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Cushing's Syndrome Secondary to Adrenocorticotropin-Independent Macronodular Adrenocortical Hyperplasia due to Activating Mutations of *GNAS1* Gene

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ACTH-independent macronodular adrenal hyperplasia (AIMAH) is an uncommon cause of Cushing's syndrome characterized by bilateral nodular adrenocortical hyperfunction in the presence of suppressed ACTH levels. We investigated whether activating mutations in the ACTH receptor (*MC2-R*) or $G_{s\alpha}$ (*GNAS1*) genes might be involved in AIMAH genesis. Five women with Cushing's syndrome due to AIMAH, confirmed by histological studies, and no signs of McCune-Albright syndrome were selected for molecular analysis of these genes. The single exon of the *MC2-R* gene and exons 8 and 9 of the *GNAS1* gene were amplified by PCR in genomic DNA from adrenal nodules and peripheral blood. Direct sequencing revealed only *MC2-R* wild-type sequences. *GNAS1* PCR products at denaturing gradient gel electrophoresis revealed abnormal migration patterns in adrenal tissues of three patients. Automatic sequencing showed two different activating mutations at codon Arg^{201} of *GNAS1*, a substitution by histidine in two cases and by serine in one case.

In conclusion, we found two different *gsp* mutations in three patients with Cushing's syndrome due to AIMAH, and we speculate whether they belong to the spectrum of McCune-Albright syndrome or whether these are the first reported cases of AIMAH due to *gsp* mutations. (*J Clin Endocrinol Metab* 88: 2147–2151, 2003)

ACTH-INDEPENDENT MACRONODULAR adrenal hyperplasia (AIMAH) is a rare and distinct cause of Cushing's syndrome associated with multiple adrenal nonpigmented benign nodules (1–5). The pathogenesis of AIMAH is not completely understood, and its cause has been considered to be heterogeneous. The clinical presentation is usually sporadic, but there is a familial type, which illustrates the expanding clinical spectrum of AIMAH (6–10). Several studies have shown that the regulation of cortisol secretion of Cushing's syndrome secondary to AIMAH may be mediated by inappropriate, illicit, or ectopic adrenal expression of membrane hormone receptors. These include ectopic membrane receptors for gastric inhibitory polypeptide, β-adrenergic agonist, or LH/human chorionic gonadotropin, and also the altered activity of eutopic membrane receptors, such as vasopressin and serotonin (11–22). The molecular mechanisms responsible for the abnormal expression and function of these membrane hormone receptors are still unknown (22–24).

Bilateral adrenocortical nodular hyperplasia can also be present in McCune-Albright syndrome (MAS), but this finding is rare and not always associated with Cushing's syndrome (25–29). Activating mutations in the *GNAS1* gene, lead to constitutive steroidogenesis in the affected adrenal nodules that carry the mutation (30). These mutations, called *gsp*, are dispersed in a mosaic pattern in the tissues, and the greatest number of *gsp*-containing cells are present in the abnormal areas of affected tissues. These observations and the lack of heredity confirmed the hypothesis that the mosaic pattern is derived from a postzygotic somatic mutation (26). Two previous studies of AIMAH not associated with MAS did not find activating mutations in the *GNAS1* gene (31–32).

In the adrenal gland, ACTH activates its receptor by inducing a conformation change in the hormone-receptor complex. The activated receptor interacts with heterotrimeric $G_{s\alpha}$ protein and promotes the steroidogenesis molecular pathway (33). The possibility of an ACTH-receptor activating mutation as the cause of AIMAH was reported in a single patient with a homozygous mutation in the *MC2-R* gene; this mutation, tested *in vitro*, increased basal and ACTH-stimulated cAMP production (34–35). Light *et al.* (36) failed to identify this type of mutation in one case with bilateral nodular hyperplasia. Because *MC2-R* is coupled to the G protein signaling pathway, mutations affecting these genes may be considered candidate causes of AIMAH. We hypothesized that activating mutations in *MC2-R* or *GNAS1* genes might be involved in the genesis of AIMAH.

The aim of this study is to screen a group of five women with Cushing's syndrome secondary to AIMAH for activating mutations in *MC2-R* and *GNAS1* genes.

Abbreviations: AIMAH, ACTH-independent macronodular adrenal hyperplasia; DGGE, denaturing gradient gel electrophoresis; MAS, McCune-Albright syndrome.

