Malignant Gastric Carcinoid Causing Ectopic ACTH Syndrome: Discrepancy of Plasma ACTH Levels Measured by Different Immunoradiometric Assays

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Abstract. Discrepancy of plasma ACTH levels measured by different immunoradiometric assays (IRMA) in a case with malignant gastric carcinoid causing ectopic ACTH syndrome was examined by gel chromatography and immunohistochemical analysis. A 49-year-old male was found to have a large gastric tumor, with muscle wasting, hypertension, diabetes and hypokalemia caused by hypercortisolemia. His plasma ACTH levels, although initially elevated, were found to be almost in normal ranges. The discrepancy of plasma ACTH levels was proven to be due to different IRMA kits used; the initial assay was performed by a kit that could recognize high-molecular weight (HMW) form as well as ACTH(1-39), but the later assay by another kit that could recognize only ACTH(1-39). Pathological examination of the gastric tumor was consistent with the diagnosis of malignant carcinoid. Immunohistochemical study revealed that immunoreactivity of proopiomelanocortin (POMC) was positive within the tumor cells, whereas those of ACTH and prohormone convertase 1/3 were negative. Molecular sieving analysis of patient's plasma by gel chromatography coupled with ACTH radioimmunoassay which could recognize HMW form and ACTH(1-39) and two different IRMAs revealed that the predominant form of ACTH was HMW form with a minor peak of ACTH(1-39). This is a rare case of ectopic ACTH syndrome caused by malignant gastric carcinoid with preferential production of HMW form of ACTH, possibly due to unprocessed POMC.

Key words: Ectopic ACTH syndrome, Gastric carcinoid, Proopiomelanocortin (POMC), Immunoradiometric assay (IRMA)

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THE etiology of Cushing's syndrome can be divided into two groups; ACTH-dependent and ACTH-independent one. Among the ACTH-dependent group, pituitary-dependent Cushing's syndrome (Cushing's disease) is the most common (60–80%), while 15%

cases are non-pituitary tumors secreting ACTH (ectopic ACTH syndrome) [1–4]. In ectopic ACTH syndrome, circulating ACTH and cortisol levels can be extremely elevated. Approximately 20% of ectopic ACTH syndrome are due to indolent tumors, such as bronchial carcinoids [5, 6]; they present with the typical clinical and biochemical features similar to Cushing's disease. Thus, the principal diagnostic dilemma is the distinction of pituitary-dependent Cushing's disease from the indolent ectopic ACTH-producing tumors [2].

ACTH is synthesized as a precursor molecule,

termed proopiomelanocortin (POMC). In the human pituitary, POMC is enzymatically processed to pro-ACTH, N-terminal POMC (N-POC), β-lipotropic hormone (β-LPH), ACTH(1-39), and β-endorphin. Processing of POMC in the pituitary takes place by the protease prohormone convertase (PC) 1/3 located within mature dense core granules via the regulated secretory pathway [7, 8].

The presence of high molecular weight (HMW) form of ACTH (so-called 'big ACTH') in plasma and tumor extract was first demonstrated in a patient with ACTH-producing thymoma [9]. However, the complexity of chromatographic methods limited the identification of ectopic ACTH syndrome [10–12]. By using an IRMA that recognizes different sites of the POMC molecule, White *et al.* reported that plasma levels of HMW form of ACTH in patients with ectopic ACTH syndrome were greater than those in patients with Cushing's disease, suggesting the impaired processing of POMC by the tumors [13]. While POMC gene is also expressed in a number of extra-pituitary tissues [14], the regulation of POMC gene expression and post-translational processing in tumors associated with the ectopic ACTH syndrome remain unclear.

We herein describe a case with ectopic ACTH syndrome caused by a malignant gastric carcinoid, with apparently discrepant plasma ACTH levels as determined by different IRMAs. This was later proved to result from the predominant production and secretion of HMW form of ACTH by the tumor as revealed by gel chromatography coupled with different ACTH assays as well as immunohistochemical analysis.

