Intrafamilial phenotypic variability and consequences of non-compliance with treatment in Congenital Adrenal Hyperplasia and Congenital Hypothyroidism within a single family

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Established facts

- Congenital Adrenal Hyperplasia may show genotype-phenotype discordance; nevertheless, intra-familial phenotypic variability is unusual.

- Non-adherence to treatment in Congenital Adrenal Hyperplasia may be associated with early puberty, signs of virilization and short final height.

Novel insights

- Coexistence of Congenital Adrenal Hyperplasia and Congenital Hypothyroidism due to mutations in the gene encoding Thyroglobulin (TG) has never been reported in the same family originating from non-consanguineous parents.

- Congenital Adrenal Hyperplasia may be characterized by marked genotype-phenotype discordance, even within the same family.

- Poor compliance to medications in Congenital Adrenal Hyperplasia may be associated with the development of an adrenal adenoma.

- We describe a novel mutation in the *TG* gene, causing Congenital Hypothyroidism.

Abstract

Background: Coexistence of congenital adrenal hyperplasia (CAH) and congenital hypothyroidism (CH) due to *TG* mutation in the same non-consanguineous family is rare.

Case series: We report four siblings born to unrelated parents, the father being an asymptomatic carrier of homozygous p.V281L and heterozygous p.I172N *CYP21A2* mutations. Sibling 1 had salt-wasting CAH (*CYP21A2* genotype Intron 2 splice/p.I172N and p.V281L). She also had CH (*TG* genotype p.R296/p.T1416Rfs*30) and learning difficulties. Poor compliance and multifactorial morbid obesity were associated with short stature, precocious puberty, hirsutism, amenorrhea, insulin insensitivity and a possible adrenal adenoma. Sibling 3 (*CYP21A2* and *TG* genotype similar to sibling 1) is a boy who presented with salt-wasting CAH, CH, and developmental delay. He was also overweight and underwent precocious puberty. Although Siblings 2 and 4 (both females) share the same *CYP21A2* genotype (I2-splice/p.V281L), the former only had biochemical evidence of CAH, while the latter presented at the age of 9.8 years with a past history of pubarche at 7 years and an advanced bone age.

Conclusions: We report the unusual occurrence of two rare autosomal recessive diseases, CAH and CH. Our cases highlight the phenotypic variability of CAH and CH due to *TG* mutations, even within a single family, and illustrate the importance of optimal disease control.

Background

Congenital adrenal hyperplasia due to 21-hydroxylase deficiency is caused by autosomal recessive mutations in the *CYP21A2* gene. Although this disorder can be viewed as a continuum of disorders, phenotypically, it can be divided into classical and non-classical (NC) forms, with the former presenting as salt-wasting (SW) or simple-virilising (SV) CAH. More than 100 *CYP21A2* mutations have been reported. However, molecular genetic testing for nine common mutations and deletions detects approximately 80%-98% of disease-causing alleles (1).

Thyroglobulin (TG) is exclusively synthesized in the thyroid gland. Autosomal recessive mutations in *TG* lead to permanent CH. The combination of CAH and CH occurring in the same individual has not, to our knowledge, been reported in the literature.

Methods

Hormone assays

Serum cortisol, testosterone, ACTH, DHEA, DHEA-S, androstenedione, FT4 and TSH were measured using competitive chemiluminescent immunoassay (Immulite 2000 Siemens Diagnostics). During follow-up, the median time from intake of last hydrocortisone dose until blood sampling was 4.75 hours. Plasma renin activity (PRA) was measured using RIA.

GnRH stimulation test has been performed by measuring plasma LH and FSH concentrations at baseline and 20 and 60 minutes after IV injection of GnRH 2.5 micrograms/kg (maximum 100 micrograms).

Reference ranges for TSH and FT4 were 0.55 – 6 mU/L and 10.2-20.6 pmol/L, respectively.

Genetic testing

CYP21A2 gene deletion and chimeric genes were detected using a commercially available multiplex-ligation probe amplification strategy following the manufacturer's protocol (mrc-Holland, Amsterdam, The Netherlands). *CYP21A2* point mutations were detected by targeted multiplex minisequencing after allele-specific PCR amplification of the *CYP21A2* gene.

