CLINICAL STUDY

Heterogeneity in the molecular basis of ACTH resistance syndrome

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Abstract

Objective: ACTH resistance syndromes are rare, autosomal, and genetically heterogeneous diseases that include familial glucocorticoid deficiency (FGD) and triple A syndrome. FGD has been shown to segregate with mutations in the gene coding for ACTH receptor (MC2R) or melanocortin 2 receptor accessory protein (MRAP), whereas mutations in the triple A syndrome (AAAS, Allgrove syndrome) gene have been found in segregation with triple A syndrome. We describe the clinical findings and molecular analysis of MC2R, MRAP, and AAAS genes in five Brazilian patients with ACTH resistance syndrome.

Design and methods: Genomic DNA from patients and their unaffected relatives was extracted from peripheral blood leucocytes and amplified by PCR, followed by automated sequencing. Functional analysis was carried out using Y6 cells expressing wild-type and mutant MC2R.

Results: All five patients showed low cortisol and elevated plasma ACTH levels. One patient had achalasia and alacrima, besides the symptoms of adrenal insufficiency. The molecular analysis of FGD patients revealed a novel p.Gly116Val mutation in the MC2R gene in one patient and p.Met1Ile mutation in the MRAP gene in another patient. Expression of p.Gly116Val MC2R mutant in Y6 cells revealed that this variant failed to stimulate cAMP production. The analysis of the AAAS gene in the patient with triple A syndrome showed a novel g.782_783delTG deletion. The molecular analysis of DNA from other two patients showed no mutation in MC2R, MRAP, or AAAS gene.

Conclusions: In conclusion, the molecular basis of ACTH resistance syndrome is heterogeneous, segregating with genes coding for proteins involved with ACTH receptor signaling/expression or adrenal gland development and other unknown genes.

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Introduction

Adrenocorticotropic (ACTH) resistance syndromes include familial glucocorticoid deficiency (FGD) and triple A syndrome (AAAS), also known as Allgrove syndrome (1). These syndromes are rare autosomal recessive disorders characterized by early onset of primary adrenocortical insufficiency (2). FGD was first described by Shepard and colleagues (1959) (3) in two sisters with Addison’s disease without mineralocorticoid deficiency. Clinical onset is usually in the first year of life, but may occur later, and is accompanied by failure to thrive, hypoglycemia, convulsions, and skin pigmentation (2).

FGD is a genetically heterogeneous disease. FGD type 1 (OMIM 202200) includes patients with mutation in the ACTH receptor gene (MC2R), which accounts for 25% of FGD patients (1). The MC2R is a member of the melanocortin receptor family in class A of the seven transmembrane domain G protein-coupled receptors (GPCRs), and signals through the cAMP pathways by stimulating adenylyl cyclase activity. The MC2R maps to 18p11.2 and comprises two exons; exon 1 is untranslated and exon 2 encodes the entire sequence for the MC2R protein (4). Since the first report of MC2R mutation (5), several other mutations in the coding region of this gene have been described in segregation with FGD (for review, see (1)).

More recently, mutations in MRAP, a gene encoding a small single-transmembrane domain protein known as melanocortin 2 receptor accessory protein, have been described in a subset of patients with ACTH resistance syndrome, comprising FGD type 2 (OMIM no. 607398) (6, 7). Human MRAP is located at 21q22.1 and codes
for two MRAP isoforms, MRAP-α and MRAP-β, which differentially regulate the function of MC2R (8). MRAP mutations are present in 20% of patients with FGD (6). In more than 50% of FGD patients, there is no known molecular genetic defect. In three of these families, linkage was shown to markers on chromosome 8q (OMIM% 609197), confirming the genetic heterogeneity of the disease (9). The disease linking to chromosome 8q was defined as FGD type 3.