Methods

Gel exclusion chromatography

Analysis of molecular size of circulating immunoreactive ACTH and β-endorphin was performed by gel exclusion chromatography as reported [15]. Extraction of plasma samples was performed using Sep-Pak C18 cartridge (Waters Inc., MA). 0.5 ml-aliquots of extracted and non-extracted plasma were applied to a 1×50 cm Sephadex G75 superfine column (Amersham Biosciences Corp., NJ) eluted with phosphate buffer, pH 7.4, at a rate of 6 ml/hr; 1 ml-fraction was collected.

Immunoradiometric assays (IRMA) and radioimmunoassays (RIA)

Measurement of ACTH was performed by two ACTH IRMA kits; Yuka® (Mitsubishi Chemical, Tokyo) and Allegro® (Nichols Institute Diagnostic, CA). Concentrations of ACTH and β-endorphin were determined by conventional specific radioimmunoassays (RIA) [9, 15, 16]. The anti-ACTH antibody used fully recognizes ACTH(1-24) and ACTH(1-39) and POMC, but not with α-MSH, β-endorphin or corticotropin-like intermediate lobe peptide (CLIP); the sensitivity was 2 pg/ml. The anti-β-endorphin antibody used fully recognizes β-endorphin and β-LPH, but not with α-MSH or ACTH(1-39); the sensitivity was 5 pg/ml.

Immunohistochemistry

Immunohistochemial study of ACTH and POMC was performed by Envision method (DAKO: Carpinteria, CA) using anti-ACTH monoclonal antibody (1:200; DAKO) and anti-POMC monoclonal antibody (1:100; Biogenesis, Poole, UK) [17], and that of PC 1/3 was performed using anti-PC 1/3 polyclonal antibody (1:400; Chemicon Int., CA) by avidin-biotin peroxidase complex (ABC) method [18].

Case Report

A 49-year-old man with chronic fatigue and anorexia, noticed a solid mass in the upper abdomen which gradually increased in size during 6 months. He visited Nakano General Hospital in June 2001, where computed tomography (CT) scan of the abdomen revealed a large gastric tumor (12×15 cm). At open laparotomy, the tumor was invasive to the liver and the spleen and too large to be removed, only biopsy was performed. Pathological diagnosis was consistent with malignant gastric carcinoid. Despite treatment with 5-fluorouracil, he complained of progressive malaise, muscle weakness, thirst and polyuria, and found to have hypertension (160/100 mmHg), hyperglycemia (438 mg/dl), and hypokalemia (2.8 mEq/l). Endocrine data showed elevated plasma levels of ACTH (220 pg/ml) and cortisol (45.98 μg/dl). The patient was referred to our hospital for further endocrine evaluation of suspected Cushing's syndrome.

Physical examination on admission revealed Cushin-

goid features, such as central obesity, marked muscle wasting of the extremities, and pigmentation of the skin and the nailbed. A large, solid abdominal mass (20×15 cm) was palpable in the abdomen. Initial laboratory data (Table 1) showed mild leukocytosis with eosinopenia, profound hypokalemia, hyperglycemia, elevated serum levels of lactate dehydrogenase (LDH) and alkaline phosphatase, and metabolic alkalosis. Serum levels of tumor markers, such as α-fetprotein, neuron specific enolase, and CA19-9, were all elevated. Endocrine examination revealed that both plasma cortisol levels and urinary excretion of free cortisol (UFC) were markedly increased; plasma cortisol level was not suppressed after overnight high-dose (8 mg) dexamethasone suppression test. Unexpectedly, plasma

ACTH level determined at our hospital was low (5 pg/ml), despite the high level (220 pg/ml) determined at previous hospital. Plasma levels of gastrin and glucagon as well as urinary excretion of 5-hydrokyindole acetic acid were also elevated. Abdominal CT scan revealed the presence of a huge heterogenous tumor ($15 \times 15 \times 20$ cm), occupying the whole abdominal cavity with infiltration to the abdominal wall (Fig. 1). Magnetic resonance imaging of the brain showed no abnormal lesion in the pituitary.