Genetic investigation of CH was performed through a customized targeted Agilent SureSelect pull-down array, designed to screen 1,188 genes, including TG, TPO, TSHR, DUOX2, DUOXA2, IYD, SLC5A5 and SLC26A4. Dataset calls were annotated with dbSNP v137 rsIDs and allele frequencies computed from several datasets including: 1000 Genomes Phase I (1KG, n=2,818), UK10K whole-exome sequencing studies (n=4,975) and Exome Aggregation Consortium r0.3 (ExAC) (n=60,706). Rare variants were selected that affected the protein coding sequence with SIFT or Polyphen pathogenicity prediction 'possibly damaging' or above for missense variants. All likely pathogenic variants were confirmed by Sanger sequencing.

Subjects

Parents (Figure 1, I:1 and I:2)

Parental medical history is unremarkable. They are unrelated and the father is Palestinian, while the mother is white British. The father reached a final height of -1.43 SDS, while the mother's height is -0.84 SDS, with a midparental height of -1.25 SDS. Molecular analysis of *CYP21A2* showed that the mother was heterozygous for the I2-splice mutation, while the father had an apparent normal *CYP21A2* copy number and carried the mutations p.V281L and p.I172N on one allele, and a second p.V281L mutation on the other. Sequencing of *TG* showed a p.R296* mutation in the father and a novel p.T1416Rfs*30 mutation in the mother.

Sibling 1 (Figure 1, II:3)

This female child was born at term by vaginal delivery with a weight of 3.1 kg and presented at birth with virilised genitalia (Prader 3). Androgen profile performed on day 3 showed markedly increased concentrations of 17-Hydroxyprogesterone (17-OHP) (276nmol/L; nr 0-5) (Table 1.), Testosterone (34.2nmol/L; nr<2.9), Androstenedione (>35pmol/L; nr 3-8) and DHEAS (11umol/L; nr 0.5-4.1). Urinary steroid profile was dominated by 17-OHP metabolites. Therefore, she was diagnosed as having a classic form of CAH and was started on hydrocortisone on day 3 of life. On day 23 her sodium concentration was 132mmol/L, with a potassium of 5.5mmol/L, and a raised plasma renin activity (PRA) of 8.4pmol/ml/hr (nr 1.1-2.7); she was therefore commenced on fludrocortisone and sodium supplements. Neonatal screening revealed an elevated TSH of 213mU/L and subsequent venous sampling confirmed a diagnosis of CH (TSH 310.2mU/L and FT4 <8pmol/L on recall), thus requiring Levothyroxine replacement on day 20 of life (starting dose 15ug/kg/day) (Table 1.).

The patient was compound heterozygous for the maternal c.290-13C>G (I2-splice) and the paternal c.515T>A (p.I172N) and c.841G>T (p.V281L) mutations. Sequencing of *TG* revealed compound heterozygous paternal c.886C>T (p.R296*) and maternal c.4246_4255delACGTTCCCAG (p.T1416Rfs*30) mutations. She had moderate learning difficulties and poor compliance with medications, as demonstrated by chronically elevated concentrations of 17-OHP (mean value among periodic measurements since diagnosis 72.8nmol/l), PRA (mean value among periodic measurements since diagnosis 5.86pmol/ml/hr) and TSH (mean value among periodic measurements since diagnosis 31.56mU/L). Nevertheless, she did not experience adrenal crises during follow-up.

At the age of 6.5 years (weight 28.3 kg, +2.36 SDS; height 114 cm, -0.54 SDS) her Tanner stage was PH (pubic hair) 1 with breast stage (B) 2, and an advanced bone age of 9 years. Ultrasound of the pelvis demonstrated a pubertal uterus, measuring 3.9 x 0.7 x 1.4 cm and GnRH stimulation

