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Cyclical Cushing's Syndrome in a Patient with a Bronchial Neuroendocrine Tumor (Typical Carcinoid) Expressing Ghrelin and Growth Hormone Secretagogue Receptors

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A 56-yr-old woman was referred with a diagnosis of Cushing's disease. Hypertension and severe hypokalemia were present and high urinary free cortisol/cortisone ratio was detected, raising a suspicion of an ectopic ACTH syndrome. Inferior petrosal sinus sampling, thoracic computed tomography, and octreotide scans were negative. Remission and relapse periods lasting 3–4 months were observed during the 3.5 yr of follow-up. Finally a thoracic computed tomography scan showed a basal paracardic nodule in the left lung. After surgery, a well-differentiated neuroendocrine tumor (typical bronchial carcinoid) was diagnosed, staining positively for ACTH. RT-PCR revealed expression of proopiomelanocortin, CRH receptor, and V3 vasopressin receptor. Somatostatin receptor type 1, 2, 3, and 5 mRNA was detected only in tumoral tissue. Interestingly, we observed the simultaneous presence of ghrelin and both GH secretagogue (GHS) receptors (1a and 1b) mRNA in tumoral tissue but not in the normal lung. This finding correlates with the *in vivo* ACTH hyperresponsiveness to hexarelin (a GHS).

This is the first report of a cyclical ectopic ACTH-secreting tumor with an *in vivo* ACTH response to hexarelin coupled with the tumoral expression of ghrelin and GHS receptors. This finding might imply an autocrine/paracrine modulatory effect of ghrelin in bronchial ACTH-secreting tumors. (*J Clin Endocrinol Metab* 88: 5834–5840, 2003)

THE PRESENCE OF a subgroup of patients with Cushing's syndrome (CS) who show periodic changes in cortisol (F) secretion is well known. In the majority of cases, cyclical CS is caused by an ACTH-secreting pituitary adenoma, by adrenal adenoma, and by ectopic ACTH secretion only rarely.

The first description of a cyclical CS was by Bailey (1) in 1971 in a patient with a bronchial neuroendocrine tumor.

Since then, other patients with ectopic ACTH syndrome have been reported to be affected by cyclical hypercortisolism (2–9). The neuroendocrine mechanisms underlying the periodicity of F overproduction still remain to be elucidated.

In this paper, we describe a case of cyclical CS caused by a corresponding bronchial carcinoid tumor presenting an ACTH hyperresponsiveness to hexarelin, a synthetic GH-secretagogue (GHS). Molecular features of this ACTH-producing tumor, including studies on receptors expression, are also presented.

Case Reports

A 56-yr-old woman was referred to us in July 1996, from the Neurosurgery Department, with the diagnosis of Cushing's disease, and pituitary surgery was planned. The diagnosis was made in another hospital, 3 months before, on the basis of clinical appearance, high urinary free F (UFF) [>1000 μg/24 h (2759 nmol)], ACTH at 360 pg/ml (79.3 pM), high levels of serum F [50 μg/dl (1380 nM)], and indirect signs (gland asymmetry and minimal stalk deviation) of a pituitary lesion, on the magnetic resonance imaging (MRI) study (Fig. 1A).

Hypertension resistant to multiple antihypertensive therapy and severe hypokalemia that required iv K supplementation were present. The patient had facies rubeosa, hirsutism, and central adiposity, with thin limbs and legs' edema.

We confirmed the high levels of UFF both by RIA [2512 μg/24 h (6930.6 nM] and HPLC [3345 μg/24 h (9228.8 nM)]. At the first evaluation, serum F was suppressed by dexamethasone (8 mg) overnight [>50%; 39 (107.6) → 11 μg/dl (30.3 nM)], but this was not observed at subsequent evaluations [<50%; 50 (137.9) → 49 μg/dl (135 nM)] (Table 1).

Serum F and plasma ACTH levels did not change in response to CRH and 1-disamino-β-D-arginine vasopressin (DDAVP) stimulations [CRH = F:11.4 (314.6) → 11.8 μg/dl (325.7 nM); ACTH: 150 (33.1) → 141 pg/ml (31.0 pM); DDAVP = F: 11.4 (314.6) → 11.1 μg/dl (306.2 nM); ACTH: 149 (32.8) → 159 pg/ml (35.0 pM)].

