and function of adrenal receptors for various hormones may regulate cortisol production in ACTH-independent hypercortisolism *(6–16)*.

Here, we report two patients, a mother and her son, the former presented with clinical features of Cushing's syndrome with AIMAH, and the latter with no stigmata of Cushing's syndrome except for bilateral adrenal swelling compatible to AIMAH. To date, there have been only a few case reports describing ACTH-independent macronodular adrenal hyperplasia occurring in two generations of a family *(17,18)*. This report presents with a rare case of familial AIMAH, and unique endocrinological features of the two patients including expression of ectopic or abnormal membrane hormone receptors on the mother's resected adrenal tissues.

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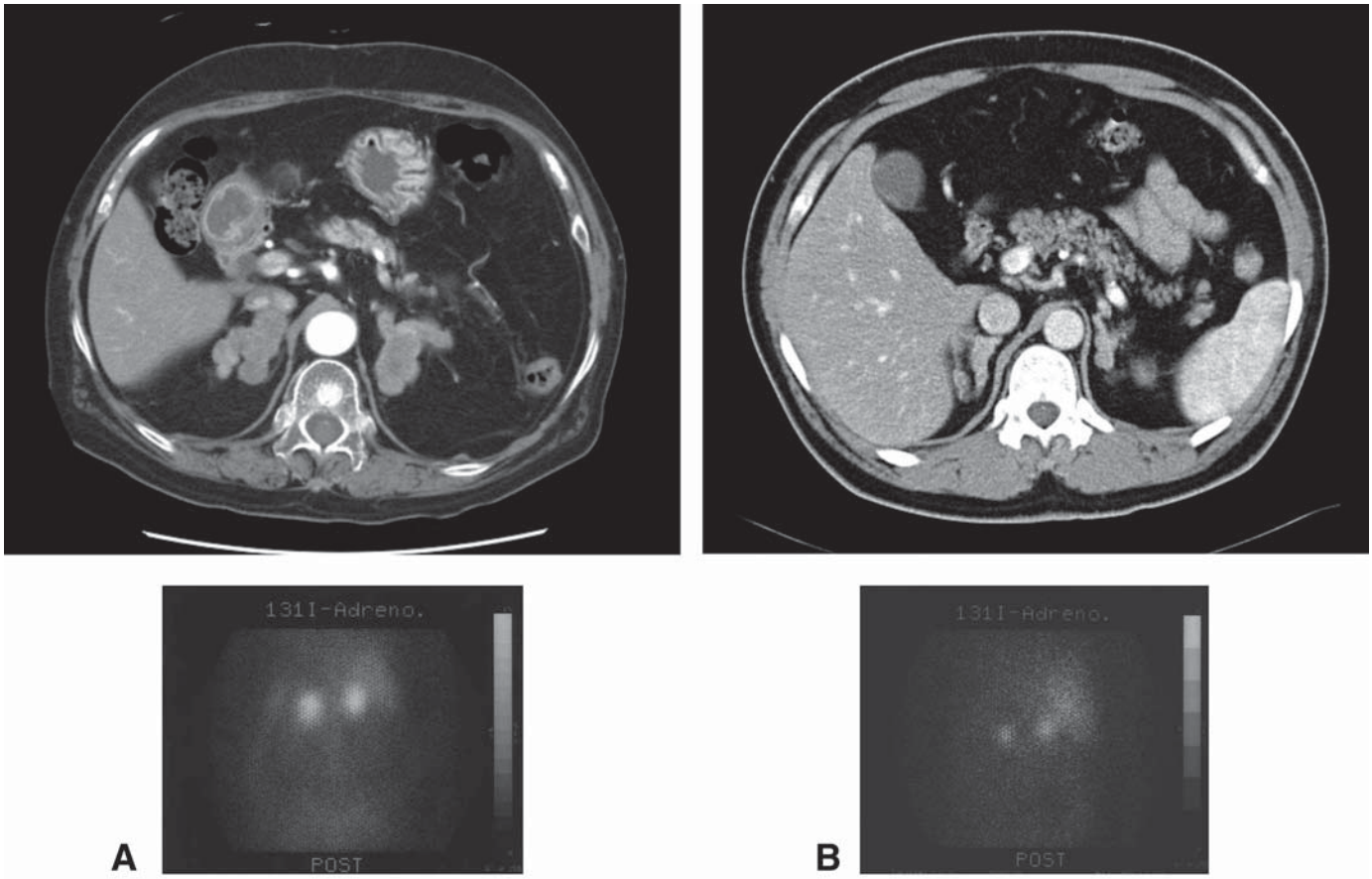