Since he had severe hypercortisolemia enough to cause hypertension, diabetes, hypokalemic metabolic alkalosis and immunocompromised state, treatment with oral administration of metyrapone (2.0 g/day) was started to control his hypercortisolemia. His clinical,

Table 1. Laboratory and endocrine data

Laboratory data			
Complete blood count		Alkaline phosphatase	375 U/l
White blood cells differential	$12800/\mu l$	Total bilirubin	0.6 mg/dl
(Neu 91%, Ly 3%, Mo 6%, Eo 0%, Ba 0%)		Total cholesterol	235 mg/dl
Red blood cells	$419 \times 10^4/\mu l$	Triglyceride	177 mg/dl
Hemoglobin	12.6 g/dl	Fasting plasma glucose	279 mg/dl
Platelets	$26.7 \times 10^4/\mu l$	Hemoglobin A_{1c}	7.5%
Chemistry		CRP	0.8 mg/dl
Total protein	5.5 g/dl	Atrial blood gas (ambient air)	
Albumin	3.2 g/dl	pH	7.526
Blood urea nitrogen	20 mg/dl	PaO_2	59 Torr
Creatinine	0.7 mg/dl	$PaCO_2$	46.3 Torr
Sodium	145 mEq/l	BE	13.4 mEq/l
Potassium	2.5 mEq/l	HCO_3^-	38.3 mmol/l
Chloride	92 mEq/l	Tumor markers	
Calcium	9.1 mEq/l	α-fetprotein	2304 ng/ml
Phosphorus	3.0 mg/dl	CA19-9	219.9 U/ml
Lactate dehydrogenase	800 U/l	CEA	4.1 ng/ml
Asparate aminotransferase	23 U/l	NSE	17 ng/ml
Alanine aminotransferase	26 U/l	Pro-GRP	25.1 pg/ml
γ-glutamyltranspeptidase	97 U/l		

CRP; C-reactive protein, CA19-9; carbohydrateantigen 19-9, CEA; carcinoembryonic antigen, NSE; neuron specific enolase, Pro-GRP; pro-gastrin releasing peptide

Endocrine data			
Plasma hormones		Aldosterone	2.5 ng/ml
ACTH (previous hospital)	220 pg/ml	Gastrin	292 pg/ml
ACTH (our hospital)	5 pg/ml	Glucagon	126 pg/ml
cortisol	45.98 μg/dl	Urine hormones (24-hours)	
after overnight dexamethasone (8 mg)	36.43 μg/dl	Free cortisol	1460 μg
suppression test		17-KS	15.3 mg
5-HIAA	8.4 ng/ml	17-OHCS	48.1 mg
Renin activity	0.4 ng/ml/h	5-HIAA	5.5 mg

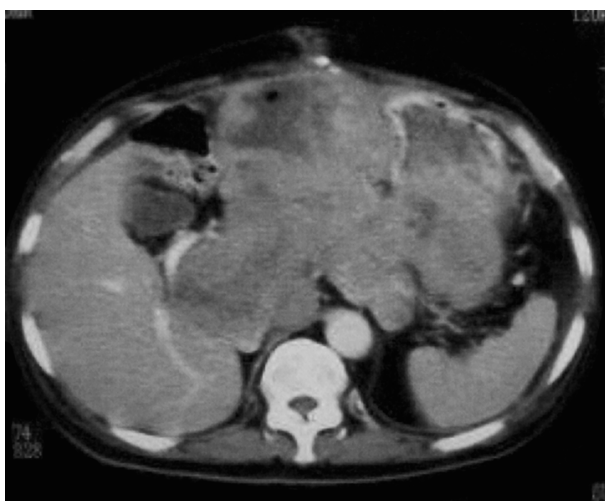17-KS; 17-ketosteroid, 17-OHCS; 17-hydroxycorticosteroids, 5-HIAA; 5-hydroxyindole acetic acid

Fig. 1. Computed tomography (CT) of the abdomen.
A huge tumor occupied intraperitoneal space, invading to abdominal wall, pancreas, and duodenum.
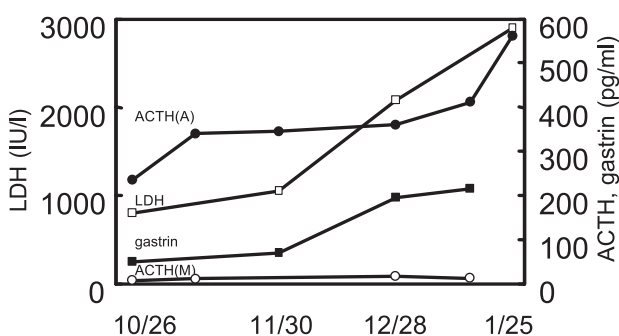

