Adrenocorticotropin-Independent Macronodular Adrenocortical Hyperplasia Associated with Multiple Colon Adenomas/Carcinomas Which Showed a Point Mutation in the *APC* Gene

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We report a male Japanese with corticotropin (ACTH)-independent macronodular adrenocortical hyperplasia (AIMAH) associated with multiple colon adenomas/carcinomas. The plasma cortisol level was elevated with no diurnal rhythm and was not suppressed with dexamethasone. Basal plasma ACTH was unmeasurable but subnormally increased after administration of metyrapone or corticotropin releasing hormone. Both adrenals were resected and weighed 90g; the histopathologic findings were similar to those of AIMAH as previously reported. At least 21 colon lesions which were adenomas or carcinomas, were resected endoscopically or surgically. This is the second reported case of the association of AIMAH with multiple colon polyps. An *APC* gene point mutation was detected in the colon cancer tissue by polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP)/direct sequencing analysis at the putative splice acceptor site consensus sequence. However, no abnormality of *APC* gene was detected in the adrenocortical hyperplastic tissue. The possible etiological coexistence of these two diseases is discussed. (Internal Medicine 36: 536–542, 1997)

Key words: Cushing's syndrome, adrenocortical nodular hyperplasia

Introduction

Nodular adrenocortical hyperplasia is not common for the etiology of Cushing's syndrome and consists of a few clinicopathological types. However, its classification has not been well defined. The clinical and pathological entity of primary pigmented adrenocortical micronodular dysplasia (PPAMD) has been established (1). Although the clinical and pathological characteristics of some cases of macronodular adrenocortical hyperplasia have become clear, their etiology has not been clarified yet. One such hyperplasia was presented as corticotropin (ACTH)-independent bilateral macronodular adrenocortical hyperplasia (AIMAH) by Aiba et al (2). We report a patient with AIMAH who was associated with multiple colon polyps; the association is extremely rare. We tried to clarify the etiological possibility of this coincidence in our patient.

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Materials and Methods

All blood samplings were performed after at least one hour of bed rest.

Hormone measurement

Plasma cortisol and dehydroepiandrosterone were measured by Gammacort Cortisol KitTM (Baxter-Travenol) (reference range, 110–505 nmol/l) and COAT-A-COUNT DHEATM (Diagnostic Product Co., Los Angeles, CA, USA) (2.8–24.3 nmol/l), respectively. Plasma ACTH was measured by commercially available immunoradiometric assay kit, ALLEGRO HS-ACTHTM (Nichols Institute, San Juan Capistrano, CA, USA), with a reference range of 2–11 pmol/l. Urinary free cortisol and 17-hydroxycorticosteroids (17OHCS) were measured by Gammacort Cortisol KitTM and Porter-Silber's method, with a reference range of 83–276 nmol/day and 2.7–7.3 mg/day, respectively. The method of measuring plasma gastric inhibi-

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tory polypeptide (GIP) was previously reported elsewhere (3) and its reference range was 167 ± 12 pg/ml.

Pathological examinations

Resected adrenals, biopsied specimens of colon, resected colon and autopsied specimens were fixed in 20% formalin neutral buffer solution and embedded in paraffin. Each three micron-thick section of paraffin-embedded tissues was stained for hematoxylin-eosin and examined light-microscopically.

PCR-SSCP/direct sequencing analysis for the APC gene mutation in the adrenocortical hyperplastic tissue and co-

lon cancer tissue (Fig. 1a, b)

The APC gene mutation was examined as described (4). Briefly, high molecular weight DNA was extracted by proteinase K digestion/phenol-chloroform extraction method from the tissues of hyperplastic lesion of the left adrenal gland, the well-differentiated adenocarcinoma at the rectosigmoid region and the non-tumorous colonic mucosa of the patient. PCR reaction mixture contains 50–100 ng of genomic DNA, 1.0 μ M each of 2 primers, 0.2 mM each of 4 deoxynucleotide triphosphates, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.125 units of Taq polymerase and 0.2 μ l of [$\alpha^{32}P$] (10mCi) in a volume of 0.5 μ l. Seventeen sets of primers were

Figure 1. A. Frequency of the APC gene mutations in familial adenomatous polyposis reported in the literature (4, 5, 26–51). The last and longest exon 15 was divided into 11 PCR-suitable segments from 15–1 to 15–11 and the remaining 3' half of the exon, designated >15–11. Both the somatic and germ-line mutations were counted. B. Exonic regions analyzed in this study. PCR was targeted to the exonic regions marked by double-head arrows. The primer sequences for exons 4–6, 8, 13 and exon 15 fragments 15–1 to 15–6 were published in ref. (7), exon 14 in ref. (52) and exon 15 fragments 15–7 to 15–10 in ref. (4). The primer set for exon 15 fragment 15–11, 5'-CTGCCATGCCAACAAAGTCA-3' and 5'-GAGCCTCATCTGTACTTCTGC-3', was newly designed in this study.