High circulating values of proopiomelanocortin (POMC) (430 U/ml, normal >100) and high UFF/free cortisone (UFF/UFE) ratio were detected (6.56: normal < 0.76) (Table 1).

Interestingly, ACTH hyperresponsiveness to hexarelin (a synthetic GHS) was observed [378 (83.2) → 1121 pg/ml (246.8

Abbreviations: CS, Cushing's syndrome; CT, computed tomography; CV, coefficient of variation; DDAVP, 1-disamino-β-D-arginine vasopressin; F, cortisol; GHS, GH secretagogue; hCRH, human CRH; 11HSD2, 11 hydroxysteroid dehydrogenase type 2; HSSTR, human somatostatin receptor; MRI, magnetic resonance imaging; NSE, neuron-specific enolase; POMC, proopiomelanocortin; UFE, urinary free cortisone; UFF, urinary free cortisol.

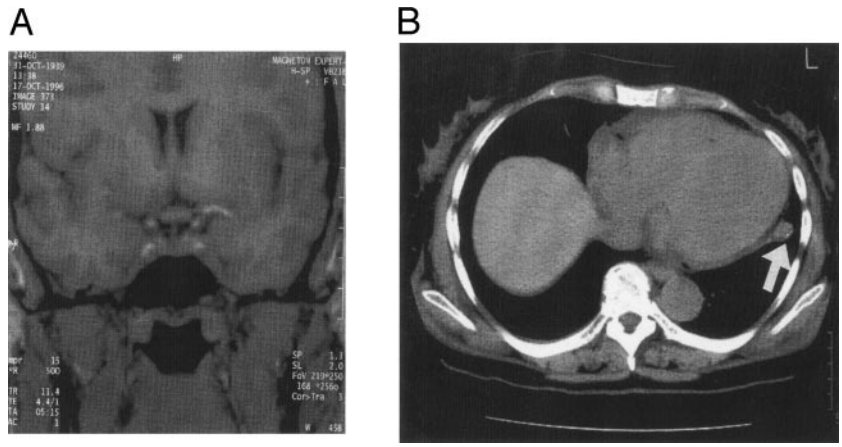

FIG. 1. A, MRI showing indirect sign of pituitary microadenoma; B, CT scan showing a nodule (1 cm in diameter) in the left lobe of the lung.

TABLE 1. Clinical and biochemical data of the patient pre- and 2 months after surgery

	Pre surgery	Post surgery
SBP/DBP (mm Hg)	160/100	130/80
BMI (kg/m^2)	29	26
Na pl. (mEq/liter)	143	142
K pl. (mEq/liter)	2	3.9
Fasting glucose (mg/dl)	119	76
F 0800 h (μg/dl)	50	10
F after Dex (1 mg)	39	1.2
F after Dex (8 mg)	49	
RIA-UFF (μg/24 h)	2512	93
HPLC-UFF (μg/24 h)	3345	27
UFF/UFE	6.56	0.58
Upright PRA (ng/ml·h)	0.1	1.4
NSE (ng/ml) v.n. 0–11	26	4
Osteocalcin (ng/ml)	2	9.6
POMC (U/ml) v.n. > 100	430	
Central (C) ACTH at BIPSS basal and after CRH (pg/ml)	100->94 (left) 100->95 (right)	
Peripheral (P) ACTH at BIPSS basal and after CRH (pg/ml)	131->98	

To convert values of plasma sodium to mmol/liter, multiply by 1; of plasma K to mmol/liter, multiply by 1; of plasma glucose to mmol/liter, multiply by 0.05551; of plasma F to nanomoles per liter, multiply by 27.59; of UFF to nmol, multiply by 2.759; of PRA to μg/liter/h, multiply by 1; of NSE to μg/liter, multiply by 1; of osteocalcin to nmol/liter, multiply by 0.17; of ACTH to pmol/liter, multiply by 0.2202. v.n., Normal values; Dex, dexamethasone; PRA, plasma renin activity; BIPSS, bilateral inferior petrosal sinus sampling; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; pl, plasma.