Fig. 2. Changes of plasma levels of ACTH and tumor markers during the clinical course.
Plasma ACTH concentrations were simultaneously measured by two different ACTH-IRMA kits: Allegro® (A) and Yuka® (Y).

laboratory and endocrine data were all consistent with the diagnosis of Cushing's syndrome caused by an ectopic ACTH-producing gastric tumor, only except for the low plasma ACTH levels. He decided to have a second surgery in the hope that tumor may be resectable. However, open laparotomy revealed a fragile, hemorrhagic huge tumor with massive invasion to the pancreas, duodenum, and abdominal wall, which made surgical resection of the tumor impossible. Postoperatively, he was treated with weekly injection of α-interferon (6×10^6 IU/week), but the tumor was progressively enlarged in parallel to elevation of serum tumor markers (LDH, gastrin); plasma ACTH levels remained almost within normal range (Fig. 2). He was

transferred back to Nakano General Hospital, and expired on February 10, 2002. Autopsy was not performed.

Hormonal Studies

The apparent dissociation of plasma ACTH levels measured at the previous and our hospital prompted us to investigate its cause. This was later proved to be due to different ACTH assay kits used. Previous hospital laboratory employed Allegro®-IRMA kit yielding high plasma ACTH levels, whereas our hospital laboratory employed Yuka®-IRMA kit yielding low-normal ACTH levels, in the same patient's plasma samples whenever simultaneously determined (Fig. 2). The possible non-specific interference of patient's plasma in Yuka®-IRMA kit could not be completely excluded.

To determine the size heterogeneity of patient's plasma ACTH, Sephadex G75 gel exclusion chromatography was performed using different ACTH assay methods. Using two different ACTH-IRMA kits, gel chromatography of non-extracted patient's plasma revealed that a major peak of HMW form eluting after void volume and a minor peak of ACTH(1-39) as determined by Allegro® kit (Fig. 3a), but only a minor peak of ACTH(1-39) as determined by Yuka® kit (Fig. 3b). Using two conventional RIAs for ACTH and β-endorphin, gel chromatography of non-extracted patient's plasma showed essentially similar elution pattern to that as determined by Allegro® kit, with a major peak of HMW form and a minor peak of ACTH(1-39), while a major peak of β-LPH appeared between HMW form and ACTH(1-39) with a minor component eluting in HMW form; there was no distinct peak of β-endorphin (Fig. 4a). In contrast, gel chromatography of extracted patient's plasma revealed a minor peak of ACTH(1-39) with a negligible HMW form, and a distinct peak of β-LPH (Fig. 4b).

Immunohistochemical Studies

Microscopical examination of the biopsied gastric tumor specimens revealed closely clustered tumor cells with marked atypia surrounded by fibrous stroma (Fig. 5a). The tumor cells occasionally formed glandular lumina. These features were consistent with the pathological diagnosis of malignant gastric carcinoids.
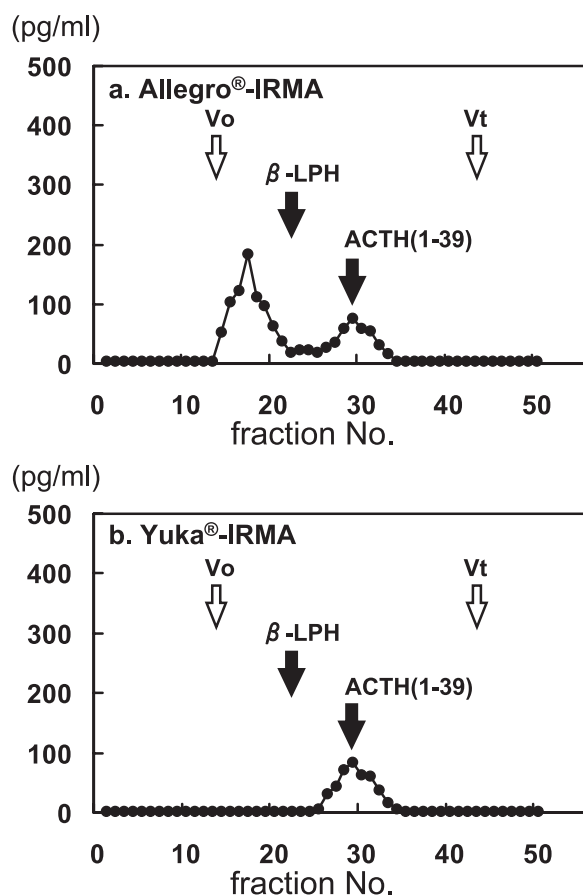
Fig. 3. Sephadex G75 gel chromatography of patient's plasma using two different ACTH IRMA-kits.
Non-extracted plasma sample (0.5 ml) was applied to Sephadex G75 (1 × 50 cm) gel chromatography; ACTH concentration was determined by (a) Allegro®-IRMA and (b) Yuka®-IRMA, respectively. Arrows indicate elution positions of ACTH(1-39), β-LPH, Vo (void volume), and Vt (total volume), respectively.