used in this study, which together analyzed the exons 4, 5, 6, 8, 13, 14 and the amino-terminal half of exon 15 of the human *APC* gene. The primers cover 45.5% of the *APC* gene exons, including the mutation cluster region from codon 1,286 to 1,514 (5), and most of the reported somatic and germ-line mutations have been mapped within this region (Fig. 1). The thermal cycles profile was 30 seconds at 94°C, 30 seconds at 55°C and 1 minute at 72°C for total 40 cycles. The PCR product was diluted 1:50 in 0.1% NaDodSO₄ and 10 mM EDTA, mixed 1:1 with 95% formamide dye and applied on a 5% polyacrylamide gel containing 5% glycerol. Electrophoresis was performed for 3 hours at 40W with the water jacket temperature set at 15°C. The gel was dried at 80°C and exposed to Kodak XAR film at room temperature.

DNA fragments corresponding to the bands on the PCR-SSCP analysis were excised from the dried gel and eluted in water (6). The eluted DNA was then PCR-amplified and sequenced by the dideoxy chain termination method using a 5'-³²P labelled deoxyoligonucleotide as a primer and Pfu polymerase (dsDNA Cycle Sequencing System, GIBCO, Gaithersburg, MD, USA). The primers used for sequencing were the same as those for the PCR-SSCP. The multiple bands with varying intensity were observed for each DNA sample on the PCR-SSCP gel. They were eluted and sequenced separately.

Case Report

A 73-year-old male Japanese had been hypertensive for 5 years, which had not been controlled well. During consultation for common cold, he was found to have glucosuria and hypokalemia. Bilateral adrenal enlargement was demonstrated by computed axial tomography (CT). He was referred to Matsunami General Hospital for further examination. On admission, he was 165 cm in height and weighed 72 kg. Blood pressure was 180/104 mmHg. Truncal obesity, thin skin and bruiseability were noticed, but buffalo hump and striae cutis were not seen. Neither spotted nor diffuse pigmentation was noticed. The cardiac ultrasonography findings were unremarkable. Iron deficiency anemia (red blood cell $3.3 \times 10^{12}/l$, Hb 100 g/l, Ht 0.30, Fe 6.3 $\mu\text{mol}/l$) was revealed. Serum potassium was slightly low (3.5 mmol/l), blood urea nitrogen (11.1 mmol/l) and serum creatinine (105 $\mu\text{mol}/l$) was high and creatinine clearance was low (0.54 mL/s). Plasma glucose level increased from 4.4 mmol/l to 11.4 mmol/l at 90 minutes after oral administration of 75 g glucose. Basal plasma cortisol level at 0800h was high (632 nmol/l) and showed no diurnal rhythm; 646 at 0900h, 604 at 2000h and 657 at 2100h. Urinary excretion of free cortisol was also increased to 370 nmol/day. Plasma ACTH was not measurable (less than 1 pmol/l). Plasma cortisol after overnight-suppression test with 8 mg dexamethasone was 422 nmol/l, which was not fully suppressed. By intravenous administration of 100 μg corticotropin-releasing hormone (CRHTM, Mitsubishi Chemical, Tokyo), both plasma cortisol and ACTH increased from 524 nmol/l and less than 1 pmol/l to 662 nmol/l and 3 pmol/l, respectively. However, these increases were subnormal. Plasma cortisol also increased from

579 to 1,793 nmol/l by intravenous administration of 250 μg 1–24 ACTH (CortrosynTM, Daiichi-Seiyaku, Tokyo). During the administration of 3.0 g metyrapone in 6 divided doses (started from 0400h) daily for 2 days, the patient felt severe fatigue. Accordingly, the final dose of metyrapone at 2400h on the 2nd day was not given. The change of urinary excretion of 17 OHCS during this period was equivocal (baseline, 13.7 mg/day; the 1st and the 2nd day of metyrapone administration, 10.0 and 12.3 mg/day, respectively) and plasma ACTH level at 0800h increased from less than 1 to 4 pmol/l on the 2nd day of metyrapone administration. Only a slightly increased response of plasma cortisol to intravenous administration of arginine-vasopressin (AVP) (PitressinTM, Sankyoseiyaku, Tokyo) was shown when 0.1 U AVP was used, from 544 to 627 nmol/l, but the increase was clear when 0.2 U was used, from 510 to 742 nmol/l. In both cases plasma ACTH did not respond at all. Basal plasma DHEA and DHEA-S levels were low, 2.1 nmol/l and 3.6 $\mu\text{mol}/l$, respectively. Plasma GIP and cortisol levels were simultaneously measured before and 2 hours after breakfast for 3 days. Mean value of plasma GIP increased after breakfast from 375 to 825 pg/ml, but that of the plasma cortisol level did not change significantly, from 361 to 348 nmol/l. They showed no correlation with each other. On abdominal CT, bilateral adrenal glands were markedly enlarged with multiple large nodules. Bilateral adrenal uptake was demonstrated on ¹³¹I-iodocholesterol (¹³¹I-AdosterolTM) adrenal scintigraphy even after treatment with 3 mg dexamethasone daily for 7 days. Magnetic resonance imaging (MRI) of the pituitary gland showed no abnormal finding. These data indicated Cushing's syndrome due to AIMAH.