test confirmed central gonadotropin-dependent precocious puberty (CPP) (basal/peak LH and FSH 0.7/5.9 and 3.5/7.4 IU/L, respectively). She was therefore commenced on gonadotropin-releasing hormone analogue (GnRHa) treatment from 7 to 10.5 years of age. Although she completed puberty, she has not attained menarche as yet at 19.8 years of age. She is short (height SDS -2.60; midparental height SDS -1.41), obese (BMI SDS +4.4), and has severe hirsutism, a deep voice, polycystic ovaries, glucose intolerance and insulin insensitivity. In fact, at the last appointment, at the age of 18.9 years, blood tests showed a testosterone of 9.85nmol/L, Androstenedione >35nmol/L, and LH<0.2IU/L, while an OGTT revealed glucose concentrations at 120 minutes of 8.4mmol/L, with an insulin peak >300mU/L. Abdominal ultrasound and MRI revealed enlarged adrenals with a right adrenal mass measuring 4.7 x 3.6 x 3.6cm (figure 2), with mild diffusion restriction and avid enhancement post gadolinium administration. Her blood pressure is high-normal (130/90 mmHg) and her plasma catecholamines and urinary metanephrines are normal; therefore in the light of the imaging findings the right adrenal mass is most likely an adrenal adenoma. Adrenal mass appearances have been stable over the last 5 years and the patient has been changed to twice daily Dexamethasone treatment, in order to improve compliance and optimise control of the CAH. In addition, she is taking Flutamide and Metformin in order to mitigate the signs of severe hyperandrogenism and insulin insensitivity.

Sibling 2 (Figure 1, II:4)

This female patient was diagnosed in the course of family screening, at the age of 16,8 years, because of a high basal androstenedione of 20nmol/l (nr 3-8) and DHEAS of 13.1mmol/l (nr 0.5-4.1), with a normal PRA. She had an exaggerated 17-OHP response (peak 81.9nmol/l) to synacthen (Table 1.) , but the cortisol response was adequate (peak 722nmol/l). She is currently asymptomatic at the age of 17.8 years, and has regular menstrual cycles. Her final adult height is 154 cm (SDS-1.35) which is well within the target height range, with a weight of 55 kg (SDS +1). She was compound heterozygous for the maternal I2-splice and the paternal p.V281L mutations.

Sibling 3 (Figure 1, II:5)

This male patient was born at term by Caesarean section with a weight of 3.3 kg. The child was diagnosed with CAH on neonatal screening because of the positive family history of CAH (capillary 17-OHP >60nmol/L). Confirmatory blood tests performed on day 3 of life at a pre-symphtomatic stage revealed serum 17-OHP 276nmol/L (Table 1.), Na 142mmol/L and K 6.2 mmol/L and the child was commenced on hydrocortisone, fludrocortisone and sodium supplements. Diagnosis of CH was suspected upon neonatal screening (Guthrie TSH 213 mU/L) and confirmed on day 7 of life with a venous TSH of 248.3mU/L and FT4 of 17.6pmol/L on recall (Table 1.). Therefore thyroxine replacement therapy was commenced on the same day at a starting dose of 12.5 ug/kg/day.

He had poor compliance with medications since the first year of life with chronically raised 17-OHP (mean value of periodic measurements since diagnosis 53.9nmol/L), PRA (mean value of periodic measurements since diagnosis 7.87pmol/ml/hr) and TSH (mean value of periodic measurements since diagnosis 28.8mUI/L) and low FT4 concentrations (below 10 pmol/L, minimum 7.8 pmol/L) detected on several occasions. He too is developmentally delayed. He is a compound heterozygote for the maternal I2-splice and the paternal p.V281L and p.I172N mutations. Sequencing of *TG* revealed compound heterozygous paternal p.R296* and maternal p.T1416Rfs*30 mutations.

At the age of 9.6 years, he was started on GnRHa, having developed CPP (testicular volumes 6 ml at the age of 9.3 years; basal/peak LH and FSH 0.5/12.6 and 2.5/9.9 IU/L, respectively). At 13.8 years of age, his growth rate was 0.82cm/year, with a bone age of 15.5 years; therefore GnRHa was discontinued. At the age of 15.4 years, he had reached his near-final height of 148.5 cm (SDS-2.77). His weight was 64.2 kg (SDS+0.86), and his BMI 29.11 kg/m2 (SDS+2.51).

Sibling 4 (Figure 1, II:6)

She was referred at 9.81 years with a history of pubarche at 7 years, accelerated growth velocity (7cm/year (SDS+1.6)) and an advanced bone age of 11.4 years. She had no signs of puberty and no clitoral hypertrophy was detected at diagnosis. Basal 17-OHP was 49nmol/L. Synacthen test showed a suboptimal peak cortisol of 385nmol/l and a peak 17-OHP of 144nmol/L and thus she was commenced on hydrocortisone (Table 1.). She was compound heterozygous for the maternal I2-splice and the paternal p.V281L mutations, resulting in NC CAH. She achieved menarche at 13 years of age (height 158.1cm (SDS+0.33), weight 72.2kg (SDS+3.03), BMI 29 (SDS+2.56)).