Fig. 4. Sephadex G75 gel chromatography of patient's plasma using conventional RIAs for ACTH and β-endorphin.
Plasma extract (0.5 ml) after Sep-pak C18 was applied to Sephadex G75 (1 × 50 cm) gel chromatography; concentrations of ACTH (●) and β-endorphin (○) of (a) non-extracted plasma and (b) extracted plasma were measured, respectively. Arrows indicate elution positions of β-LPH, ACTH(1-39), Vo (void volume) and Vt (total volume), respectively.

Immunohistochemical study revealed that immunoreactivities were positive for chromogranin A, α-fetprotein, epithelial membrane antigen, and Glimelius-positive tumor cells were also noted. But the markers for smooth muscle or gastrointestinal stromal origins, including c-kit, S-100 protein, muscle actin (HHF35) and keratin, were all negative (data not shown). Immunohistochemical analysis revealed that immunostaining for ACTH(1-39) (Fig. 5b) and PC 1/3 (Fig. 5d) were negative in the tumor cells, whereas immunostaining for POMC was positive (Fig. 5c). These data strongly suggest that the tumor predominantly produce unprocessed POMC without significant cleavage to ACTH(1-39) by PC 1/3.
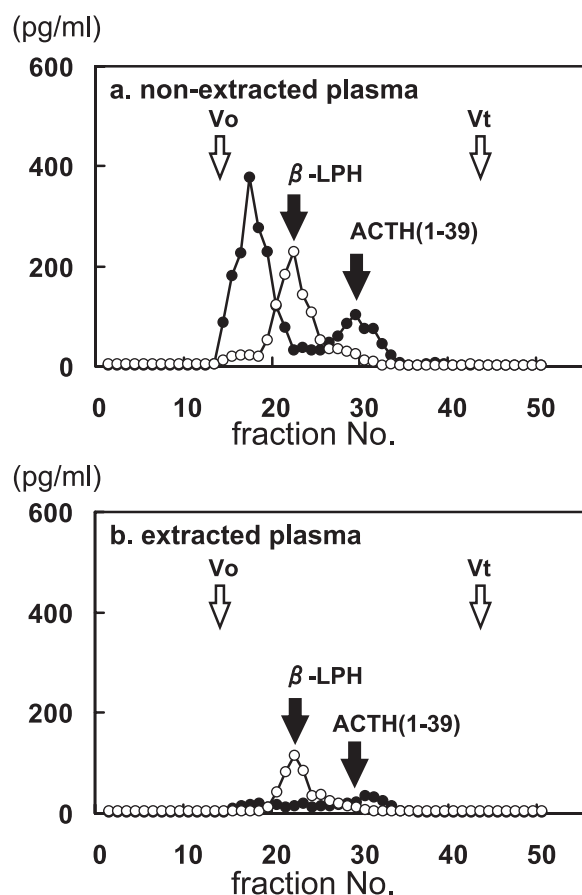
Discussion