Because of the iron deficiency anemia, colorectal examination was done, which revealed multiple polypoid lesions throughout the colon. At least 21 polyps (from 3 to 45 mm in diameter, respectively) were found and endoscopic polypectomy was performed for 15 polyps. Well differentiated adenocarcinoma was found in one of them. The two biopsied specimens from the remaining large lesions (35 and 45 mm in diameter) located in the transverse and rectosigmoid colon were also positive for adenocarcinoma.

On January 6, 1995, right adrenalectomy and partial resection of the rectosigmoid and transverse colon were performed. No gross metastasis was found. One to three months after the operation, plasma cortisol level and urinary excretion of free cortisol and 17 OHCS decreased to 248–334 nmol/l, 141–232 nmol/day and 4.9–7.3 mg/day, respectively. However, the plasma ACTH level was still suppressed, being less than 1 pmol/l. The result of 75g-oral glucose tolerance test were ameliorated, being from 4.3 mmol/l to 8.7 at peak in plasma glucose level. Hypertension and hypokalemia were still persistent.

On April 18, 1995, left adrenalectomy was performed. Prednisolone was substituted from the day of the operation and was tapered from 125 mg to 20 mg daily until the 29th day post-operative. Since discharge on June 2, 1995, the patient required 20 mg/day prednisolone for replacement therapy. In December 1995, liver metastasis of the colon cancer was suspected on

abdominal CT. He had a high fever and dyspnea following a low grade fever for about three months and was readmitted in March 1996. However, he died 4 days later. The autopsy revealed liver metastases of colon cancer and, unexpectedly, disseminated tuberculosis involving the lung, pericardium and spleen. The pituitary gland was not investigated on autopsy.

Pathological findings of adrenal glands

Resected adrenal glands were 6×2×3 cm and 25 g in the right gland and 9×5×4 cm and 65 g in the left. Histopathologic findings of both glands were similar. Cut sections of the specimens showed multiple, bulging and yellow large nodules. No normal portion of the adrenal glands could be identified macroscopically (Fig. 2). Histologic examination revealed that the nodules were relatively well-circumscribed by thin fibrous septum. Cortical cells in the nodules were composed of clear-type cells and compact-type cells. A variable number of compact-type cells formed a small nest or island-like structure (Fig. 3), and extracapsular extension was seen. The normally arranged portion of the cortex was almost lost and the adrenal medulla was thin.

Study on the mutation of APC gene in the adrenocortical hyperplastic tissue and colon cancer tissue

As shown in Fig. 4A, the PCR-SSCP analysis for the region spanning exon 8 showed an abnormal band on the colorectal cancer specimen. No other SSCP abnormalities were identified in any of the patient's samples examined by the primer set used in this study (data not shown). In general, multiple bands with varying intensities may appear on a PCR-SSCP gel, presumably representing multiple single-strand DNA conformations from both strands of the template DNA. In this particular SSCP gel, shown in Fig. 4A, three major bands were observed for the exon 8 region. Only the top band showed detectable mobility shift for the colon cancer sample. DNAs were eluted separately from all the three bands with or without mobility shift and sequenced by the direct method. All the DNA samples derived from the colon cancer sample showed A to G transition at the 8 bases upstream of the intron 7 – exon 8 boundary (only one DNA sequence is shown in Fig. 4B). The band corresponding to the normal sequence was not detectable in the colon cancer specimen on the sequencing gel.

On the other hand, the nucleotide sequence of the DNA samples obtained from the adrenal hyperplastic tissue and the non-tumorous colonic mucosa of this patient were identical to those of the control DNA from a normal unrelated subject and also to the published normal sequence on this region (7).