Subjects and Methods

The study was approved by the Ethics Committee of Hospital das Clínicas (São Paulo, Brazil), and informed written consent was obtained from all patients and normal controls. A review of 133 patients with Cushing's syndrome from 1980 through 2002 at Hospital das Clínicas in Sao Paulo, Brazil, revealed five female patients with AIMAH.

The diagnosis of Cushing's syndrome was established through measurements of 24-h urinary 17-hydroxysteroids or cortisol levels and serum cortisol levels after low and high dexamethasone doses (1.0 mg and 8.0 mg orally overnight, respectively). In all cases, several measurements of ACTH levels were performed to define the presence of suppressed ACTH levels (Table 1). The methyrapone stimulation test (six doses of 750 mg orally every 4 h) was performed in two cases (Table 1, patients 3 and 4) with measurements of urinary 17-hydroxysteroids and 17-ketosteroids. Patient 5 was screened for the presence of the most frequent abnormal adrenal hormone receptors through response to posture, to mixed meal, and to GnRH and TRH according to a previously described protocol (19).

Clinical cases

Our series consisted of five female patients with a mean age of 33 yr (Table 1). Physical examination revealed Cushing's syndrome features in all of them, such as moon face, hypertension, central obesity, abdominal striae, and muscle atrophy.

All patients had elevated 24-h urinary cortisol excretion and lack of suppression of cortisol levels after low and high dose dexamethasone test. ACTH levels were suppressed in all patients, and patients 3 and 4 did not respond to methyrapone test (Table 1). Patient 5 presented a response of cortisol levels after iv injection of 250 μg ACTH, but not after stimulation with supine posture, mixed meal, TRH and GnRH hormones, ruling out the presence of hypercortisolism dependent of the most frequent aberrant membrane hormone receptors in the adrenal gland (Table 2).

None of the patients presented signs or symptoms of MAS, such as gonadotropin-independent precocious puberty, bone polyostotic fibrous dysplasia, and *café-au-lait* spots after clinical and radiological studies.

All patients underwent adrenal computed tomography scans disclosing bilaterally enlarged adrenal glands with macronodules (up to 2.0 cm in diameter).

Bilateral adrenalectomy was performed in all patients. A single experienced endocrine pathologist (M.C.N.Z.) reviewed all adrenalectomy specimens, with specific focus on adrenal gland weight, size, and color of nodules. The histopathological analysis confirmed macronodular adrenal hyperplasia in all cases. Microscopic analysis of the glands revealed absence of internodular atrophy and any type of pigment. No heterogeneity was observed in the nodules, which had two predominant cell types: large and small cells, both with clear cytoplasms.

In all cases, magnetic resonance imaging scan findings of pituitary glands before and after bilateral adrenalectomy revealed normal glands without signs of Nelson's syndrome during a follow-up of 4–22 yr.

DNA extraction

The adrenal glands were obtained after bilateral adrenalectomy and were conserved in liquid nitrogen in two cases and in paraffin-embedded blocks in the other three cases. Three 5-μm sections of the paraffin-embedded adrenal tissue were cut from each block and mounted on glass slides. The middle section was stained with hematoxylin-eosin and reviewed by the pathologist to confirm the diagnosis and demarcate the nodular area in each case. The excess paraffin was removed, and the tissue was scraped into sterile 1.5-ml tubes. Genomic DNA from all paraffin-embedded adrenal tissues was extracted using xylene, followed by digestion with proteinase-k (37).

Genomic DNA was extracted from frozen adrenal tissues using a QIAmp DNA mini kit (QIAGEN, Hilden, Germany), and genomic DNA from peripheral blood leukocytes was also isolated from all patients and from 10 normal unrelated individuals according to standard procedures.

