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CLINICAL REPORT

Congenital adrenal hyperplasia due to two rare *CYP21A2* variant alleles, including a novel attenuated *CYP21A1P*/*CYP21A2* chimera

Qizong Lao[1] | **Deepika D. Burkardt**[1] | **Sarah Kollender**[1] | **Fabio R. Faucz**[2] | **Deborah P. Merke**[1,2]

[1]National Institutes of Health Clinical Center, Bethesda, Maryland, USA

[2]The Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA

Correspondence
Deborah P. Merke, National Institutes of Health Clinical Center, 10 Center Drive, Room 3-2740, Bethesda, MD 20892-1932, USA.
Email: dmerke@nih.gov

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Abstract

Background: Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase (21OH) deficiency is an autosomal recessive inborn error of cortisol biosynthesis, with varying degrees of aldosterone production. There is a continuum of phenotypes which generally correlate with genotype and the expected residual 21OH activity of the less severely impaired allele. *CYP21A1P/CYP21A2* chimeric genes caused by recombination between *CYP21A2* and its highly homologous *CYP21A1P* pseudogene are common in CAH and typically associated with salt-wasting CAH, the most severe form. Nine chimeras have been described (CH-1 to CH-9).

Aims: The aim of this study was to genetically evaluate two variant alleles carried by a 22-year-old female with the non-salt-wasting simple virilizing form of CAH and biallelic 30-kb deletions.

Methods: The haplotypes of the *CYP21A2* heterozygous variants, as well as the chimeric junction sites, were determined by Sanger sequencing TA clones of an allele-specific PCR product.

Results: Genetic testing revealed two rare *CYP21A1P/CYP21A2* chimeras: allele 1 matches the previously described CAH CH-1 chimera but without the P30L variant, and allele 2, termed here as novel CAH CH-10, has a junction site between c.293-37 and c.29314, which is expected to retain partial 21OH activity.

Conclusion: These two variant alleles further document the complex nature of RCCX modules and highlight that not all *CYP21A1P/CYP21A2* chimera severely impair 21OH activity.

KEYWORDS

21-hydroxylase deficiency, CAH, chimera, congenital adrenal hyperplasia, *CYP21A2*

1 | INTRODUCTION

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OHD, OMIM 201910) caused by *CYP21A2* defects (OMIM 613815) is an autosomal recessive inborn error of cortisol biosynthesis, with varying degrees of aldosterone production and corticotropin-driven adrenal androgen excess (Merke & Auchus, 2020). The majority of individuals are compound heterozygotes. The phenotype is determined by the less severely impaired *CYP21A2* allele and the expected residual 21OH activity, with some phenotypic variability described in large cohort studies (Finkielstain et al., 2011; Gurgov et al., 2017; Krone et al., 2000). The phenotypic spectrum is traditionally categorized as classic salt-wasting (SW), with marked deficiencies in both cortisol and aldosterone and life-threatening if left untreated; classic simple virilizing (SV), with cortisol deficiency and minimal aldosterone production; and the nonclassic (NC) phenotype with mild manifestations ranging from asymptomatic to late-onset hyperandrogenism. The SW and SV types are termed classic CAH and result in 46, XX atypical genitalia due to in utero excess androgens. Classic CAH is estimated to affect 1 in 15,000; NC CAH is estimated to affect 1 in 200 (Hannah-Shmouni et al., 2017).