Triple A syndrome (OMIM 231550) is a genetic disorder characterized by a triad of adrenal insufficiency, achalasia, and alacrima, first described by Allgrove et al. (1978) (10). Patients with triple A syndrome usually present with hypoglycemia due to adrenal insufficiency in the first decade of life but it can manifest as late as the third decade of life (11). Some patients may have progressive and disabling neurological manifestations due to central, peripheral, or autonomic nervous system involvement (12, 13), often associated with mild mental retardation (11). Genetic linkage analysis revealed a locus on chromosome 12q13 as a critical region in segregation with triple A syndrome (14). Subsequently, a novel gene (AAAS), encoding a protein of 547 amino acids named ALADIN (for alacrima/achalasia/adrenal insufficiency/neurologic disorder), was identified as a molecular basis of triple A syndrome (13, 15). This protein belongs to the WD repeat family of regulatory proteins, and is located in the nuclear pore complexes (16). Though the function of ALADIN is not yet known, it has been suggested that it could regulate nucleocytoplasmic transport in specific cell types and the development of specific tissues (17). Homozygous and compound heterozygous mutations in the AAAS gene have been identified in several patients with triple A syndrome from different ethnic backgrounds (18, 19).

In this study, we aimed to describe the clinical findings and molecular analysis of MC2R, MRAP, and AAAS of five Brazilian patients with ACTH resistance syndrome.

Subjects and methods

Blood samples were obtained from five patients with ACTH resistance syndrome and their relatives and also from 50 control individuals. This study was performed with informed consent and the approval of the University Hospital Ethics Committee.

DNA extraction, PCR, and sequencing

Genomic DNA was extracted from peripheral blood leukocytes collected from affected patients and unaffected relatives using QIAamp Blood Kit (Qiagen), according to the manufacturer’s protocol. PCR conditions and respective primers were based on previous reports for MC2R (20) and MRAP (6). Amplification of AAAS was performed using specific primers as reported by Katsumata et al. (21). Eight pairs of primers were used to amplify 16 exons of AAAS by PCR with the following annealing temperature: exons 1 and 2, 55 °C; exons 3 and 4, 57 °C; exons 5 and 6, 57 °C; exon 7, 57 °C; exon 8, 57 °C; exons 9 and 10, 55 °C; exons 11–14, 65 °C; and exons 15 and 16, 57 °C. Following PCR amplification of each gene, automated sequencing was performed on ABI PRISM 3100 Genetic Analyzer, using the Big Dye Terminator Cycle Sequencing Kit, according to the manufacure’s protocol (Perkin–Elmer Applied Biosystems Division, Foster City, CA, USA). The molecular analyses were compared with databank information from Ensemb:Ensembl:ENSG00000185231 for MC2R, Ensembl:ENSG00000170262 for MRAP, and Ensembl:ENSG00000094914 for AAAS.

MC2R expression vectors and stable cell line generation

The full-length coding sequence of MC2R was amplified by PCR from patient and control genomic DNA and cloned into the expression vector pcDNA3.1/V5-His TOPO (Invitrogen). The integrity of the DNA sequence was confirmed by sequencing. Mouse Y6 cells, kindly provided by Prof. Bernard Schimmer, University of Toronto, were grown in DMEM–Ham’s F-10 (vol/vol) with horse serum (15%), FBS (5%), and penicillin/streptomycin. Cells were transfected with pcDNA expression vector containing normal or p.Gly116Val MC2R using Lipofectin Reagent (Invitrogen). Cells were selected in the presence of G418 (200 μg/ml) 25 days after transfection, and resistant clones were isolated by ringing. Positive clones expressing each mutation were further cultured and tested for cAMP generation.

ACTH stimulation

Y6 cells expressing the normal or p.Gly116Val MC2R were seeded into 96-well plates and grown until 70–80% confluence. On the day of the experiment, cells were incubated with serum-free medium containing 1 mmol/l 3-isobutyl-1-methylxanthine with different concentrations of ACTH-(1–24) (10–12–10–9 M) or forskolin (10–5 M) for 60 min. After incubation, cells and medium were harvested and cAMP was determined using cAMP Biotrak Enzyme Immunoassay System (Amersham Biosciences).