Generally, gastric carcinoids are small in size and slowly-growing with benign prognosis [19]. The patient's gastric tumor described herein was unusual in that it was huge and rapidly-growing, thereby leading to fetal outcome about one year after the onset. The pathological diagnosis of malignant carcinoid tumor originated from the stomach was made from the biopsied tissue specimens obtained from laparotomy. The clinical diagnosis of ectopic ACTH syndrome caused by malignant gastric carcinoid in the present case was established based on the following reasons; 1) apparent Cushingoid appearance associated with profound mus-
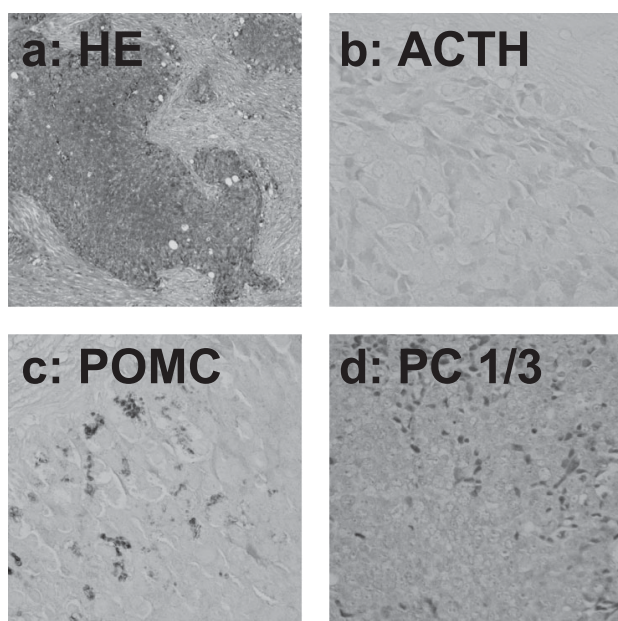
Fig. 5. Immunohistochemical analysis.
Immunohistochemical study was performed by Envision method for anti-ACTH and anti-POMC antibody and avidin biotin peroxidase complex method for anti-PC 1/3 antibody, respectively.
(a) HE staining (×25), (b) anti-ACTH (×100), (c) anti-POMC (×200), (d) anti-PC 1/3 (×200)

cle weakness, hypertension, diabetes and hypokalemic metabolic alkalosis, 2) hypercortisolemia with no suppression by high-dose (8 mg) dexamethasone, 3) presence of immunoreactivity for POMC within the tumor cells.

A 39 amino-acid residue ACTH is synthesized from a larger precursor molecule, termed POMC, via specific processing by PC 1/3 in a tissue-specific fashion [20–22]; in the normal anterior pituitary, POMC is processed to β-LPH and pro-ACTH, which is further cleaved in to an N-terminal peptide containing γ-MSH (N-POC), joining peptide, and ACTH. In contrast, aberrant processing of POMC, such as unprocessed POMC, and/or enhanced processing of ACTH into α-MSH and CLIP, by non-pituitary tumors, leads to increased proportion of circulating levels of HMW form ACTH and ACTH-related fragments in patients with ectopic ACTH syndrome [20, 21].

The apparent discrepancy of plasma ACTH levels in this case can be accounted for by the measurement by different assay kits used. In contrast to high ACTH levels determined by Allegro® assay kit, those determined by Yuka® assay kit in the same patient's plasma

samples were almost normal. Two-site IRMA kit by Allegro® employs ^{125}I-labeled antibody against N-terminal fragment(1-17) and antibody against C-terminal fragment(34-39) of ACTH, while that by Yuka® employs ^{125}I-labeled antibody against N-terminal fragment(20-23) and antibody against C-terminal fragment(35-39) of ACTH, respectively. These data strongly suggest that various circulating forms of ACTH molecule in patient's plasma could be differentially recognized by these two different IRMA kits.

Gel chromatographic analysis of non-extracted patient's plasma coupled with two different ACTH-IRMA kits revealed two distinct peaks composed of a major peak eluting earlier than ACTH(1-39), and a minor peak of ACTH(1-39) as measured by Allegro® kit, but only one peak of ACTH(1-39) as measured by Yuka® kit (Fig. 3). These data suggest that Allegro® kit can recognized HMW form as well as intact ACTH(1-39), but Yuka® kit can recognize only ACTH(1-39), but not HMW form. This was confirmed by the chromatographic analysis of non-extracted patient's plasma coupled with conventional RIA that can fully recognize POMC and ACTH(1-39) (Fig. 4a). It should be noted that patient's extracted plasma contained a small peak of ACTH(1-39) with a negligible HMW form (Fig. 4b). The apparent loss of HMW form of ACTH after extraction could be accounted for by its low recovery rate (less than 10%) of POMC by extraction using Sep-pak C18 (unpublished observation).