PM)], whereas GH values did not increase significantly (0.1 → 0.6 ng/ml). The absence of GH stimulation was not surprising because of the well-known suppressive action of hypercortisolism on pituitary function, as demonstrated also for TSH and gonadotropins, in this case. Inferior petrosal sinus sampling, performed during an active phase of the disease, showed no difference between central and peripheral ACTH concentration before and after CRH stimulation (Table 1).

Some biochemical data, the rapid onset of the clinical symptoms, and the presence of severe hypokalemia suggested, in contrast to the initial diagnosis, an ectopic ACTH syndrome.

However, thoracic/abdominal computed tomography scans were negative, and only bilateral hyperplasia of the adrenal glands was found. The Indium ^{111}Pentreotide scan was negative.

Subsequently, within a few weeks, a complete remission of symptoms and signs of hypercortisolism occurred.

Spontaneous remission and relapse periods lasting 3–4 months were observed during the 3.5 yr of follow-up, suggesting that the patient was affected by a cyclical CS (Figs. 2 and 3). In the active phases, therapy with ketoconazole, at dosages ranging from 600-1200 mg/die, and oral potassium supplementation were instituted. Two octreotide suppression tests in the active phase of the disease produced a significant F and ACTH reduction. For this reason, a brief trial of octreotide administration (100 μg sc, three/d) was started, resulting in a good control of the hypercortisolism, even if spontaneous remission could not be excluded because of the cyclic ACTH secretion. Relapses with the same clinical manifestation and severe hypokalemia became more frequent and of longer duration. In the meanwhile, the patient presented multiple vertebral collapses, related to severe osteoporosis, and had to wear an orthopedic corset.

A second MRI scan of the pituitary, 1 yr later, was negative.

Because of the increasing suspicion of a cyclic CS caused by ectopic ACTH secretion, another Indium ^{111}Pentreotide scan (6 months after the octreotide treatment) was performed; and this time, three thoracic spots were detected.

A new thoracic computed tomography (CT) scan showed the presence of a basal paracardiac nodular lesion in the left lung (Fig. 1B). Despite the absence of a corresponding uptake at a subsequent octreotide scan, the patient underwent a surgical chest exploration (July 1999).

A subpleural nodule was removed, and the histologic examination confirmed the diagnosis of a typical carcinoid tumor.

All biochemical findings and clinical features of the disease disappeared after the removal of the tumor, and all evaluations remained normal in the 3 subsequent years. The blood pressure did also normalize. ACTH values did not change significantly after hexarelin administration [18 (3.9) → 22 pg/ml (4.8 PM)], whereas a normal GH response reappeared [1.1 (1.1) → 37 ng/ml (37 μg/liter)].

Laboratory methods

Serum F levels were measured by chemiluminescence (IMMULITE, Diagnostic Product Corporation, Los Angeles, CA). The sensitivity of the assay was 0.2 μg/dl. The inter- and intraassay coefficients of variation (CVs) were 10.3 and 9%, respectively.