Discussion

The etiology of AIMAH has not been well-established. Hermus et al (8) and Smals et al (9) proposed the possibility that long-standing ACTH excess induces macronodular adrenocortical hyperplasia. However, based on their observation, patients with bilateral adrenocortical hyperplasia have measurable plasma ACTH and the urinary 17 OHCS excretion increases in

Figure 2. Resected right adrenal gland. Cut sections of the specimen showed multiple, bulging and yellow large nodules. No normal portion of adrenal gland could be identified.

Figure 3. Microscopic findings of adrenocortical nodule (HE stain, ×200). Clear-type cells formed a cord or nest-like structure and some have nuclear pleomorphism. A variable number of compact-type cells formed a small nest or island-like structure.

response to metyrapone (8). In AIMAH, the plasma ACTH level is suppressed and shows no increase after CRH or metyrapone administration (2, 10–12). In the present patient, however, plasma ACTH slightly increased after CRH or metyrapone administration, although subnormal in level. This suggests that the suppression of pituitary ACTH secretion in our patient was not as severe as that observed in AIMAH (2, 10–12). Extremely atrophic adjacent adrenocortical tissue to macro-

Figure 4. A. PCR-SSCP analysis of the APC gene for the exon 4, 5, 6 and 8 regions. Nc is the normal control DNA from an unrelated subject; N, Tm and A are non-tumorous rectal mucosa, colon cancer and adrenal hyperplasia of the patient, respectively. The arrow shows the abnormal band for the colon cancer sample on the exon 8 PCR-SSCP analysis. B. Direct sequencing of the PCR-SSCP band of the exon 8 region. Only N and Tm samples are shown. The sequence of the Nc and A samples were the same as N, which was identical as the published normal sequence (6). The sequence was confirmed on both strands. The intron sequence is presented in lower case letters and the exon sequence in upper case letters. The A to G point mutation is circled.

nodules and no evidence of pituitary adenoma in AIMAH (2, 10–14) is not consistent with the hypothesis of Kloppenborg (8, 9). An increase of plasma cortisol in response to AVP stimulation was seen in the present patient, similar to other patients with AIMAH as reported by Horiba et al (15). The absence of correlation between plasma cortisol and GIP proved that the hyperplasia in our patient was not GIP-dependent (16). Based on these findings, the clinicopathological characteristics of our patient satisfied the criteria of AIMAH, although the reason why there was incomplete suppression of ACTH secretion remains unclear.

Almost all reported adrenocortical lesions of Cushing's syndrome associated with colorectal adenomatosis were adenoma or carcinoma, but not AIMAH (17–20). The patient with AIMAH reported by Lieberman et al (14) had colon polypec-

tomy, with no mention of the number of polyps. One Japanese patient (21) had AIMAH associated with multiple colon polyps. It seems that this rare association seen in this patient (21) and our patient was not incidental. Genetic study of tumorigenesis of colon adenoma/carcinoma is now being explored extensively. Characteristic genetic findings, however, have not been reported in patients with AIMAH. A point mutation of stimulatory guanine nucleotide-binding protein ($Gs\alpha$) gene in adrenocortical nodular tissue of an infant with Cushing's syndrome due to nodular adrenal hyperplasia was reported (22), similar to McCune-Albright's syndrome (23), although the adrenal lesion was not AIMAH. Activating $Gs\alpha$ mutations in adrenocortical cells could, in theory, lead to cellular proliferation and nodule formation (22). In another report (14), no mutation of $Gs\alpha$ gene in the adrenocortical tissue was found in a patient with

AIMAH. Unfortunately, we did not examine mutations in *Gsa* gene in the present patient.

The genetic abnormality responsible for the development of familial adenomatous polyposis was identified as the inactivation of the putative tumor suppressor gene, *APC*, which stands for adenomatous polyposis coli (24). Loss of normal allele of the *APC* gene was reported in an adrenocortical carcinoma tissue from a patient with familial adenomatous polyposis (19). The A to G transition seen in the splice acceptor site of intron 7 of *APC* gene in the colon cancer tissue detected in the present patient was within the polypyrimidine tract of the splice acceptor consensus sequence, (U/C)₁₁NCAG:G, the last G being the first nucleotide of the 3' exon (25). The point mutation in the splice acceptor site may affect the normal splicing mechanism of the *APC* gene in cancer cells. In our patient, however, a point mutation of *APC* gene was found in the colon cancer sample but not in the adrenal hyperplastic tissue. The association of AIMAH with multiple colon adenoma/carcinoma is considered to be incidental, but the possibility that common etiological genetical anomaly other than *APC* gene may be present between multiple colon adenomas/carcinomas and hyperplastic adrenal cortex in AIMAH in our patient cannot be disregarded.

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