CYP21A2 encoding 21OH is mapped to chromosome 6p23.1 in a low copy repeat region termed *RCCX* with homologous genes in tandem: *RP1* (*STK19*) and pseudogene *RP2* (*STK19P*); *C4A* and *C4B*; *CYP21A2* and pseudogene *CYP21A1P*; *TNXB* and pseudogene *TNXA*. These gene pairs are error-prone during meiosis with the most common pathogenic outcome CAH. Minor conversions from *CYP21A1P* to *CYP21A2* are responsible for approximately 60% of CAH alleles; chimeric genes, mostly termed "30-kb deletions," together with major conversions, account for 30% (Merke & Auchus, 2020). 30-kb deletions are further categorized based on junction sites into nine *CYP21A1P/CYP21A2* chimeras (CAH CH-1 to CH-9) (Figure 1) and three *CYP21A1P-TNXA/TNXB* chimeras with deletion of the entire *CYP21A2* extending to the neighboring *TNXB* (CAH-X CH-1 to CH-3) (Finkielstain et al., 2011; Merke & Auchus, 2020). Junction site locations can influence gene functionality and the degree of 21OH impairment (Keen-Kim et al., 2005). Furthermore, the monoallelic presence of a CAH-X chimera disrupts both *CYP21A2* and *TNXB* which results in hypermobility-type Ehlers-Danlos syndrome (Merke et al., 2013). The vast majority of "30-kb deletions," including seven of the nine CAH chimeras and all three CAH-X chimeras, are null variants associated with a SW phenotype. To date, two CAH chimeras (CH-4 and CH-9) due to the location of their junction sites correspond to a phenotype which straddles the classic SV–NC boundary and are named attenuated CAH chimeras (Chen et al., 2012).

Here we report a case of 21-OHD SV CAH due to biallelic 30-kb deletions. We identified two rare CAH chimeric genes including a novel attenuated chimera featuring a new junction site. Our findings further document the variability and complexity of *CYP21A2* defects causative for 21-OHD CAH.

2 | MATERIALS AND METHODS

2.1 | Participants

The subject was enrolled in an ongoing Natural History Study at the NIH Clinical Center in Bethesda, MD, USA (NCT00250159) and gave written informed consent.

2.2 | Genetic analyses

CAH genotyping was initially conducted by CLIA-accredited laboratories with an assay-based multiplex mini-sequencing and conversion-specific PCR (MMCP) (Esoterix Laboratory Services, Inc), (Keen-Kim et al., 2005) and a comprehensive test based on Sanger methodologies (PreventionGenetics LLC). The haplotypes of the *CYP21A2* heterozygous variants, as well as the chimeric junction sites, were then determined by Sanger sequencing TA clones of an allele-specific PCR product with primers CYP779f/Tena32f (Chen et al., 2012) The allele-specific PCR, *CYP21A2* duplication test, and Sanger sequencing were conducted as previously described (Xu et al., 2013). The TA cloning was conducted with a TOPO XL complete PCR cloning kit (Thermo Fisher Scientific). References for *CYP21A2* and *CYP21A1P* were ENSG00000231852.2 and ENSG00000204338.4, respectively. CAH pathogenic variants on *CYP21A2* were presented in common names with Human Genome Variation Society nomenclature shown after their first appearance. A DNASTAR Lasergene software (version 17) was used for sequence analysis.

3 | RESULTS

3.1 | Clinical findings

A 22-year-old Haitian female presented with atypical genitalia at birth. She underwent clitoroplasty at age 3 years, and was treated with prednisone and fludrocortisone until age 10 years, after which she was off medications with limited access to care due to a poor social environment. She started shaving her face around age 13 years and had no