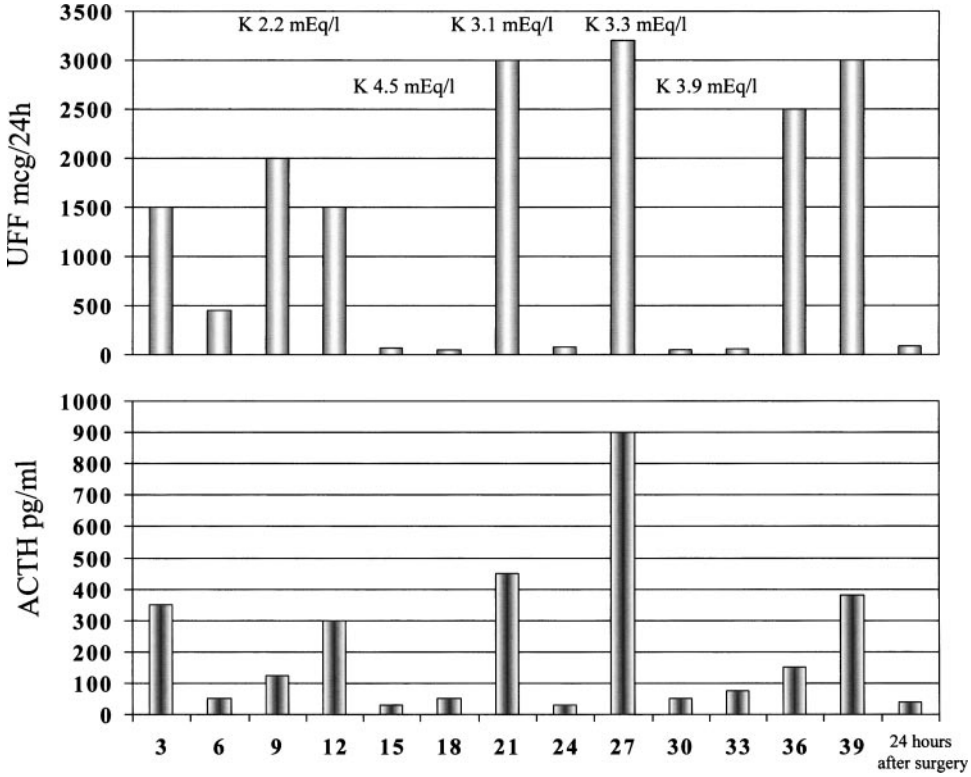

FIG. 2. Cyclicity of UFF, ACTH, and potassium levels during the time course (months) of the disease.

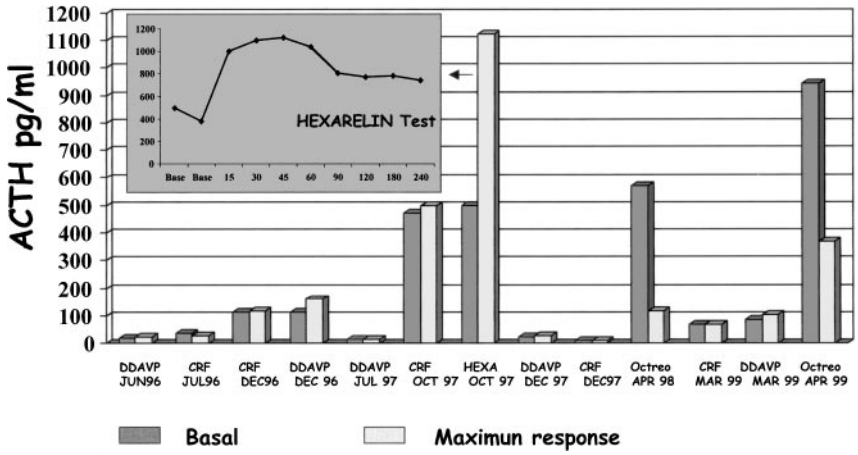

FIG. 3. ACTH levels response to dynamic tests during the time course of the disease. HEXA, Hexarelin; Octre, octreotide.

Plasma ACTH levels were measured by chemiluminescence (IMMULITE, Diagnostic Product Corporation). The sensitivity of the assay was 9 pg/ml. The inter- and intraassay CVs were 8.8% and 9.6%, respectively.

UFF and UFE were evaluated by HPLC with the Santos-Montes method (intraassay CV = 5.54% and interassay CV = 6.99% for UFF, intraassay CV = 7.95% and interassay CV = 7.86% for UFE). UFF was also measured by RIA (Diagnostic Products Corporation, Los Angeles, CA; intraassay CV = 4.05%).

The UFF/UFE ratio was used as an activity index of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD type 2), the key-enzyme of the F metabolism.

Lyophilized hexarelin was provided by Europeptides (Argenteuil, France) in vials of 100 μg.

ACTH and F measurements were determined in basal condition (0 min) and then every 15 min, up to 120 min after HEX administration (2.0 μg/kg, iv).

Human CRH (hCRH) and DDAVP were purchased from Ferring Pharmaceuticals, Ltd. (Kiel, Germany) in vials of 100 μg.

Blood samples for ACTH and F were taken in basal condition (0 min) and at 15, 30, and 60 min after CRH or DDAVP administration.

All dynamic tests started between 0830–0900 h, after overnight fasting and 30 min after venous cannulation and slow infusion of isotonic saline.