PCR protocols

PCR was used to amplify the single exon of the *MC2-R* gene and fragments of exons 8 and 9 that included codons 201 and 227 of *GNAS1* gene, as previously described (38, 39). The exons 8 and 9 were amplified in a 100-μl PCR mixture containing 0.2 mmol/liter of each of the deoxynucleotide triphosphates, 50 mmol/liter KCl, 20 mmol/liter Tris-HCl (pH 8.3), 1.5–2.5 mmol/liter $MgCl_2$, 30 pmol of each primer, and 1.25 U Taq polymerase (Pharmacia Biotech, Uppsala, Sweden). Thermal cycling was performed in a Gene amp PCR system (Perkin-Elmer Corp./Cetus, Norwalk, CT). The reaction included an initial 94 C denaturation step for 5 min, followed by 30 cycles of denaturation at 94 C (30 sec),

TABLE 2. Basal and stimulated serum cortisol levels in patient 5

Test	Basal cortisol (nmol/liter)	Cortisol after stimulus (nmol/liter)
Fasting supine - upright	579	606
Mixed meal (75 g oral glucose, plus an isocaloric protein-rich meal)	606	20 (Lunch)
ACTH, 250 ug iv	606	1075
TRH, 200 ug iv	524	579
GnRH, 100 ug iv	551	579

TABLE 1. Clinical, hormonal, and molecular data of five female patients with Cushing's syndrome due to AIMAH

Patients	1[a]	2[a]	3[b]	4[b]	5[b]
Age at diagnosis (yr)	25	34	33	42	37
Time of disease prediagnosis (yr)	1	5	1	5	5
Follow-up after adrenalectomy (yr)	9	9	22	17	4
Basal 24-h urinary cortisol (nmol/d)	1710	1439	1572	3588	2132
Basal 24-h urinary 17-OH and 17-KS (nmol/d)			36/27	46/22	
17-OH/17-KS after metyrapone test (μmol/d)			69/41	69/45	
Cortisol levels after 8 mg of dexamethasone (nmol/liter)	441	799	441	1296	524
Basal ACTH levels (pmol/ml)	<2.2	<2.2	<2.2	<2.2	<2.2
Weight of right adrenal gland (g)	5	45	23	25	25
Weight of left adrenal gland (g)	10	25	20	95	30
gsp mutation in both adrenal nodule tissues (exon 8)	Absent	R201S	R201H	Absent	R201H
gsp mutation in peripheral blood-exon 8	Absent	Absent	Absent	Absent	Absent
gsp mutation in peripheral blood and both adrenal nodule tissues (exon 9)	Absent	Absent	Absent	Absent	Absent
Mutation in *MC2-R* in blood and both adrenal nodule tissues	Absent	Absent	Absent	Absent	Absent

17-OH, 17-Hydroxysteroids; 17-KS, 17-ketosteroids.
[a] DNA extracted from fresh frozen adrenal tissue.
[b] DNA extracted from paraffin-embedded adrenal tissues.

annealing at 60 C (45 sec), and extension at 72 C (45 sec), with a final extension step of 10 min at 72 C. PCR products were examined on 2% agarose gel electrophoresis.

Denaturing gradient gel electrophoresis (DGGE)

The melting profiles of the amplified fragments were generated using computer algorithms of Lerman *et al.* (40). Melting maps predicted that the coding regions of the *GNAS1* gene analyzed would all be contained in a single low-melting domain. Hence, any mutations that occur within those regions are likely to be detected. We improved *gsp* somatic mutation detection, combining a second DGGE of the PCR products obtained from the heteroduplex and mutant homoduplex bands excised separately from the first DGGE (41, 42). Forty microliters of PCR products were required for the DGGE analysis. Electrophoresis was performed for 15 h at 80 V at 60 C on 10% acrylamide gel with increasing denaturant concentrations (37–67% for exon 8, and 40–90% for exon 9 of *GNAS1*). Gels were stained with ethidium bromide. For comparative purposes, samples of normal controls and mutant controls (R201C and Q227R) were included.

Direct sequencing

DNA sequencing was performed on all PCR products of *MC2-R* and all PCR products of *GNAS1* displaying abnormal migration patterns on DGGE. The PCR products were pretreated with an enzymatic combination of exonuclease I and shrimp alkaline phosphatase (United States Biochemical Corp., Cleveland, OH) and directly sequenced using the Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA) in an ABI PRISM 310 automatic sequencer (Perkin-Elmer Corp.).