Fig. 1. (**A**) Upper panel: An abdominal computed tomography scan of case 1. Lower panel: An adrenal scintigram imaging with ^{131}I-adosterol of case 1. (**B**) Upper panel: An abdominal computed tomography scan of case 2. Lower panel: An adrenal scintigram imaging with ^{131}I-adosterol of case 2.

Case Reports

Case 1

A 68-yr-old woman was referred for evaluation of Cushing's syndrome because of her cushingoid feature and an abnormal abdominal computed tomography (CT) scan, which revealed bilateral adrenal tumor. She had been treated with antihypertensive agents over the previous 15 yr. Diabetes mellitus and hyperlipidemia had developed during the preceding 1–2 yr. She had complained of easy bruisability associated with fatigue, leg edema, and generalized weakness, predominantly involving the proximal musculature of the extremities. Physical findings included mild facial plethora, moderate centripetal obesity (weight, 59.1 kg; height, 147.5 cm; body mass index, 27.2), proximal muscle weakness, multiple bruises, and high blood pressure (190/90 mm Hg), but no abdominal striae.

The absence of a circadian rhythm was demonstrated by the stable values of plasma cortisol of 532, 502, and 510 nmol/L at 0900, 1300, and 2100 h, respectively, whereas ACTH was undetectable by immunoradiometric assay (less than 0.8 pmol/L throughout the day; normal, 0.93–10.2 pmol/L), and morning plasma cortisol was not suppressed by 1 mg overnight dexamethasone (466 nmol/L). Free urinary cortisol level was 197 nmol/d (normal, 31–220 nmol/d). Urinary excretion of 17-hydroxycorticosteroids and 17-ketosteroids was not suppressed after the administration of 2 mg dexamethasone/d for 2 consecutive days, or after 8 mg for 2 d. CT scan and magnetic resonance imaging (MRI) revealed bilateral huge macronodular adrenals (Fig. 1A). Adrenal scintigram imaging with ^{131}I-adosterol disclosed uptake of the isotope in the area corresponding to both adrenals. After investigation, bilateral adrenalectomy was performed; the left adrenal measured 7.0 × 6.8 × 4.4 cm (115 g) and the right adrenal measured 6.4 × 4.9 × 3.4 cm (59 g). Both adrenals were diffusely enlarged with alternation of clear and acidophilic micronodules, without internodular atrophy. Final clinical and pathological diagnosis was AIMAH.

Case 2

A 38-yr-old man (a son of case 1) consulted us because he had been hypertensive and obese for the previous several years. Physical examination revealed obesity (weight, 81.9 kg; height, 164 cm; body mass index, 30) but no facial plethora, cervicodorsal fat pads, abdominal striae, bruisability, or muscle weakness. There was no other physical symp-

