A rare *CYP21A2* mutation in a congenital adrenal hyperplasia kindred displaying genotype–phenotype non-concordance

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Abstract

Congenital adrenal hyperplasia (CAH) owing to 21-hydroxylase deficiency is caused by the autosomal recessive inheritance of mutations in the gene *CYP21A2*. *CYP21A2* mutations lead to variable impairment of the 21-hydroxylase enzyme, which, in turn, is associated with three clinical phenotypes, namely, salt wasting, simple virilizing, and non-classical CAH. However, it is known that a given mutation can associate with different clinical phenotypes, resulting in a high rate of genotype–phenotype non-concordance. We aimed to study the genotype–phenotype non-concordance in a family with three sibs affected with non-classical CAH. All had hormonal evidence of non-classical CAH, but this phenotype could not be explained by the genotype obtained from commercial *CYP21A2* genetic testing, which revealed heterozygosity for the maternal 30 kb deletion mutation. We performed Sanger sequencing of the entire *CYP21A2* gene in this family to search for a rare mutation that was not covered by commercial testing and found in the three sibs a second, rare c.1097G>A (p.R366H) mutation in exon 8. Computational modeling confirmed that this was a mild mutation consistent with non-classical CAH. We recommend that sequencing of entire genes for rare mutations should be carried out when genotype–phenotype non-concordance is observed in patients with autosomal recessive monogenic disorders, including CAH.

Keywords

CYP21A2; genotype; phenotype; p.R366H; congenital adrenal hyperplasia

Introduction

Congenital adrenal hyperplasia (CAH) is a highly prevalent rare monogenic disorder with an overall incidence of 1 in 15,000 live births.[1] Around 90% of CAH cases are due to

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mutations in the gene *CYP21A2*, all of which reduce the activity of the enzyme 21-hydroxylase, but to varying extents. Three clinical phenotypes thus arise: salt-wasting, simple virilizing (the two grouped as classical CAH), and non-classical CAH. The diagnosis of CAH due to 21-hydroxylase deficiency is made biochemically by performing a 60-minute cosyntropin stimulation test. Plotting the 0- and 60-minute levels of 17α-hydroxyprogesterone (17-OHP) on a published nomogram distinguishes classical from non-classical CAH.[1, 2]

Around 150 disease-causing *CYP21A2* mutations have been described. To predict the extent of functional loss that each mutation induces by altering the conformation of 21-hydroxylase, we recently performed computational modeling by plugging in mutations into the humanized crystal structure of 21-hydroxylase.[3] In doing so, we were able to broadly predict clinical phenotypes arising from specific structural changes induced *in silico* by mutations. Namely, if the mutation disrupted heme or substrate binding, or rendered the enzyme unstable, a salt-wasting form of CAH could be expected. On the other hand, if the mutation disrupted conserved hydrophobic patches, it led to simple virilizing CAH; but if the enzyme remained relatively intact, non-classical CAH was a likely outcome.

Correlating genotype with clinical phenotype due to each specific mutation is indeed highly relevant to the management of CAH, including counseling regarding offspring and, possibly, prenatal therapy. To this end, we recently completed a comprehensive study of genotype–phenotype concordance in 1507 families.[4] We found that non-concordance between genotype and phenotype was high, at 39%, strongly suggesting that mechanisms other than the mutation *per se* altered the activity of 21 hydroxylase.

Here, we report genotype–phenotype non-concordance in a family with CAH that was explained by a second, rare mutation p.R366H in *CYP21A2*. Computational modeling suggests that this mutation would only mildly affect 21-hydroxylase activity, a mechanism consistent with the hormonal phenotype of non-classical CAH. That p.R366H is a mild mutation is further concluded from an unrelated female non-classical CAH patient who carries p.I172N/p.R366H compound heterozygous *CYP21A2* mutations.