Discussion

We report the unusual co-occurrence of two rare autosomal recessive conditions, CAH due to *CYP21A2* mutations and CH due to mutations in *TG* in a family originating from unrelated parents. Our cases represent a striking description of genotype-phenotype variability of CAH in members of the same family. Mutations resulting in complete inactivation of 21-hydroxylase result in a SW phenotype, while those that reduce enzyme activity to 2% cause the SV phenotype and those that reduce activity to more than 10% cause the NC phenotype (2). Of note, the wide range of clinical manifestations of 21-hydroxylase deficiency represent a disease continuum and there is often a fine line between the NC and SV types of CAH, as there is between SW and SV CAH in the absence of an actual SW crisis. The phenotype of CAH should be predicted by the least severe mutation. However, a study including 1507 families with CAH demonstrated a direct genotype-phenotype correlation in fewer than 50% of affected individuals. The I2-splice mutation activates cryptic splice acceptor sites, resulting in severe enzyme impairment. Although this constitutes the most frequent mutation causing SW-CAH, a genotype-phenotype discordance may be due to alternate splicing allowing variable enzyme activity (1).

The p.I172N mutation disrupts a hydrophobic interaction which is crucial in maintaining the enzyme conformation, thus affecting relatively severely the enzyme activity (3). However, in a recent study, 76% of the individuals who were compound heterozygotes for this mutation and a

severe mutation had the SV phenotype while 23% had the SW phenotype, and 1% even had a NC form (1). The p.V281L mutation causes mild enzyme impairment, due to changes in the enzyme tertiary structure (4). Patients carrying a severe *CYP21A2* mutation and the p.V281L mutation usually present with the NC form of CAH, but there is evidence suggesting that these patients have higher 17OHP concentrations which might predict a more severe phenotype as compared to those who are homozygous for the p.V281L mutation, regardless of gender and age (5). In our family, siblings 1 and 3 presented with a severe SW form, when a SV or even a NC form would have been predicted. Moreover, although the specific combinations of mutations in the father and in sibling 2 should result in a NC form, they were asymptomatic, indicating a so-called cryptic form of NC CAH (6). On the other hand, sibling 4, despite genotypic similarity to sibling 2, presented with signs of hyperandrogenism. Several hypotheses have been suggested to explain genotype-phenotype discordance. Subtle variations in transcriptional regulation or downstream protein translation may account for reduced enzyme activity. Hence the marginal enzymatic function of the p.I172N mutant may not always be sufficient to prevent salt wasting. In studies relying, as in our case, on detection of a panel of common mutations, additional mutations might be missed (1). Finally, genetic or environmental factors and individual androgen sensitivity may influence phenotype (7). Therefore, it has been suggested that selected cases with genotype-phenotype discordance may benefit from whole exome sequencing (1).

CAH was poorly controlled in our patients, mainly due to poor compliance. Despite a prompt suppression of early-onset puberty, siblings 1 and 3 reached a final height <2 SD below the midparental height, mainly attributable to advanced bone maturation. Although CPP is a well-known complication of CAH, on the other hand the age of menarche in inadequately treated girls is late (8), and a small proportion of women do not undergo menarche (9). Such patients with CAH are often obese and have insulin insensitivity and polycystic ovaries. All siblings from our kindred (except for sibling two) presented with overweight/obesity, which probably has a multifactorial pathogenesis. In the light of poor compliance to glucocorticoid replacement therapy, steroid

treatment may not have a major role; on the other hand it can be hypothesized that inadequately treated hypothyroidism may have contributed to weight gain in siblings 1 and 3. However, other factors such as CAH itself, CAH-related adrenomedullary hypofunction, familial or environmental factors might have played a role in the pathogenesis of obesity (10). Of note, in our patients obesity had an early onset, thus contributing to the advanced bone age, precocious sexual maturation, insulin insensitivity and hyperandrogenism.