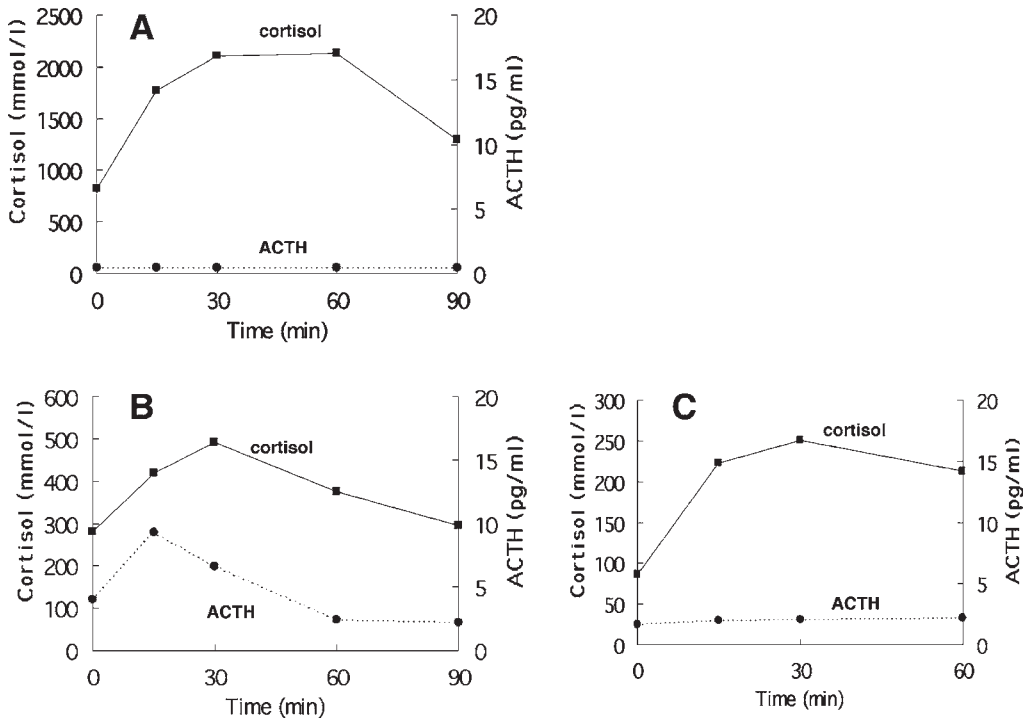

Fig. 2. The AVP–(ACTH)–cortisol axis examinations in both patients. Solid square: plasma cortisol level, solid circle: plasma ACTH level. (**A**) AVP administration test in case 1. AVP increased plasma cortisol rapidly without any increase of ACTH (<0.8 pmol/L). (**B**) AVP administration test in case 2. AVP increased plasma cortisol with some increase of ACTH. (**C**) AVP administration test in case 2 under dexamethasone suppression. AVP also increased plasma cortisol without increase of ACTH.

toms that suggested hypercorticism. Blood pressure was 164/90 mmHg; 75g-oral glucose tolerance test showed mild glucose intolerance (fasting blood glucose, 5.2 mmol/L; after 120 min, 9.1 mmol/L). An abdominal CT scan unexpectedly revealed bilateral adrenal tumors. Although smaller in size, they had much morphological resemblance compared with those of case 1, suggesting AIMAH (Fig. 1B). Adrenal scintigram using ^{131}I-adosterol was also performed, showing a similar uptake pattern. However, endocrine work-up did not match the features of Cushing's syndrome or even "subclinical" Cushing's syndrome; (1) a circadian rhythm of plasma cortisol presented with the values of 215, 133, and 77 nmol/L at 0900, 1300, and 2100 h, respectively; (2) morning plasma cortisol was suppressed by 1 mg overnight dexamethasone (52 nmol/L); (3) free urinary cortisol level was 27 nmol/d (normal, 31–220 nmol/d). Serum ACTH values were 2.0, 1.55, and 1.25 pmol/L at 0900, 1300, and 2100 h, respectively (normal, 0.93–10.2 pmol/L), and the morning level was suppressed by 1 mg overnight dexamethasone. His blood pressure could be controlled in normal range and obesity improved with a diet therapy with restrictions of salt (8 g/d) and energy (1800 cal/d). A biopsy incision to confirm AIMAH was not performed. Provocative tests for ACTH and cortisol were performed.

The pedigree of the family revealed no consanguinity. Siblings or the other son of the case 1 did not show the clinical symptoms of Cushing's syndrome. Abdominal CT scan has not been performed for them because their consent could not be obtained.

Results

Clinical Studies

1. The CRH–ACTH–cortisol axis in both cases: In case 1, there was no ACTH or cortisol response in the CRH provocative test, but the plasma cortisol level was elevated from 466 to 1917 nmol/L in the ACTH test. (ACTH was administered subsequently to 1 mg overnight dexamethasone suppression.) In case 2, ACTH and cortisol responded normally to the administration of CRH, from 2.8 to 10.5 pmol/L and from 334 to 571 nmol/L, respectively. Cortisol increased from 52 to 590 nmol/L in response to ACTH stimulation after 1 mg overnight dexamethasone suppression.
2. AVP–(ACTH)–cortisol axis in both cases: In case 1, administration of 10 U of AVP increased plasma cortisol rapidly from 816 to 2128 nmol/L without any increase of ACTH (<0.8 pmol/L) (Fig. 2A). AVP stimulation also increased the cortisol level in case 2 (281 to 491 nmol/L), but it was accompanied by an ACTH increase from 4.0 to 9.3 pmol/L (Fig. 2B).