Histologic evaluation

The pathological specimen was dissected and fixed in 10% formalin for 48 h and embedded in paraffin. Monoclonal anti-

bodies were used for immunohistochemistry studies: anti-chromogranin A clone DAK-A3; anti-neuron-specific enolase (anti-NSE) clone BBS/NC/VI-HI4; anti-synaptophysin clone SY38; anti-ACTH clone 02A3, all purchased by Dakopatts (Dako Corporation, Carpinteria, CA).

Molecular biology evaluation

Molecular biology studies were performed in the tumor to characterize its corticotroph phenotype and to look for somatostatin receptors, ghrelin, and ghrelin receptor gene expression. Before the start of these studies, informed consent of the patient was obtained.

Bronchial carcinoid tissue and normal peritumoral tissue, used as negative control, were collected at surgery and stored at −80 C until processed.

Total RNA was extracted using a monophasic solution of phenol and guanidine isothiocyanate (TRIzol reagent, Life Technologies), followed by extraction and precipitation with isopropanol. The amounts of RNA were quantified using a UV spectrophotometer (Beckman Inc.) at 260 nm. The integrity of mRNA was verified by electrophoresis in 2% agarose gel. The extracted RNA (200 ng) was denaturated by incubation for 10 min at 70 C, followed by rapid cooling. Single-stranded cDNA was synthesized by mixing RNA with RT buffer 1X [Tris-HCl (25 mM, pH 8.3), MgCl2 (5 mM), KCl (50 mM), dichlorodiphenyltricloroethane (2 mM)], 0.55 mM deoxynucleotide triphosphate mix (10 mM of each deoxynucleotide triphosphate) (Invitrogen), random primers (0.7 ng/liter), and 2U Avian Myeloblastosis Virus Reverse Trascriptase (Finnzymes Oy, Finland). The following steps were at 1 h and 10 min at 42 C, and 5 min at 95 C.

RT products, were amplified by the PCR with 25 pmol of each primer (Table 2), deoxynucleotide triphosphate mix (10 mM of each deoxynucleotide triphosphate) (200 M), Taq Buffer 1X (Tris-HCl, 10 mM, pH 8.8; Triton X-100, 0.1%; MgCl2, 1 mM; and 2 U Taq DNA polymerase (Finnzymes) in a total vol of 25. The reaction was carried out under the following conditions: 35 cycles of 95 C (1 min), 60 C for human somatostatin receptor (HSSTR)1 and HSSTR3, 62 C for ghrelin and HSSTR5, 64 C for HSSTR2 and 66 C for GHS-R1a, 68 C for GHS-R1b (1 min) and 72 C (1 min). PCR products were electrophoresed in 1.8% agarose gel, stained with ethidium bromide, and visualized with a Gel Doc 1000 (Bio-Rad Laboratories, Inc., Richmond, CA).

Results

Histological and immunohistochemical studies (Figs. 4 and 5)

The surgical specimen consisted of an atypical lung resection containing a subpleural nodule, 9 × 4 mm in diameter. The cut surface of the nodule appeared reddish, with small areas of hemorrhage. No necrosis was detected. The nodule was well circumscribed but not encapsulated. At histological examination, the tumor showed an organoid nesting growth pattern; the tumor cells had uniform cytologic features, with moderate eosiniphilic, finely granular cytoplasm: nuclei were round or oval, with finely granular chromatin and inconspicuous nucleoli. A trabecular pattern and spindle-cell histology were observed only focally. Mitotic figures were hard to find; Ki-67 antigen positivity was observed in less than 3% of the nuclei. The tumor was considered a well-differentiated neuroendocrine tumor (typical carcinoid) by the histologic criteria established by Solcia *et al.* (2000). The immunohistochemical study for the demonstration of neuroendocrine differentiation was performed on paraffin-embedded sections by using the panel of antibodies listed in the *Histologic evaluation* section.

The proliferative activity was evaluated with the monoclonal antibody directed against Ki-67 (MIB-1) anti-MIB-1 antibody (Immunotech, clone 02A3).