Gel filtration of the extracted and non-extracted patient's plasma coupled with β-endorphin RIA that equally recognizes β-LPH and β-endorphin showed almost similar elution pattern with a major peak of β-LPH without a distinct peak of β-endorphin (Fig. 4). These data suggest that β-LPH produced by and secreted from the tumor could not be further processed into β-endorphin by PC 2.

Immunohistochemical study using anti-ACTH and anti-POMC antibodies clearly demonstrated that positive immunostaining for POMC, but negative immunostaining for ACTH(1-39) and PC 1/3 within the tumor cells. Although the failure to detect immunoreactivities for ACTH and PC 1/3 by immunohistochemical method does not completely exclude their expressions by the tumor, our results appear to be compatible with the notion that the tumor synthesizes predominantly unprocessed POMC due to impaired expression of PC 1/3. It has been reported that both PC 1/3 and PC 2

immunoreactivities were widely distributed in normal neuroendocrine cells, including pituitary, gastrointestinal tract, pancreas, thyroid, and adrenals, as well as in the neuroendocrine tumors [18]. PC 1/3 has been suggested to be sensitive markers for neuroendocrine differentiation because of its positive correlation with the expression of the secretory granules [23]. Thus, the negative staining of PC 1/3 in the present tumor may be accounted for by undifferentiated neuroendocrine tumor. However, PC 1/3 could be expressed little, if any, by the tumor, because the tumor also secretes predominantly β-LPH derived from POMC via specific cleavage by PC 1/3. However, the absence of β-endorphin in the patient's plasma suggests the tumor does not significantly express PC 2 that specifically cleaves β-LPH into β-endorphin and β-LPH as well as ACTH into α-MSH and CLIP, respectively [7, 24].

Biological activities of ACTH precursors remain unanswered simply due to the lack of their standard preparations and the adequate assay methods. For example, 'big ACTH' has been reported to be undetectable by *in vivo* bioassay [25] or only 3–5% of ACTH(1-39) by *in vitro* assay [26]. By contrast, pro-ACTH has been reported to be equipotent with ACTH(1-39) by rat adrenal cell [13] or 8–33% of ACTH(1-39) by cytochemical assay [10]. The predominant HMW form of ACTH in plasma associated with profound hypercortisolemia as in the present case is very similar to that of our previous case of 'big ACTH'-producing gastric carcinoid causing clinically overt Cushing's syndrome [27]. Taken together, circulating ACTH precursors, including POMC and pro-ACTH, when present at high concentration, and/or be cleaved at the peripheral level, *i.e.* locally at the site of adrenal cortex, could induce steroidogenic activity *in vivo*.

Non-pituitary tumors more frequently synthesize and secrete large amount of ACTH precursors than pituitary tumors. A retrospective analysis of 86 patients with ACTH-dependent Cushing's syndrome showed that 34/35 patients with pituitary tumors had low plasma levels of ACTH precursors, while 51 patients with non-pituitary tumors had large excess of ACTH precursors in the circulation [28]. Our study suggests that the discrepant plasma ACTH levels measured simultaneously by two different IRMA kits could distinguish patients with ectopic ACTH syndrome from Cushing's disease. If plasma ACTH levels measured by Allegro® kit are greater than those by Yuka® kit as in the present case, ectopic ACTH syndrome with preferential secretion of HMW form ACTH appears more likely. By contrast, if plasma ACTH levels determined by two different kits are comparable, Cushing's disease with preferential secretion of ACTH(1-39) is a likely diagnosis. Thus, simultaneous measurement of plasma ACTH levels by two different assay kits could be a useful endocrine screening test for differential diagnosis of ACTH-dependent Cushing's syndrome.

In conclusion, we report herein a rare case of malignant gastric carcinoid with ectopic production of HMW form of ACTH causing Cushing's syndrome, in whom discrepant plasma ACTH levels measured by different IRMA kits proved to be diagnostically useful.

Acknowledgment

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