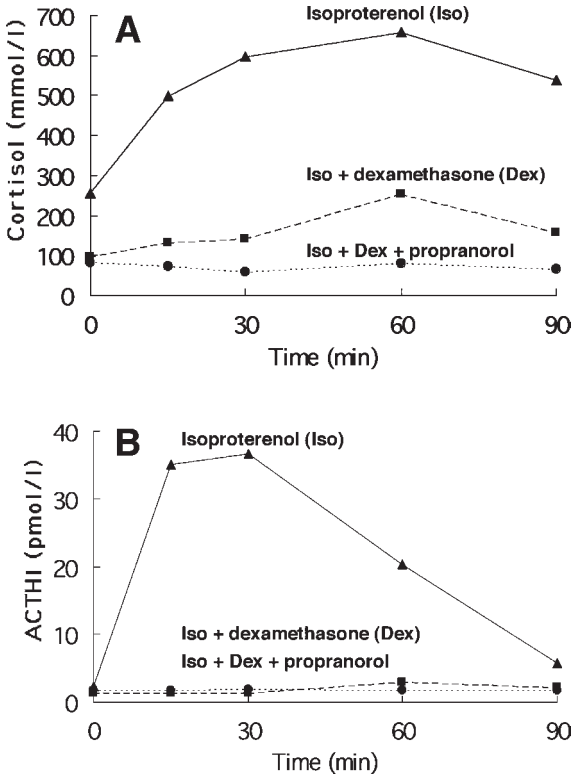

Fig. 3. The β-adrenaline–(ACTH)–cortisol axis examinations in case 2. (**A**) Cortisol responses and, (**B**) ACTH responses to isoproterenol infusion under various conditions. Solid triangle; isoproterenol only; solid square; isoproterenol under dexamethasone infusion; solid circle; isoproterenol under dexamethasone infusion with propranolol pretreatment.

Under dexamethasone suppression, AVP stimulation also increased plasma cortisol from 86 to 251 nmol/L without a significant ACTH increase (from 1.7 to 2.1 pmol/L) (Fig. 2C). On the other hand, administration of dDAVP did not result in any stimulation of ACTH or cortisol in both patients (data not shown).

3. β-Adrenaline–(ACTH)–cortisol axis in case 2 (Fig. 3): A single isoproterenol infusion test resulted in a cortisol response from 256 to 659 nmol/L (Fig. 3A) with a sharp increase of ACTH from 2.3 to 36.7 pmol/L (Fig. 3B). Dexamethasone treatment during infusion of the β-adrenergic agonist suppressed ACTH response (from 1.4 to 2.9 pmol/L), but did not suppress cortisol response (from 97 to 254 nmol/L) (Figs. 3A,B). Under this dexamethasone suppression, propranolol pretreatment revealed a complete suppression of the isoproterenol-induced cortisol response (Figs. 3A,B).
4. Other provocative tests: GnRH (LHRH; 10 U iv) did not stimulate ACTH or cortisol secretion despite a 3.5- or 19-fold increase in plasma LH (from 20.9 to 70.9 or 2.9 to 56 mIU/mL) in case 1 or case 2, respectively. Mosapride (a $5HT_4$ receptor agonist) or an oral glucose administration did not stimulate ACTH or cortisol secretion in case 2, either (data not shown).

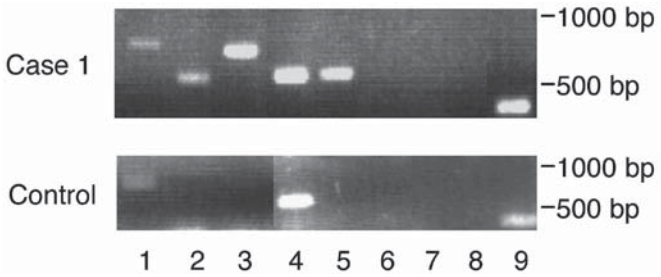

Fig. 4. RT-PCR of various receptors mRNA. Lane 1, PCR products for V1a receptor (718 bp); lane 2, V1b (492 bp); lane 3, V2 (657 bp); lane 4, ACTH (513 bp); lane 5, LH/hCG (518 bp); lane 6, β-adrenalin; lane 7, GIP; lane 8, $5\text{-}HT_4$; lane 9, β-actin. Upper panel: case 1; lower panel: control.