Results

Clinical and molecular findings

Patient 1 The female patient from consanguineous parents was born at term, with a birth weight of 3600 g and length of 50 cm. In the neonatal period, she had seizures associated with hypoglycemia and, at 1 year of age, she had respiratory distress and seizures. At the age of 4 years, she was admitted with fever, pneumonia,
vomiting, seizures, hypoglycemia (blood glucose: 25 mg/dl), and hypothermia (35.5 °C). On examination, height was above the 97th percentile and generalized excessive skin pigmentation was noticed. No clinical evidence of deficient tear production or achalasia was observed. The parents revealed that her older brother died at 10 months after an episode of convulsion. Biochemical analysis showed cortisol deficiency (0.7 µg/dl) with elevated ACTH (940 pg/ml) plasma levels. There was no cortisol response to ACTH stimulation test. Plasma 17-hydroxyprogesterone levels were 100 ng/dl, within the reference range. Since the diagnosis of FGD, the patient has been maintained on glucocorticoid therapy and appears to have normal growth, free from infections or hypoglycemia, and reduction of skin pigmentation.

The molecular analysis of this patient revealed a novel mutation in exon 2 of the MC2R, with a homozygous substitution of guanine by thymine (g.1042G>T) that results in a substitution of glycine by valine in codon 116 (p.Gly116Val; Fig. 1), which is located at the third transmembrane domain. Her parents and her younger healthy brother were heterozygous for p.Gly116Val mutation. Y6 cells expressing wild-type MC2R showed a dose–response pattern of cAMP generation when stimulated with different doses of ACTH, however, p.Gly116Val MC2R failed to generate cAMP (Fig. 2).

**Patient 2** The female patient from non-consanguineous parents was born at term with a birth weight of 4500 g and length of 55 cm. She showed neonatal jaundice and, at 11 months old, she had convulsions with hypoglycemia and history of recurrent infections. On examination, at 1 year and 10 months, she had height above the 97th percentile, generalized skin pigmentation, and no remarkable neurological findings. Biochemical analysis showed undetectable basal cortisol with elevated plasma ACTH level. There was no cortisol response to ACTH stimulation test. Glucocorticoid treatment improved her symptoms.

The molecular analysis of MC2R gene of this patient showed no alteration. MRAP gene analysis showed a homozygous mutation with a substitution of guanine by adenine (g.727G>A; Fig. 3) in the initiation codon (p.Met1Ile). Her parents were heterozygous for the same MRAP mutation.

**Patient 3** The female patient was born from consanguineous parents, and at 16 years of age, showed body weight loss, nausea, vomiting, hyporexia, excessive pigmentation of the tongue and skin, weakness, adynamia, and dizziness. She also presented with lower limb weakness. Her history revealed that she had always cried with few tears and the Schirmer test confirmed alacrima. Achalasia was diagnosed by manometry. The laboratory findings revealed elevated ACTH plasma levels (2140 pg/ml), low serum cortisol (<1.2 µg/dl), low serum DHEA-S (<3.9 µg/dl), and elevated plasma renin activity (19.9 ng/ml per h) with serum sodium and potassium of 132 and 5.2 mEq/l, respectively. MRI of the brain and spinal cord revealed bone deformity of the foramen magnum and left atlas arch, causing a slight cervicomedullary compression (Fig. 4). Upon gluco- and mineralocorticoid replacement, she had an improvement of the symptoms. She died of unknown causes at the age of 17 years.

The molecular analysis of this patient showed no alterations in MC2R and MRAP genes. The analysis of AAAS revealed a novel g.782_783delTG base deletion in exon 1 (Fig. 5), which introduces a premature stop codon at position 19. Her mother was heterozygous for the same AAAS gene mutation.