Results and discussion

The proband (p) was first evaluated at age 8.8 years for precocious puberty (Tanner III pubic hair with increased testicular volume) and rapid growth. Evaluation revealed marked advancement in skeletal maturation (12.5 to 13 years at a chronological age of 9.3 years). Central precocious puberty was confirmed biochemically by a serum luteinizing hormone (LH) level of 0.323 mIU/mL, measured using an ultrasensitive assay (ICMA). Serum 17-OHP values by liquid chromatography–mass spectrometry (LC/MS/MS) rose from 136 ng/dL to 4700 ng/dL with the 60-minute cosyntropin stimulation testing (Table 1). Using the 17-OHP nomogram, the patient was diagnosed biochemically with non-classical CAH and treated with a luteinizing hormone-releasing hormone (LHRH) analog and glucocorticoids. Genetic testing for *CYP21A2* mutations was not performed at the time.

The proband presented to our clinic for a second opinion at age 10.7 years due to poor adrenal hormonal control and advanced skeletal maturation (13.75 years), which was

compromising his adult height. After counseling, the proband (p) was treated with an aromatase inhibitor, his glucocorticoid regimen was adjusted, and desired adrenal control was achieved. Currently, the patient, age 12 years, is being treated with an LHRH analogue, aromatase inhibitor, and hydrocortisone (10 mg/m^2); he continues to have minimal interval skeletal maturation with a significant improvement in his predicted adult height. Commercial testing for 10 common *CYP21A2* mutations (analyzed by mini sequencing for four different PCR products) revealed a heterozygous 30 kb deletion inherited from his mother. The father did not appear to carry any *CYP21A2* mutation.

As heterozygosity of the deletion mutation could not explain the non-classical CAH phenotype, the entire family was invited for a thorough evaluation, with informed consent, consisting of a clinical examination, hormone measurements, cosyntropin stimulation test, repeat PCR, and Sanger sequencing of full length *CYP21A2*. Notably, the older sibling (a) tested genetically and hormonally similar to the proband (p). However, he had a relatively delayed puberty (onset 13 years), somewhat delayed skeletal maturation (14.1 years at a chronological age of 15.1 years), and was a Tanner stage V for pubic hair, with a testicular volume of 20 cc bilaterally. He had always been short compared with his peers, growing along the 5th to 8th percentiles for age and sex. Owing to the growth concerns, a thorough growth evaluation was performed, including a thyroid function test, celiac disease screen, blood count, comprehensive metabolic panel, glycosylated hemoglobin, insulin-like growth factor-1 (IGF-1), IGF binding protein-3, and arginine-clonidine growth hormone provocative tests were all unremarkable. Serum 17-OHP (LC/MS/MS) rose from 649 ng/dl to 5500 ng/dl on the 60-minute cosyntropin stimulation test (Table 1); this was diagnostic of non-classical CAH *per* the nomogram (Fig. 1A).[2]

The proband's second brother (b) displayed an identical hormonal profile, with 17-OHP values (LC/MS/MS) rising from 419 ng/dl to 5780 ng/dl upon a 60-minute cosyntropin stimulation testing (Table 1), compared with the nomogram; this again established the diagnosis of non-classical CAH (Fig. 1A). Clinically, the patient was growing along the 50th percentile for age and sex and was reported to have an onset of puberty at age 11 years; his skeletal age was consistent with his chronological age.

Thus, while the three brothers were diagnosed as non-classical CAH, they were found only to harbor the maternally inherited heterozygous *CYP21A2* deletion mutation. This non-concordance between the hormonal phenotype and heterozygous genotype (otherwise consistent with a carrier state) led us to perform Sanger sequencing of the entire *CYP21A2* locus of all family members. Sequencing revealed that the three brothers (p), (a), and (b) inherited a rare p.R366H mutation from the father in addition to the *del* mutation from the mother; the former was not detected by routine genetic testing. The three sibs thus harbored a compound heterozygous mutation p.R366H/*del*. All three brothers carry exactly the same CYP21A2 sequence; no variation was detected in the coding region, proximal promoter, and introns. Sequencing also confirmed that the father carried the p.R366H mutation. Sibs (d) and (e), in contrast, had a normal clinical examination as well as baseline adrenal hormonal profiles, and carried no mutations in the *CYP21A2* gene (Fig. 1B and Table 1). In addition, we independently confirmed the heterozygous deletion of the *CYP21A2* gene in the three sibs and the mother (m), who is of Italian, Irish and German non-Jewish descent.