The tumor cells were diffusely and strongly positive for

TABLE 2. Primers used in the PCRs

Gene and gene accession no.	Forward primer	Reverse primer	Product length (bp)
Ghrelin AF296558	GCTTCCTGAGCCCTGAACAC	GCTGCAGAAGCAAGCGAAAA	350
GHS-R1a HSU60179	CTGGACCTCGTTCGCCTCTGG	CACCCGGTACTTCTTGGACAT	705
GHS-R1b HSU60181	GCACCACCACCAACCTCTACC	GCGAGAGAAAGCCTGAGCGCG	607
POMC NM000939	CCTGCCTGGAAGATGCCGAGAT	TGCTGCCGCTGCTGCTGCTGTT	304
CRF-R1 NM004382	CATCCGGTGCCTGCGAAACA	GGCCCTGGTAGATGTAGTCG	386
HSSTR1 NM001049	TATCTGCCTGTGCTACGTGC	GATGACCGACAGCTGACTCA	217
HSSTR2a AF184174	CAACCAACACCTCAAACCAGA	CCTGTGTACCAAGCCCCAGAT	541
HSSTR3 NM001051	TCAGTCACCAACGTCTACATCC	ACGCTCATGACAGTCAGGC	188
HSSTR5 NM001053	CTGTCTCTGTGCATGTCGCTG	TCACAGCTTGCTGGTCTGCAT	600
β-Actin NM001101	TGACGGGGTCACCCACACTGT GCCCATCTA	CTAGAAGCATTTGCGGTGGAC GATGGAGGG	661
V3 L37112	TCTCGGGTCAGCAGCATCAAC	ACCCCCACAGCAGGCAAGG	285

V3, Vasopressin receptor type 3.

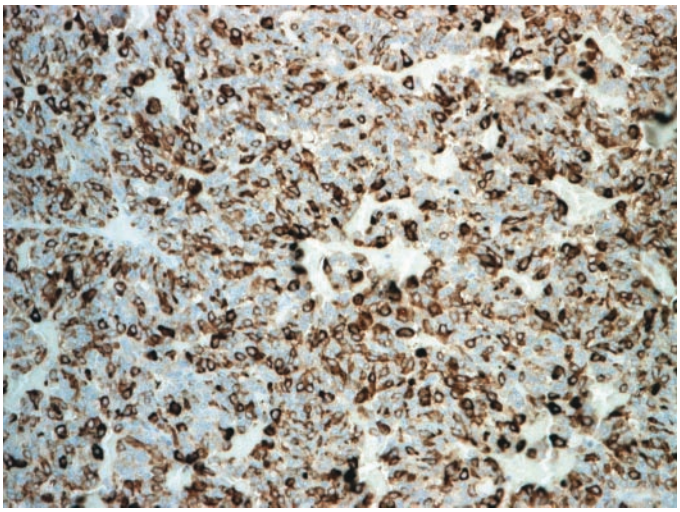

FIG. 4. The tumor cells are relatively small, round to polygonal, with a moderate amount of eosinophilic cytoplasm. Nuclei are regular, and there are not atypical features (hematoxylin-eosin, ×100).

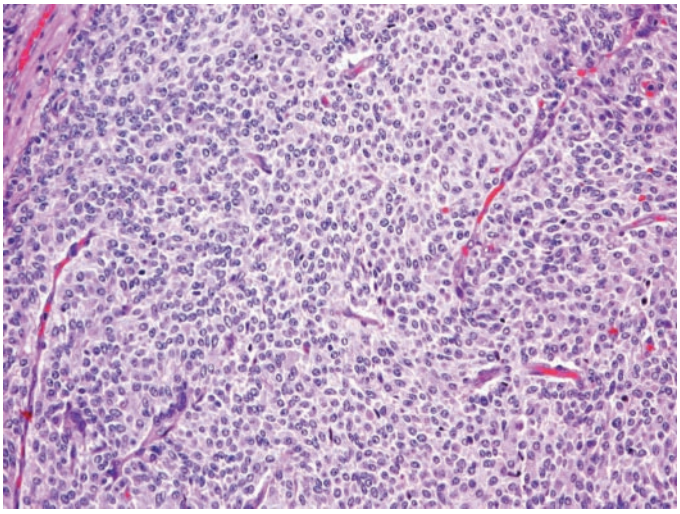

FIG. 5. The tumor cells are diffusely and strongly positive with antibody to ACTH (×200).

chromogranine A, NSE, synaptophysin, and ACTH. Eight percent of the nuclei were positive for the MIB-1 antibody.