**Patient 4** The female patient from consanguineous parents was born at term with a birth weight of 3000 g. At 2 years of age, she had pneumonia and convulsions, which were followed by recurrent infections. At the age of 6 years, she was diagnosed as having rheumatic fever. No clinical evidence of deficient tear production or achalasia was noted. She had four dead brothers (two died of pneumonia before 2 years old and two died of unknown causes) and five healthy siblings. At the age of 7 years, she was admitted with excessive skin pigmentation, dehydration, and nausea. Laboratory analysis showed undetectable plasma cortisol levels with no responses after ACTH stimulation. Basal ACTH plasma levels were high (10 670 pg/ml). Sodium, potassium, and glucose plasma levels were 140, 4.1, and 49 mg/dl respectively. A second laboratory re-evaluation, at the age of 13 years, confirmed the
undetectable basal and ACTH stimulated plasma cortisol. Plasma 17-hydroxyprogesterone and aldosterone levels, and plasma renin activity were 30 ng/dl, 6.2 ng/dl, and 2.6 ng/ml per h respectively. Computerized tomography showed adrenal atrophy with no other abnormality. She has been on glucocorticoid therapy since 7 years of age with improvement of the symptoms. The molecular analysis of \textit{MC2R}, \textit{MRAP}, and \textit{AAAS} genes of this patient showed no alterations.

**Patient 5** The female patient, born from non-consanguineous parents, showed seizures associated with hypoglycemia and jaundice, and skin pigmentation in the neonatal period. On examination, at the age of 10 months, her height was above the 97th percentile and she had mild skin pigmentation. Biochemical analysis showed undetectable basal cortisol levels (0.1 µg/dl) with elevated plasma ACTH (2970 pg/ml). Plasma 17-hydroxyprogesterone, aldosterone, plasma renin activity, and sodium and potassium were 4 ng/dl, 4.8 ng/dl, 1.6 ng/ml per h, 137 mEq/l and 5.3 mEq/l respectively. Ultrasonography showed no urogenital morphological abnormality. Glucocorticoid treatment improved her symptoms. The molecular analysis of \textit{MC2R}, \textit{MRAP}, and \textit{AAAS} genes of this patient showed no alterations.

None of the mutations described in the present study were found in control individuals.

**Discussion**

In the present study, we report the clinical findings and molecular analysis of four patients with FGD and one patient with triple A syndrome. We found mutations in the \textit{MC2R}, \textit{MRAP}, and \textit{AAAS} genes in three patients and no molecular alterations in these genes in two patients.

With the exception of patient 4, all patients with FGD showed the typical clinical findings of severe cortisol deficiency during the neonatal period. Patient 4 showed pneumonia and convulsions, followed by recurrent infections at 2 years of age. In spite of the early clinical manifestations observed in all FGD patients, the disease was diagnosed only during infancy or childhood, as previously reported (2). The delay of the diagnosis of FGD may be due to the non-specific symptoms and absence of salt loss, and also due to the rarity of the disease. Three out of four patients with FGD had stature above the 97th percentile, a clinical finding already reported in the literature, although its association with ACTH receptor function has not yet been clarified (22).

Patient 3 presented at 16 years of age with the typical clinical findings of triple A syndrome, which were
improved by glucocorticoid and mineralocorticoid treatment. Manifestations of ACTH resistance in patients with triple A syndrome may present in a dramatic fashion with hypoglycemia and seizures or may be less severe and not necessitate glucocorticoid replacement until the teenage years or later (23), as observed in this patient. Mineralocorticoid deficiency may be present in about 15% of patients with triple A syndrome, as demonstrated by the elevated plasma renin activity in patient 3. Moreover, this syndrome is frequently associated with a variety of neurological disorders, such as progressive central, peripheral, and autonomic nervous system abnormalities, in 60% of triple A syndrome patients (14, 23). In accordance with these features, patient 3 showed lower limb weakness, which was improved by steroid treatment, suggesting that this clinical finding, in this case, might be related to gluco- and mineralocorticoid deficiencies, as previously reported in patients with Addison’s disease (24). The causal relationship between medullary compression present in this patient and AAAS mutation remains to be elucidated, since this manifestation has not been described in other triple A patients from the literature.