Detection of mRNA for Various Hormone Receptors by Reverse Transcriptional (RT)-PCR Analysis

RT-PCR analysis of RNA from adrenal tissue of a normal control showed amplified bands for V1a receptor in addition to ACTH receptor (positive control), whereas that from AIMAH tissues of case 1 revealed expression of V1b, V2, and LH/hCG receptors in addition to V1a and ACTH receptors (Fig. 4). Bands for β-adrenergic, glucose-dependent insulinotropic peptide (GIP), glucagons-like peptide-1 (GLP-1), or $5\text{-}HT_4$ receptors were not detected in either case 1 or normal control (Fig. 4).

Discussion

It is known that inherited Cushing's syndrome is very rare except for primary pigmented nodular adrenal dysplasia *(19)*. AIMAH mostly occurs in a sporadic form, although there have been a few cases of genetic transmission reported *(17,18)*. In this report, we have demonstrated a woman and her son with AIMAH, possibly sharing expression of vasopressin receptors and/or β-adrenergic receptor in their adrenal glands. Thus, it adds evidence for existence of the genetic inheritance in some cases of AIMAH.

In this case report, the 68-yr-old mother (case 1) presented the clinical features of Cushing's syndrome with AIMAH, whereas the 38-yr-old son (case 2) exhibited no signs of Cushing's syndrome except for bilateral adrenal tumor and mildly elevated blood pressure. Because histopathological investigations have not been completed in case 2, a diagnosis of AIMAH may not be confirmed. However, in addition to the morphological characteristics of bilateral adrenal swelling (Fig. 1B), his cortisol secretion was stimulated by either exogenous AVP or isoproteronol administration under dexamethasone suppression (Figs. 2 and 3), and such responses have not been observed in normal subjects *(8)*. These observations strongly suggest that he should be diagnosed as AIMAH, which has been inherited from case 1, and is now in the very early stage of development.

Autonomous glucocorticoid production resulting from adrenal incidentalomas without specific signs and symptoms of Cushing's syndrome is termed subclinical Cushing's syndrome, and cases have been increasing in number *(20, 21)*. Recently, subclinical AIMAH has also been described *(22)*, and this new clinical entity of AIMAH will be detected more frequently hereafter, for hypercortisolism in most AIMAH patients has been suggested to be milder compared with those with unilateral adrenal adenoma *(4)*. Cortisol secretion in subclinical Cushing's syndrome is not completely under the regulation of endogenous ACTH, which means insufficient cortisol suppression by the dexamethasone administration. Because the plasma cortisol level of case 2 was suppressed by low dose (1 mg) of dexamethasone, it seems inappropriate to place him even in the category of "subclinical." This patient may be the first case of AIMAH that was discovered before the adrenals revealed autonomous glucocorticoid production. It is of much interest to observe the natural clinical course of case 2, whether the adrenal masses would enlarge and gain the autonomy of glucocorticoidogenesis; on the other hand, it would be of great benefit for this patient if interventions were possible to hinder the growth of both adrenal's hyperplasia and the progress of overt Cushing's syndrome.

Molecular and genetic mechanisms of this disease are largely unknown, but recent reports from several groups have indicated that abnormal or ectopic expression and function of adrenal receptors for various hormones might regulate cortisol production, leading to a hypothesis that it plays an important role in the development of AIMAH. Moreover, most of patients reported with this disorder had been related to expression of least one of those abnormal or ectopic receptors *(9,22)*. The growing list of hormones or cytokines that can be the ligands of these aberrant receptors includes vasopressin *(13,23)*, GIP *(12,24–26)*, LH *(27)*, catecholamines *(28)*, serotonin/5HT$_4$ *(10)*, angiotensin II *(29)*, or interleukin-1 *(14)*.