We also studied a 20-year-old unrelated female (z) born with normal female genitalia, who was diagnosed at age 10 years with non-classical CAH after presenting with resistant acne. Sanger sequencing of the *CYP21A2* gene demonstrated that the patient was a compound heterozygote for the exon 4 c.515T>A (p.I172N) and the rare p.R366H mutation (Table 1).

To examine the extent to which the p.R366H mutation altered 21-hydroxylase structure *in silico*, we mapped the mutation to our humanized model of CYP21A2.[3] The R366 residue (β4 sheet) normally forms a salt-bridge with D111 (B'-C loop); this ion-pair interaction holds the B'-helix in position allowing for appropriate gating of binding sites (Fig. 1C). At this position, the enzyme normally harbors a positively charged residue, such as Arg or Lys, both of which are relatively well conserved across the steroid-synthesizing P450 family. A mutation in this residue, p.R366C, completely disrupts the salt-bridge interaction, causing protein inactivation and salt-wasting disease.[3] In contrast, a p.R366H mutation results in the loss of one of two hydrogen bonds, thus weakening, but not abolishing the ion-pair interaction. Our previous study has shown that mutations disrupting hydrogen bonding produce milder, non-classical disease.[3] We calculated the free energy change in protein stability, for the mutations and found that the G value to be 0.79 and 1.01 kcal/mol for R366H and R366C mutations respectively. We therefore predict that the p.R366H mutation is less detrimental.

The p.R366H mutation of *CYP21A2* has been reported once before, by Ghizzoni, *et al*,[5] who studied the relationship between 17-OHP levels and *CYP21A2* genotype. Their patient with established non-classical CAH was a compound heterozygote harboring an exon 7 c. 841G>T (p.V281L) mutation on the maternal allele and a p.V281L + p.R366H double mutation on the paternal allele. However, as the p.V281L mutation is known to result in non-classical CAH, the authors were unable to assess a role for p.R366H in this setting.

That p.R366H is a mild mutation is testified on several grounds. First, in our kindred, a non-classical presentation is noted despite a ~50% reduction in 21-hydroxylase expression from the *del* mutation. Second, the absence of a virilizing phenotype in the unrelated female displaying a compound heterozygous p.I172N/p.R366H mutation, wherein p.I172N alone is known to cause simple virilizing CAH, indicates that p.R366H must indeed be a mild mutation. Finally, our computational analysis predicts a potential reduction, as opposed to elimination, of 21-hydroxylase activity with the p.R366H mutation *in silico*. However, it is interesting that, while p.R366H in our kindred is consistent biochemically with non-classical CAH in all three affected siblings, the respective clinical profiles are distinct with varying Tanner scores and extents of growth retardation. We do not have a clear explanation for this phenotypic variation.

In summary, genotype–phenotype discordance is not uncommon in autosomal recessive monogenic disorders, and specifically, poses diagnostic challenges in CAH. As opposed to genotyping using a panel of common *CYP21A2* mutations, our data highlight the importance of sequencing the full gene to identify rare mutations in cases where the clinical/biochemical picture is non-concordant with genotype.[6] The identification of novel genetic mutations, which we can now model computationally to obtain preliminary insights at the protein level, is particularly critical for perinatal counseling.