The molecular analysis of MC2R from patient 1 revealed the p.Gly116Val homozygous mutation. Her parents and her healthy younger brother were heterozygous for this mutation. Glycine 116 of MC2R is located in the third transmembrane domain, which is

![Figure 4](image-url) (A) Axial CT scan of the skull base. Bone deformity of the foramen magnum and left atlas arch (*), causing a slight compression of cervicomedullary junction (white arrow). (B) Axial T2-weighted MR image, posterior cranial fossa level. Left vertebral artery slightly dilated and tortuous (white arrow), causing small medullary compression (*).

![Figure 5](image-url) Representative direct sequencing of AAAS gene showing the homozygous deletion g.782_783delTG in exon 1. Her mother was heterozygous for g.782_783delTG mutation.
the most buried transmembrane domain in the structural arrangement of the GPCR (25), playing a critical role in receptor activation (26). In fact, mutagenesis and biochemical studies have demonstrated that activation of several GPCRs by ligand binding causes changes in the orientation of transmembrane domains 3 and 6 (27).

Glycine is a member of a group of small and weakly polar amino acids that are conserved among the class A GPCR family, other members being alanine, serine, cysteine, and threonine (28). In the case of MC2R, there is an interspecies conservation of glycine 116, as shown in Fig. 6. Moreover, in the melanocortin receptor family at the equivalent position to glycine 116, there is a serine residue in human MC1R and an alanine residue in human MC3R, MC4R, and MC5R (29). In addition, more than 80% of group conservation of these small and weakly polar amino acids among other class A GPCR members has been described (28). These amino acids play an important role in mediating helix–helix contact in the GPCRs, which is essential for receptor stabilization in an inactive conformation in the absence of ligand (30). It is interesting to note that changing of serine 120, also a weakly polar amino acid located at the third transmembrane domain of MC2R, to a positively charged arginine (p.Ser120Arg) was shown to be associated with FGD (31). Functional study of p.Ser120Arg mutated MC2R demonstrated loss of function of the receptor, with no cAMP production after ACTH stimulation (32). Therefore, the substitution of glycine at codon 116 by valine, a hydrophobic amino acid, in the ACTH receptor, as found in patient 1, is likely to impair its function, leading to ACTH resistance.

To investigate the functional effect of p.Gly116Val mutation, we carried out in vitro ACTH stimulation using Y6 cells transfected with wild-type and mutant receptors. Wild-type MC2R showed cAMP generation in a dose–response pattern, though a decrease of cAMP generation was observed at the highest dose of ACTH. This pattern of response has been described for other GPCRs (33, 34), indicating loss or decrease of agonist efficacy in receptor activation. It is possible that desensitization of the MC2R may account for this, although it is notable that the wild-type receptor does not show this response except possibly at high ACTH concentrations, and in mouse Y1 cells, this receptor has been shown to desensitize relatively slowly in response to ACTH (35). Functional study of p.Gly116Val mutated MC2R showed a decreased cAMP generation when stimulated with ACTH. Therefore, this result confirms that the p.Gly116Val mutation affects signal transduction leading to ACTH resistance.

The MC2R molecular analysis of patient 2 did not show alteration of this gene. However, the molecular analysis of MRAP demonstrated a homozygous mutation with a substitution of guanine by adenine (g.727G>A) in the initiation codon (p.Met1Ile). This missense mutation was previously described by Metherell et al. (6) in nine patients from eight families. In their study, they showed that MRAP and MC2R interact and are co-localized in the endoplasmic reticulum and plasma membrane.