We have investigated the possible involvement of some of these receptors in vivo. First, the involvement of AVP–(ACTH)–cortisol axis was examined in both patients. In case 1, administration of AVP increased plasma cortisol in 2.6-fold with undetectable ACTH level (<0.8 pmol/L). In case 2, AVP stimulated cortisol level by 1.7-fold, with an ACTH increase from 4.0 to 9.3 pmol/L. Even under dexamethasone suppression, this cortisol stimulation induced by AVP still remained in 2.6-fold without significant ACTH increase. These results strongly suggested that AVP promoted cortisol secretion from their adrenals, at least in part, without ACTH pathway. Second, β-adrenaline–(ACTH)–cortisol axis was also studied in case 2. Isoproterenol increased plasma cortisol by 2.6-fold with a sharp increase of ACTH from 2.3 to 36.7 pmol/L. Dexamethasone pretreatment did not suppress the cortisol response (2.6-fold increase) although ACTH response was eliminated. In addition, the β-adrenergic antagonist pretreatment under dexamethasone suppression revealed a complete suppression of ACTH and cortisol responses. These data also indicated that this cortisol stimulation resulted, at least partly, from the direct effect of isoproterenol to the adrenal receptors. Finally, other potential secretagogues including dDAVP, mosapride (5-HT$_4$ agonist), endogenous LH induced by GnRH, or oral glucose administration on behalf of the provocation by endogenous GIP did not stimulate cortisol hypersecretion in vivo.

RT-PCR analysis of the resected adrenal tissues of case 1 revealed expression of receptors for V1a, V1b, V2, and LH/hCG, but not of those for β-adrenalin, GIP, GLP1, or 5HT$_4$. Among these results, the expression of V1b, V2, or LH/hCG receptor was considered to be ectopic, while that of V1a receptor to be eutopic, comparing with the RT-PCR results obtained from normal control. For case 2, since we have not performed a biopsy incision of the adrenals, the expression of these receptors has not been examined.

These results pose several questions. Discrepancy between the responses to exogenous ligands and the expression of the receptors for LH/hCG, V2 and β-adrenalin remains unexplained, but there may be a hint in the latest case that we have experienced. The case is another 45-yr-old male patient that is not related to this family and had no family history of adrenal disorder, who undewent bilateral adrenalectomy for the treatment of overt Cushing's syndrome owing to AIMAH. We have performed the same RT-PCR analysis in this patient, and expression of aberrant receptors was observed in the same combination as in case 1: positive for V1a, V1b, V2, and LH/hCG, and negative for β-adrenalin, GIP, GLP-1, or 5-HT-4 (manuscript in preparation). It is possible that a common pathogenesis underlies both familiar AIMAH in the present study and this unrelated patient, which may be such as abnormalities of some unknown adrenal transcriptional factors that regulate or modify, in common, the gene transcriptions of the G-protein coupled receptors of V1b, V2, and LH/hCG, or possibly including V1a and β-adrenalin. In addition, some of the transcripted membranous receptors such as LH/hCG or V2 might not be translated to receptor proteins, or if being translated, might not have function to secrete by ligand stimulations. Further study will be needed to address these questions.

There are possibilities of pharmacological treatments to control hypercortisolism in case 2, such as suppression of possible endogenous ligands for the abnormal receptors or administration of specific antagonists for them. The clinical course of case 2 should be carefully observed and therapeutic approaches described above should be challenged to hinder the growth of both adrenal's hyperplasia and the progress of overt Cushing's syndrome.

In conclusion, we reported a rare case of familial AIMAH. In vivo and in vitro findings suggested that abnormal or ectopic expression and function of adrenal receptors for AVP, catecholamines, and LH/hCG, which might play a role in

Table 1
Oligodeoxynucleotides Used to Amplify RT-PCR Products from Various Receptor mRNAs