Materials and methods

All patients and their family members gave informed consent for DNA analysis. Genomic DNA was isolated from peripheral blood leukocytes (DNeasy, Qiagen). Table 2 shows the oligonucleotides used for *CYP21A2* genetic analysis. Briefly, *Taq*I restriction fragment length polymorphism of the 779F/Tena32F PCR product was used to detect 30 kb deletion, as previously described.[7] To sequence *CYP21A2*, two PCR fragments were amplified from genomic DNA using primer pairs 779F/ex6a and ex3s/ex10a, respectively. The *CYP21A2*-specific ex6a and ex3s primers avoid amplification of the *CYP21A1P* pseudogene. Sanger sequencing using primers 1399F, in2a, in4a, in4s, in6s, and in8s provides complete sequence information of the *CYP21A2* coding region, all intervening introns, 260 bases upstream of the start codon, and 32 bases downstream of the stop codon.

For *in silico* analysis, we have recently generated a model of human CYP21A2 based on the crystal structure of the bovine homolog.[3] These two sequences share a homology of ~79% over 450 amino acids, rising to 95% in the heme binding region. To rationalize consequences of the R366H and R366C mutations, we introduced these changes into our humanized model and calculated the free energy of stability using the ICM mutagenesis program.[8] The free energy change in protein stability, after introduction of a mutation, was calculated as:

$$
\begin{aligned}
\Delta\Delta G &= \Delta G_{\mathrm{mutant}} - \Delta G_{wt} \\
\Delta G &= \Delta G_{\mathrm{folded}} - \Delta G_{\mathrm{unfolded}}
\end{aligned}
$$

The calculations were based on the free energy of unfolded and misfolded states, and was approximated by a sum of the residue specific energies, which were derived empirically using a large set of experimental data.[8]

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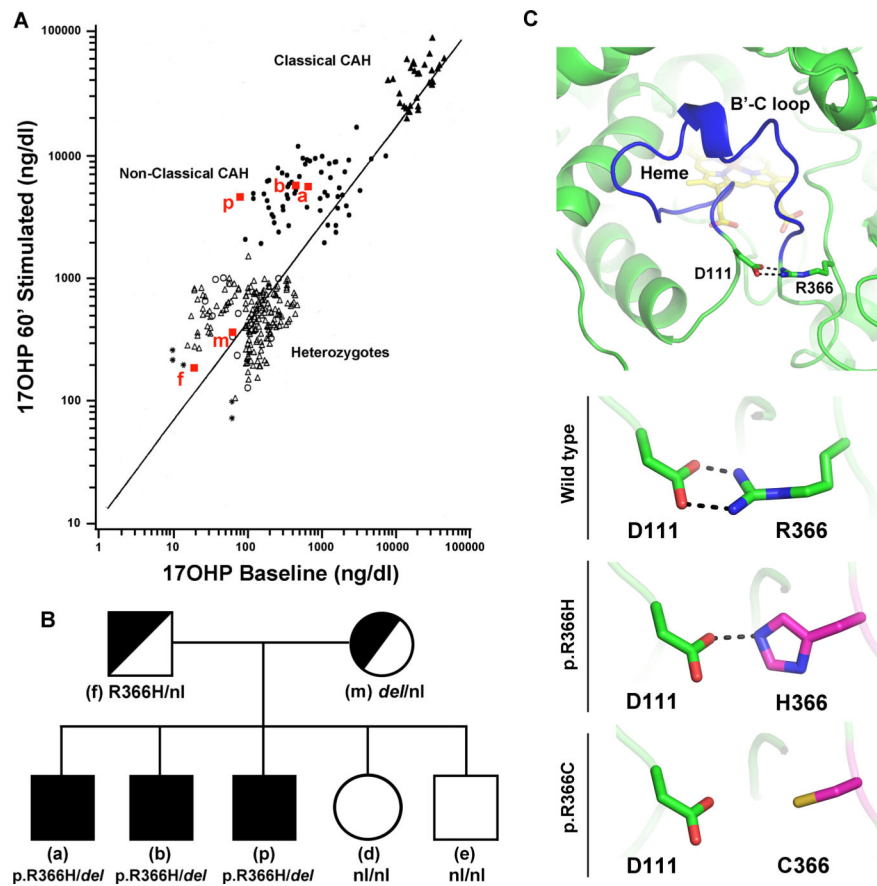
Figure 1.