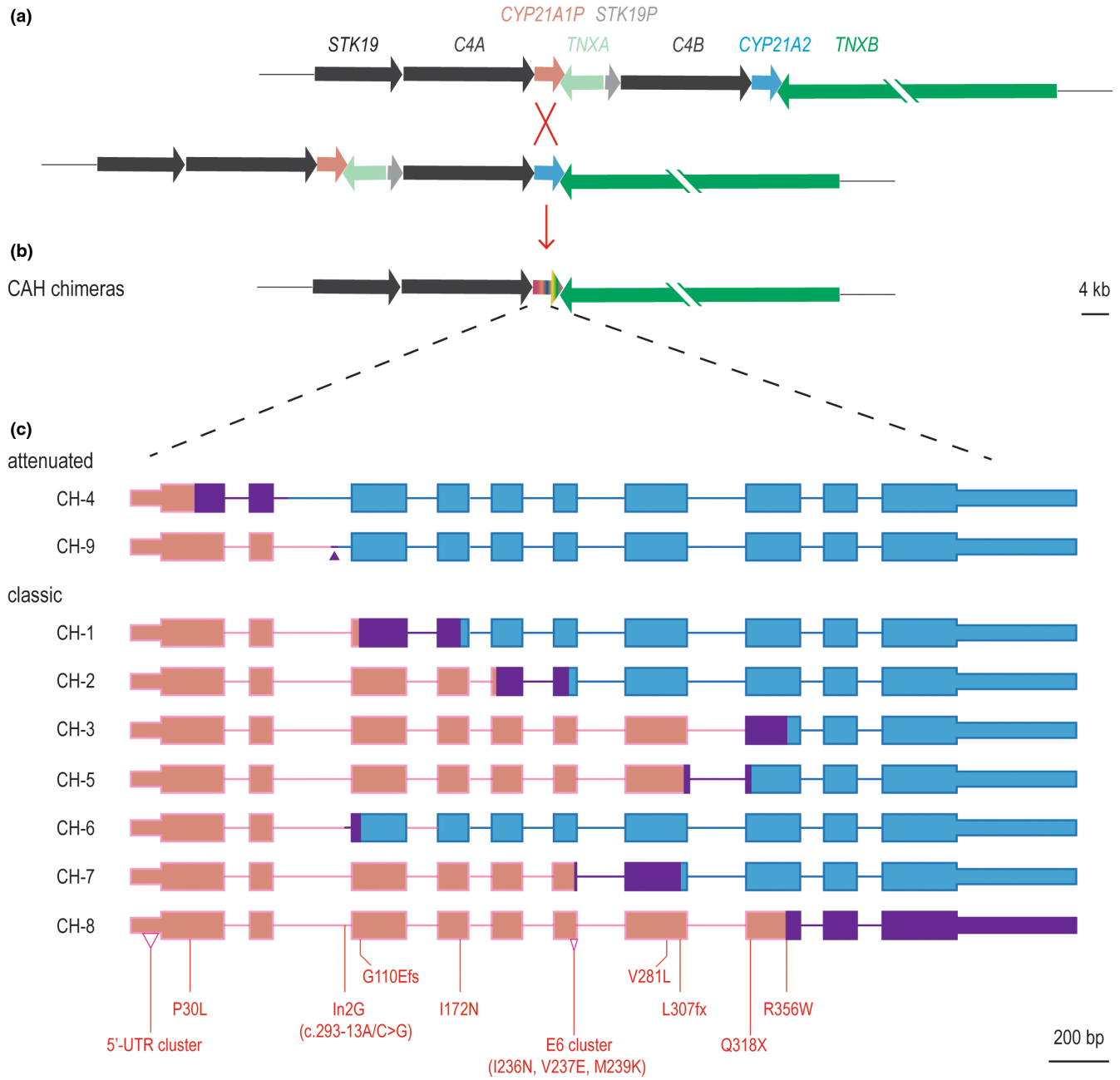


FIGURE 1 *CYP21* chimeras and their junction sites. (a) Unequal crossover between *CYP21A1P* and *CYP21A2* during meiosis, pseudogenes are shown in muted colors. (b) CAH chimeras, shown in chameleon as the unequal crossover outcome. (c) Schematic of all previously known CAH chimeras: gene body of *CYP21A1P*-origin and *CYP21A2*-origin are in pink and blue, respectively, and junction sites are in purple. The junction site of CAH CH-9, which is only 7 bp in size, is marked with a purple triangle. The variants of *CYP21A1P*-origin commonly used to determine the CAH chimera subtype are marked on CAH CH-8, which carries all of these variants (Chen et al., 2012). Genes are shown in scale except *TNXB*, whose size is 68 kb.