In addition to these complications, sibling 1 presented with a right adrenal mass, with radiological features consistent with an adenoma. The prevalence of adrenal adenomas in patients with CAH varies in different case series from 82% of cases (11-12) to 0% (13), with reports of adrenal adenoma also in NC CAH (5). The aetiology of such complications is unclear, but the correlation found between adrenal nodules and adrenal volume points toward a role of excess ACTH stimulation (14). The role of genetics in the pathogenesis of adrenal tumours is uncertain. Results of the CaHASE study in 153 adults with CAH showed no association between health outcomes of CAH and genotype (15). On the other hand, studies on subjects with adrenal tumours showed an increased incidence of *CYP21A2* mutations, and an exaggerated 17OHP response after ACTH stimulation in up to 70% of cases, thus suggesting a pathogenic role of impaired 21-hydroxylase activity (16, 17). Finally, other factors, such as a direct effect of sex steroids, LH and angiotensin II, might be involved (18).

Our cases are complicated by the coexistence of poorly controlled CH in the 2 siblings with the most severe form of CAH. One of the TG mutations (p.R296*) is known to be pathogenic [19], while the maternally inherited c.4246_4255delACGTTCCCAG (p.T1416Rfs*30) mutation is novel. The first mutation consists of a C-to-T transition at position 886 in exon 7, resulting in a premature stop codon [19]. Such a mutation truncates TG before the carboxy-terminal acetylcholinesterase homology (ACHE-like) domain, affecting conformational maturation and cellular export of TG [20]. Although functional studies for the p.T1416Rfs*30 mutation are not available, the mutant TG is predicted to lack the ACHE-like domain, thus supporting a pathogenic consequence.

Although TG mutations are usually associated with a goitre, which may develop from the antenatal period to an adult age [21, 22], this was absent in our hypothyroid siblings. The biochemical spectrum of TG mutations is broad, ranging from severe cases to moderate hypothyroidism and even normal thyroid function. Of note, sibling 1 displayed severe hypothyroidism at diagnosis, while sibling 3 had normal FT4 concentrations at diagnosis, but experienced hypothyroxinaemia during follow-up. When adequately treated, patients with moderate hypothyroidism do not have physical impairment and/or mental retardation; therefore, learning difficulties in our hypothyroid patients are probably a consequence of noncompliance with thyroxine therapy since the first few months of life. Intrafamilial variability of CH due to TG mutations has been previously reported [19], and it is probably attributable to variability of phenotypic expression of the defective TG protein [23].

To conclude, our cases highlight the importance of optimal disease control and exemplify the genotype-phenotype discordance of CAH and CH in members of the same family. This must be borne in mind when predicting phenotype in prenatal testing and when evaluating relatives of CAH patients. In selected cases, whole-exome or genome sequencing may shed further light. Finally, establishing the molecular basis of CH may guide follow-up, and confirm recurrence risk.

Declaration of interest

The authors declare that there is no conflict of interest.

List of Abbreviations: CAH Congenital Adrenal Hyperplasia; CH Congenital Hypothyroidism; TG Thyroglobulin; NC Non-classical; SW salt-wasting; SV simple-virilising; 17-OHP 17-Hydroxyprogesterone; PRA plasma renin activity; CPP central precocious puberty; GnRHa gonadotropin-releasing hormone analogue; ACHE-like carboxy-terminal acetylcholinesterase homology.

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Figure legends

Figure 1. Family pedigree: I:2 and the siblings II:3–II:6 are affected with CAH, with phenotypic variability. I:2 also carries the p.R296* mutation in *TG*. I:1 and the siblings II:1-II:2 are carriers of *CYP21A2* and *TG* mutations. II:3 and II:5 are also affected with CH. II:4 is wild type for TG genotype, while II:6 has not been screened for *TG* mutations.

Figure 2. Axial (A) and coronal (B) abdominal MRI sections showing a 4.7-cm well-defined mass arising from the medial lobe of the right adrenal gland. The mass is isointense to the liver in T1-weighted (A) and hyperintense to the liver in T2-weighted scans (B). The lateral limb of the right adrenal gland is enlarged. Both limbs of the left adrenal gland are enlarged, in the absence of defined masses, in keeping with the diagnosis of congenital adrenal hyperplasia.