RNA studies

RT-PCR confirmed the immunohistochemical positivity for ACTH (POMC) and also revealed low expression of CRH receptor and vasopressin receptor (V3). Somatostatin receptor types 1, 2, 3, and 5 were detected only in tumoral tissue. Interestingly, we observed the simultaneous presence of ghrelin and both types of GHS receptor (1a and 1b) mRNA in tumoral tissue but not in normal peritumoral lung tissue (Fig. 6).

Discussion

We describe a case of cyclic ectopic ACTH secretion caused by a bronchial carcinoid identified only after a long period of clinical, biochemical, and radiological follow-up.

The diagnosis of CS, and especially of the source of the

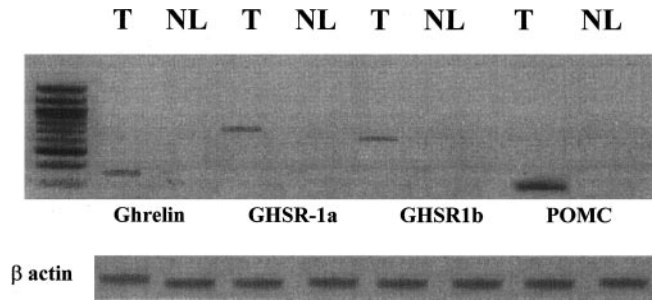

FIG. 6. Ghrelin, ghrelin receptor 1a and 1b, POMC, and actin mRNA expression in tumor (T) and in normal peritumoral lung tissue (NL). GHSR, GH-secretagogue receptors.

excessive ACTH secretion, is often challenging. Endocrine testing and clinical evaluation do not always yield conclusive results in the differential diagnosis between pituitary and ectopic ACTH secretion, especially when there is an erratic F hypersecretion (10).

In the majority of cases, the CS is caused by an ACTH-secreting pituitary adenoma, and only in a few cases by ectopic ACTH secretion (11). To date, three cases of cyclical ACTH-secreting bronchial tumors have been described in the literature (1–2, 12).

In fact, biochemical evaluation can be contradictory because of the periodic hormonogenesis present in cyclical CS. Abnormal responses to several tests, and especially paradoxical responses to dexamethasone, have been reported by several authors (12–19).

The mechanisms of cyclicity are still unknown. One of the explanations, at least in cyclical pituitary tumors, could be a cyclical change in the central dopaminergic tone as a trigger for periodic ACTH secretion (18). The recent report of a case of ectopic occult CS in which a temporary remission was observed during an acute pulmonary infection is also of great interest (20). The clinical presentation of these patients is extremely variable. The cycles of excessive F production can last from several weeks to several months, whereas the inactive phase can last from 1 or 2 months to several years.

In our case, remission and relapse periods, without an evident trigger, lasting 3–4 months, were observed during 3.5 yr of follow-up before surgery; and therefore, diagnostic evaluations were contradictory.

In particular, a dexamethasone (8 mg) overnight suppression test yielded confusing results because F suppressed initially, but not in a second evaluation, and there was not a paradoxical response, which has been described in cyclical CS even though only in pituitary forms (19). On the other hand, lack of responses of F and ACTH to hCRH and/or DDAVP was suggestive of an ectopic ACTH source.

Overall, it is not surprising that it took several years to establish a definitive diagnosis, because diagnostic tests with a high degree of specificity in the differential diagnosis of CS are still not available. Cases of ectopic ACTH syndrome, with F suppression after a high dose of dexamethasone and ACTH stimulation by hCRH/DDAVP, have already been described (20–26).

In this patient, the presence of a severe hypokalemic-alkalosis and hypertension, even if intermittent, and the hor-

monal data (see Table 1) were consistent with the diagnosis of an ectopic CS.

In fact, patients with ACTH ectopic secretion often present with an apparent mineralcorticoid excess syndrome caused by 11HSD2. The F/cortisone (UFF/UFE ratio), which was very high in our patient, is an index of 11HSD2 activity and can therefore be considered a useful tool in the differential diagnosis of the CS. The data in the literature regarding the cause of 11HSD2 impaired activity are contradictory. The impaired activity of 11HSD2 can be caused by substrate saturation because of very high levels of F and/or by enzyme inhibition by ACTH (27, 28).