Results

Molecular analysis

The single exon of *MC2-R* and the exons 8 and 9 of *GNAS1* were adequately amplified by PCR from both adrenal nodules and from blood samples of the 5 patients and 10 normal controls. All fragments showed the expected size on a 2% agarose gel. Direct sequencing revealed only wild-type sequences of *MC2-R* in all samples analyzed. DGGE analysis of PCR products (exons 8 and 9) from blood samples was normal in all patients and normal controls. Both mutant controls in exons 8 and 9 of *GNAS1* displayed abnormal migration patterns, as expected. Analysis of PCR products of exon 8 of *GNAS1* revealed an abnormal pattern of migration, suggestive of a heterozygous mutation, in the adrenal nodule tissues of patient 2 (Fig. 1A) and patients 3 and 4 (Fig. 1C). The migration pattern of adrenal nodule tissues of patient 2 was different from the mutant control (R201C; Fig. 1A).

Automatic sequencing of fragments that showed an abnormal migration on DGGE identified a heterozygous transversion from base C to A in the exon 8, determining a substitution of amino acid arginine (**C**GT) by serine (**A**GT) at codon 201 in both adrenal tissues of patient 2 (Fig. 1B). In the adrenal tissues of patients 3 and 5, the automatic sequencing revealed a heterozygous transition at the same codon, 201, which resulted in a change of the amino acid arginine (C**G**T) by histidine (C**A**T) in both adrenal tissues (Fig. 1D). These mutations were not present in DNA obtained from blood samples of all patients.

Discussion

In several patients with Cushing's syndrome due to AIMAH, the increase in cortisol secretion is mediated by the overexpression of several membrane receptors. The mechanism underlying this promiscuous expression is unclear, and a mutation in the promoter region of the genes of the ectopically expressed receptors, or a more generalized defect that leads to dedifferentiation of adrenocortical cells, is speculated (22–24).

Inactivating mutations of tumor-suppressor gene *PRKAR1A,* the most abundant regulatory subunit of protein kinase A and a principal cAMP-signaling modulator have recently been reported in Cushing's syndrome due to sporadic primary pigment nodular adrenocortical disease, consistent with the proliferative role of the cAMP pathway in the adrenal gland (43).