A rare p.R366H mutation in *CYP21A2* in a kindred with congenital adrenal hyperplasia. An established nomogram showing basal and stimulated serum 17-hydroxyprogesterone levels, with measured values in proband (p), and his male siblings (a and b) indicative of non-classical congenital adrenal hyperplasia **(A)**. Family pedigree shows *CYP21A2* genotypes, as determined by Sanger sequencing **(B)**. Mapping of the newly discovered p.R366H mutation to our humanized model of wild-type CYP21A2 **(C)**.[3] By forming a salt-bridge with D111, R366 (β4 sheet) holds the B'-helix in position that is implicated in gating (highlighted in blue). The p.R366H mutation results in the loss of one of two hydrogen bonds (dashed lines), thus weakening, but not abolishing the ion-pair interaction. In contrast, a loss of both hydrogen bonds in the p.R366C mutation disrupts enzyme stability to cause salt wasting disease.[3]

Table 1

Summary of family

	Father (f)	Mother (m)	Brother (a)	Brother (b)	Proband (p)	Sister (d)	Brother (e)	Unrelated (z)
Current age (years)	46	46	16	14	12	9	7	20
Symptoms	None	None	Short	None	Precocious puberty and rapid growth	None	None	Acne, normal external genitalia
Puberty onset (years)	14	12 (menarche)	13	11	8	N/A	N/A	12 (menarche)
Bone age/chronological age (years)	N/A	N/A	14.1/15.1 (delayed)	13.7/13.2	12.5–13/9.3 (advanced)	N/A	N/A	N/A
Current height (cm) (%ile)	186 (90th)	155.2 (10th)	162.5 (5th)	173.2 (77th)	149.3 (39th)	131 (28th)	116 (11th)	162.5 (50th)
Z score (SDs)	+1.29	−1.25	−1.6	+0.74	−0.3	−0.57	−1.22	−0.03
Mid-parental target height (cm) (%ile)	N/A	N/A	177.1 (50th)	177.1 (50th)	177.1 (50th)	164.1 (50th)	177.1 (50th)	161 (25–50th)
Predicted height (cm) (%ile) [c]	N/A	N/A	169–172 (10–25th)	181.6 (75th)	160–165 (<5th)	N/A	N/A	N/A
Baseline 17-OHP (ng/dL)	19	61	649	419	79	13[a]	11[a]	3840[b] (at age 10)
Cosyntropin stimulated 17OHP (ng/dL)	198	375	5500	5780	4700	N/A	N/A	N/A
***CYP21A2* sequencing**	p.R366H/nl	de/nl	p.R366H/del	p.R366H/del	p.R366H/del	nl/nl	nl/nl	p.I172N/p.R366H

N/A: not available

[a] Normal range for Tanner I (age <9.2 years) = less than 83 ng/dL

[b] Normal range for Tanner II (age 9.2–13.7 years) = 11–98 ng/Dl

[c] Height prediction according to Gruelich and Pyle

Table 2

Oligonucleotides used for *CYP21A2* genetic analysis

Primer	Sequence
779F	5′-AGGTGGGCTGTTTTCCTTTCA-3′
Tena32F	5′-CTGTGCCTGGCTATAGCAAGC-3′
ex6a	5′-AGCTGCATCTCCACGATGTGA-3′
ex3s	5′-CGGACCTGTCCTTGGGAGACTAC-3′
ex10a	5′-GGAGCAATAAAGGAGAAAC-3′
1399F	5′-GATGTGGAACCAGAAAGCTG-3′
in2a	5′-CCTGGGCAACATAGCAAGA-3′
in4a	5′-AGGACAAGGAGAGGCTCA-3′
in4s	5′-TGCAGCATCATCTGTTACCT-3′
in6s	5′-ACTCTGTACTCCTCTCCCCA-3′
in8s	5′-ATGAGTGAGGAAAGCCCGA-3′

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