Our case report confirms that localization of ectopic ACTH-secreting tumors is extremely difficult, and there is not a single imaging technique with an optimal accuracy. Bronchial carcinoid tumors are small, classically ranging from 0.3–1.3 cm (29) and, as such, may prove particularly difficult to localize, even after CT or MRI scan (30). The combinations of conventional radiology and ^{111}In-pentreotide scintigraphy should be employed and eventually repeated during the follow-up, because a single negative finding doesn't exclude the diagnosis.

In the above reported patient, the finding of indirect signs of pituitary lesion on MRI scan, consistent with the initial diagnosis of Cushing's disease, was a confounding factor, particularly in the presence of a negative chest CT examination and negative octreotide scan. It is worth mentioning that indirect signs of pituitary lesions on MRI are reported as an incidental finding in 10–20% of the healthy population.

Some authors (20, 31, 32) have described successful carcinoid localization with ^{111}In-pentreotide scintigraphy. Neuroendocrine cells often express somatostatin receptors (33, 34), and patients with ectopic ACTH syndrome have occasionally benefited from octreotide treatment (35–37).

In our case, we performed two octreotide suppression tests in the active phase of the disease, and a significant F reduction was observed. For this reason, a brief trial of octreotide administration was started, resulting in a good control of hypercortisolism even if spontaneous remission could not be excluded because of the ACTH cyclic secretion. Furthermore, expression of SS1, SS2, SS3, and SS5 receptor mRNA in tumoral tissue was demonstrated. On the other hand, the octreoscan was negative on several occasions. This is not surprising, because it is well known that the octreoscan has limited sensitivity for the localization of sources of ectopic ACTH secretion.

Recently, ghrelin and ghrelin receptor expression has been described in neuroendocrine tumors (38), and specific GHS receptors have been demonstrated in ectopic ACTH-secreting tumors (39). Ghrelin and synthetic GHS possess not only GH-releasing activity but a clear stimulatory effect on ACTH and F secretion, too (40). Previous reports saw ACTH hyperresponsiveness only in ACTH-secreting pituitary microadenomas and not in ectopic tumors, suggesting that this test could have been useful in differentiating the site of ACTH-secreting tumors, even though only two cases of ectopic ACTH-secreting tumors (one bronchial carcinoid tumor and one microcitoma) were tested (40–41). The relationship between hypercortisolism and GHS-R expression has been described in the literature, but the data are contrasting. In the paper from Tamura *et al.* (42), glucocorticoids enhance GHS-R expression in the rodent pituitary, whereas Kaji *et al.* (43) showed that glucocorticoids down-regulate human ghrelin receptor gene expression through a transcriptional mechanism.

To our knowledge, this is the first reported case, where an ACTH hyperresponsiveness to GHS (hexarelin) and a mRNA expression of ghrelin receptors, 1a and 1b, in the tumoral tissue were found. Therefore, the *in vivo* response was supported by molecular studies, even though *in vitro* studies would have been useful to prove a cause-effect relationship between GH secretagogues and F secretion. After surgical removal of the tumor, the ACTH hyperresponsiveness to hexarelin was not present any more, and a normal response of GH appeared.

Previous studies on GHS-R and ghrelin mRNA expression have shown variable and controversial results. In a very recent study (44), ghrelin and ghrelin 1b receptor mRNA were shown to be present in normal lung tissue, whereas ghrelin1a receptor mRNA was absent. Our case is the first to demonstrate, in an ectopic ACTH secreting tumor, simultaneous expression of ghrelin and of both ghrelin receptors, 1a and 1b, mRNA. No expression of ghrelin and ghrelin receptor mRNA was present in the peritumoral normal lung tissue. Even if this finding can be attributed to lower sensitivity of semiquantitative PCR toward real-time PCR, our data are still suggestive of a much higher expression of such molecules in the tumor.

The interesting molecular features of the tumor, coupled with the clinical behavior, might imply an autocrine/paracrine modulatory effect of ghrelin in bronchial ACTH-secreting tumors.

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