Because ACTH stimulates cortisol synthesis and secretion

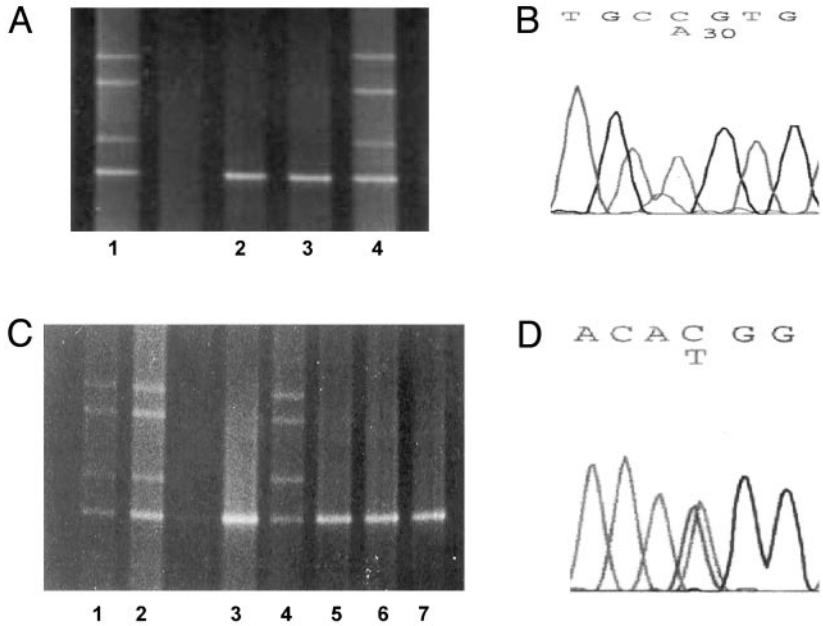

FIG. 1. A, DGGE analysis of PCR products of exon 8 from adrenal nodule tissue of patient 2. Lane 1 shows the mutant control (R201C), and lanes 2 and 3 show the normal control and peripheral blood of patient 2, respectively (normal homoduplex band). Lane 4 shows the nodule of right adrenal with a four-band pattern (normal homoduplex, mutated homoduplex, and heteroduplex bands), characterizing a heterozygous pattern. B, Automatic sequencing (with sense primer) of the PCR product of exon 8 of *GNAS1* gene of the adrenal nodules from patient 2 identified a heterozygous mutation (**C**GT→ **A**GT), at codon Arg^{201} (R201S). C, DGGE analysis of PCR products of exon 8 from adrenal nodule tissue of patient 3. Lanes 1 and 4 show results of left and right adrenal nodules, with a four-band pattern; lanes 3 and 5–7 show normal pattern of peripheral blood of patient 3 and normal controls, respectively; lane 2 shows the pattern of migration of the mutant control (R201C). D, Automatic sequencing (with antisense primer) of the PCR product of exon 8 of *GNAS1* gene of the adrenal nodule tissues from patient 3, identified a heterozygous mutation (C**G**T→ C**A**T), at codon Arg^{201} (R201H).

mediated by the activation of $G_{s\alpha}$, we investigated the possibility of activating mutations in the *MC2-R* and *GNAS1* genes as the cause of the AIMAH.

Abnormalities in the coding region of the *MC2-R* gene were not present in our series or in a single case previously reported (36), suggesting that activating mutations in the ACTH-receptor gene do not represent a frequent mechanism of Cushing's syndrome due to AIMAH.

Naturally occurring mutations in codons 201 and 227 that alter GTPase activity in *GNAS1* gene have been described in autonomous hormone-producing tumors. Mutations involving substitution of either cysteine or histidine and rarely serine for arginine at codon 201 or arginine for glutamine at codon 227 were first described in GH-producing pituitary tumors (44–46). Similar *gsp* mutations have been described in functional thyroid adenomas and rarely in adrenal tumors, ovarian and testicular Leydig cell tumors (32, 39, 47, 48). Somatic missense mutations affecting the residue Arg^{201}, which is important for the GTPase turn-off reaction, lead to the constitutively active form of $G_{s\alpha}$ protein that represents the molecular pathogenesis of MAS (27). This syndrome, in addition to the classical triad, may also present several endocrinopathies due to hormonal oversecretion from adrenal cortex, thyroid, and pituitary somatotrophs. The MAS is never inherited, indicating that *gsp* mutations presumably occur during early embryonic development, leading to a widespread distribution of cells bearing the mutations with a wide range of phenotypes, depending on the tissues involved (28). Cushing's syndrome due to nodules in the adrenal glands in MAS is a rare situation, and in all cases reported, the clinical presentation of Cushing's syndrome occurred during the first years of life (49–53).

We have identified through an optimized DGGE followed by direct sequencing the presence of two different activating mutations (R201S and R201H) in the α-subunit of the stimulatory guanine nucleotide protein in three patients with Cushing's syndrome due to AIMAH without MAS features. These activating $G_{s\alpha}$ mutations in adrenocortical cells increase the levels of cAMP and might lead to cellular proliferation and nodule formation with autonomous cortisol secretion.

The mutation R201S is rare and was previously described in two GH-secreting tumors, in one papillary thyroid tumor, and in one case with a panostotic bone disease; it has never been described in MAS (45, 48, 54, 55).

Previous studies analyzing two cases (32, 33) and more recently a study analyzing 13 more cases had negative results looking for *gsp* mutations in AIMAH through direct sequencing (56). These negative results could be related to the methodology used since regarding somatic mutations, the identification of the mutant allele depends on its amount that can be decreased due to the contamination with normal surrounding healthy tissue. In this situation, the mutated DNA could lie below the detection level of direct sequencing. In our experience, DGGE was more sensitive for detecting somatic point mutation than direct sequence (41, 42). In addition, DGGE was more sensitive for detection of somatic mutation than direct sequence in an elegant study screening TSH somatic receptor mutations in DNA from toxic thyroid nodules (57). The authors found that the minimum amount of mutated DNA required to be detected through DGGE was 3–6%, whereas the amount of mutated DNA to be detected through sequencing was 25–50% (57).

In conclusion, we found two different *gsp* mutations in three of five patients with Cushing's syndrome due to AIMAH. We could speculate whether our patients may be part of the spectrum of MAS with a late somatic mutation, leading to a defect in only one cell type, or whether they are the first reported cases of isolated AIMAH in which the *gsp* mutations are involved in its molecular pathogenesis.

Therefore, AIMAH has a heterogeneous etiology and should be included as a human disorder caused by activating mutations in *GNAS1* gene.

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