A – MC2R

| Homo sapiens | I I D S L F V L S L L G S I F S L S V I A A D R Y |
| Mas masculus | I I D C M F I L S L L G S I F S L S V I A A D R Y |
| Bos taurus | V V D S L F I L S L L G S I C S L S V I A A D R Y |
| Canis familiaris | I M D S L F I L S L L G S I F S L S V I A A D R Y |
| Rattus norvegicus | I I D C M F V L S L L G S I F S L S V I A A D R Y |
| Macaca mulatta | I I D S L F V L S L L G S I F S L S V I A A D R Y |

B – Human melanocortin receptor

| Human MC2R | I I D S L F V L S L L G S I F S L S V I A A D R Y |
| Human MC1R | V I D V I T C S S M L S S L C F L G A I A V D R Y |
| Human MC3R | I F D S M I C I S L V A S I C N L L A I A V D R Y |
| Human MC4R | V I D S V I C S S L L A S I C S L L S I A V D R Y |
| Human MC5R | V F D S M I C I S V V A S M C S L L A I A V D R Y |

**Figure 6** Alignment of the amino acid sequences of the (A) MC2R from different species and (B) human melanocortin receptors. Conserved weak polar amino acids (glycine, alanine, and serine) in a region of the receptors within the third transmembrane domain are highlighted in bold and underlined letters.
Though not frequent, mutations in the initiation codon have been reported as a cause of human diseases (36–38). It has been demonstrated that initiation at non-AUG codons is inefficient in eukaryotic cells (39). It has been shown in vitro that mutation at the initiation AU/G prevents the RNA polymerase from recognizing the translation start codon (36). Mutations in the initiation codon may lead to a translation of mutant mRNA from a subsequent in-frame initiation codon, resulting in a truncated protein with a predictable severe loss of function. In patients with β-thalassemia, more severe anemia has been associated with different mutations in the initiation codon of the β-globin gene (37). More recently, Yu et al. (40) showed that M1I mutation, due to a change of ATG to ATC, in the initiation codon of LPL gene results in a reduced catalytic activity of lipoprotein lipase. Taking into account these reports, we can assume that mutation at the initiation codon of MRAP would result in a loss of the N-terminus of the protein, which would probably be non-functional, leading to the ACTH resistance in patient 2.

The diagnosis of triple A syndrome in patient 3 was confirmed by molecular analysis of AAAS, which revealed a novel g.782_783delTG base deletion in exon 1. Her mother was heterozygous for the same AAAS gene mutation. Homozygous and compound heterozygous AAAS gene mutations have been described in 95% of the patients with triple A syndrome from different ethnic backgrounds (1, 18). The majority of mutations in AAAS gene are nonsense, frameshift, or splice site with some missense mutations (23, 41). Though the function of ALADIN is not yet well established, this protein localizes in the nuclear pore and is involved in the nucleocytoplasmic transport (17, 18, 42). Studying natural variants described in triple A syndrome, a mislocalization of mutant ALADIN proteins in the cytoplasm was demonstrated (17, 42). Storr et al. (43) showed high expression of AAAS mRNA in the adrenal cortex and central nervous system (CNS), mostly in the cerebral cortex, cerebellum, hippocampus, motor-associated nuclei of the brainstem including cranial nerve nuclei, and ventral horn of the spinal cord. This pattern of expression confirms the role of ALADIN in adrenal and CNS function and agrees with the clinical features of triple A syndrome. However, the wide variety and severity of clinical findings in triple A syndrome (1) and the absence of phenotype resembling human disease in Aaas−/− mice, indicates that additional factors may be involved in the pathogenesis of this disease (42).

Molecular analysis of DNA from patients 4 and 5 did not show mutation in MC2R, MRAP, and AAAS genes. This result is in accordance with previous report that about 50% of FGD has unknown genetic cause (1). Recently, it was shown that MC2R interacts with Nup50, a nuclear pore complex protein (44), suggesting that this interaction could be a novel mechanism of action of ACTH receptor (45). The participation of changes in Nup50 function as a cause of ACTH resistance syndrome remains to be established.

In conclusion, we describe five patients with ACTH resistance syndrome with heterogeneous clinical findings, suggesting that excluding congenital adrenal hyperplasia, this rare syndrome should be investigated in infants and children with congenital adrenal insufficiency. Furthermore, besides genes already known to be involved in the FGD pathogenesis, other accessory proteins to ACTH receptor function may also underline the molecular basis of this disease.

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