Receptor	Primer set (sense/antisense)
V_{1a}	5'-AACGGCCCACCGAGGGACGTG-3' / 5'-AGGGTGCGAGCAGGAACCCCTTT-3'
V_{1b}	5'-CTGGCTGTGCTGCTGACCCTGGG-3' / 5'-CCGTGAGCATGGTCACCGGCAGA-3'
V_2	5'-GCTGCTCTCCATAGTCTTTG-3' / 5'-GTGGGCTCCCTCACCGGGGCTG-3'
GIP	5'-AATGCCACTGCCCGTGC-3' / 5'-CCACCAAATGGCCTTGACTTC-3'
GLP-1	5'-TGCCACTGTGTCCCTCT-3' / 5'-TGCTGGGCGGCTGTGCTATACA-3'
ACTH	5'-TTCAAGAATAAGAATCTCCAGG-3' / 5'-GTGTGATGGCCCCTTTCATG-3'
β-adrenergic	5'-CTTCTTCGCTCCCTGCCTCA-3' / 5'-AGCGCGACGCCTTGGCA-3'
LH/hCG	5'-TTTTGCATGGGGCTCTATCTGCT-3' / 5'-TCGGTGAAGATGAGGATTGC-3'
5-HT_4	5'-ATTTATGGGGAGGTGTTTTG-3' / 5'-AGGTCTTCGGTAGCGCTCAT-3'

pathogenesis of AIMAH in the family. The responsible genes or germline mutations for inherited AIMAH still remain to be identified.

Materials and Methods

Clinical Studies

Each of the provocative tests was performed on a separate day after an overnight fast, in the supine position for 30 min before testing. Then five single-stimulation tests with 100 µg iv corticotropin release hormone (CRH) (Mitsubishi Pharma Corporation, Tokyo, Japan), 250 µg iv ACTH 1–24 (Cortrosyn, purchased from Daiichi Pharmacoceutical Co., Ltd, Tokyo, Japan), 10 U sc AVP (Pitressin, Sankyo Co., Ltd, Tokyo, Japan), 2.5 µg sc desmopressin acetate (dDAVP, Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan), or 100 µg iv LHRH (Tanabe Seiyaku Co., Ltd, Osaka, Japan) were performed on both patients. Following three tests using isoproterenol (Nikken Chemicals Co., Ltd, Tokyo, Japan), a 5-HT_4 agonist mosapride citrate (30 mg of oral dose) and an oral 75 g glucose administration were performed for case 2 only. Isoproterenol was infused intravenously at the rate of 20 ng/kg/min for 30 min.

Because the endogenous ACTH secretion was not completely suppressed in case 2, AVP stimulation test under dexamethasone suppression (according to the method described in ref. *8*) was performed to eliminate the influence of the patient's AVP–ACTH–cortisol axis. The effect of the isoproterenol infusion under dexamethasone suppression was also examined in case 2 to exclude the effect of his β-adrenergic–ACTH–cortisol axis. Dexamethasone infusion (1 mg/h) was started 180 min before the start of isoproterenol administration and was continued until the end of the test. In another isoproterenol test under the same dexamethasone suppression, β-adrenergic antagonist, propranolol (20 mg), was administered orally 60 min before isoproterenol infusion started.

Assays

Plasma and urinary concentrations of cortisol were measured by a commercial RIA kit (Gamma-coat, Nihon Schering Co., Ltd, Osaka, Japan), as were ACTH (IRMA Allegro, Sumitomo Seiyaku Biochemical Co., Ltd, Osaka, Japan).

Detection of mRNA for Various Hormone Receptors by RT-PCR Analysis

Primers

Primers for vasopressin V_{1a}, V_{1b}, V_2, β-adrenergic, GIP, GLP-1, 5-HT_4, LH/hCG, and ACTH receptors were designed according to a previous description *(14)* (Table 1). A set of primer pairs for β-actin was also designed as a positive standard.

RNA Preparation and RT-PCR

Total RNA was isolated from case 1's excised tumor using the TRIzol reagent (Gibco BRL cat nos. 15596-026, 15596-018). Normal adrenal cortex tissue was obtained from a normal gland (kidney removal; kindly provided from Yoshihiro Wada, MD). One microgram of total RNA from the tumor and the normal adrenal cortex were reverse transcribed, using random hexanucleotides, M-MLV reverse transcriptase, RNase inhibitor, and deoxyribonucleotide triphosphates at 37ºC for 90 min. This was followed by the addition of 400 U of M-MLV to the reaction, and the incubation was continued for another 90 min. The RT reaction was terminated with the addition of 3 U of RNase H for 30 min at 37ºC. The PCR reaction (35 cycles) was subsequently performed in a final volume of 25 µL, using 20 pmol of each primer, and 5 U of *Taq* polymerase (Applied Biosystems, NJ) at 94ºC for 30 s, 57ºC for 30 s.

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