breast development and primary amenorrhea. At 21 years, she presented to a community hospital twice with reported possible adrenal crises in the setting of infection and inflammation. Physical examination revealed normal vital signs, hirsutism of the face and trunk, a deep voice, broad shoulders, Tanner 1 breasts, Tanner 5 public hair, malar rash. She was started on dexamethasone 0.5 mg and fludrocortisone 0.1 mg daily. Rheumatological evaluation confirmed a diagnosis of systemic lupus erythematosus (SLE) with constitutional symptoms, serositis (pericarditis), Raynaud's of the hands and feet, positive serology (ANA, anti-ENA, anti-SmRNP antibody, anti-Smith [SM] antibody), low complement, and lymphopenia. Family history reported three unaffected older siblings and a younger brother with CAH who was positive on newborn screening but not on hormone replacement and had never

had an adrenal crisis. Due to social reasons, acquisition of genotyping and phenotypic data for family members was not available. At our Institution, early morning laboratory evaluation showed 17-OHP 32,760 ng/dL, androstenedione 5315 ng/dL [ref 17–175 ng/dL], testosterone 178 ng/dL [ref 8–60 ng/dL], cortisol 3 μg/dL [ref 5–25 μg /dL], and plasma renin activity within the normal range.

3.2 | Genetic findings

The subject had two indeterminate 21-OHD genetic tests from commercial laboratories. Family genotyping was indicated but unavailable. A MMCP-based test suggested biallelic 30-kb deletions. Sanger sequencing of CYP779f/Tena32f PCR product detected a cluster of pre-coding variants of *CYP21A1P* origin (c.-210T>C, c.-199C>T, c.-190dup, c.-126C>T, c.-113G>A, c.-110T>C and c.-103A>G), all in homozygous status supporting biallelic 30 kb deletion. Compound heterozygous variants of *CYP21A1P* origin were also detected, including P30L (c.92C>T, p.Pro31Leu), In2G (c.293-13A/C>G) and G110Efs (c.332_339del, p.Gly111Valfs*21). The heterozygous status of the compound variants, especially that of P30L, did not support the existence of biallelic 30-kb deletions.

We conducted TA cloning to differentiate the two alleles of the CYP779f/Tena32f PCR product. Plasmids carrying each allele were subject to Sanger sequencing. Eight plasmid clones were analyzed and two CAH chimera alleles (haplotypes) were detected. A *CYP21A2* duplication test with negative result indicated these two chimeras were responsible for the subject's CAH phenotype (data not shown). Allele 1 sequence matched chimera CAH CH-1 except for the missing the P30L variant; allele 2 had all variants of *CYP21A1P* origin spanning from the pre-coding region till c.293-38A of intron 2, only 24 bp upstream of c.293-13A/C where the In2G (c.293-13A/C>G) variant occurs (Figure 2a,b).

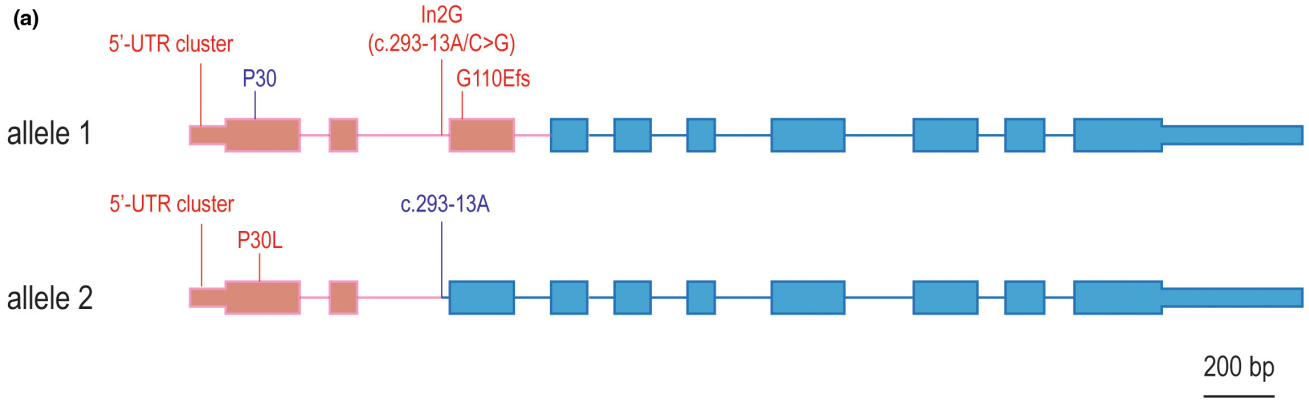
4 | DISCUSSION

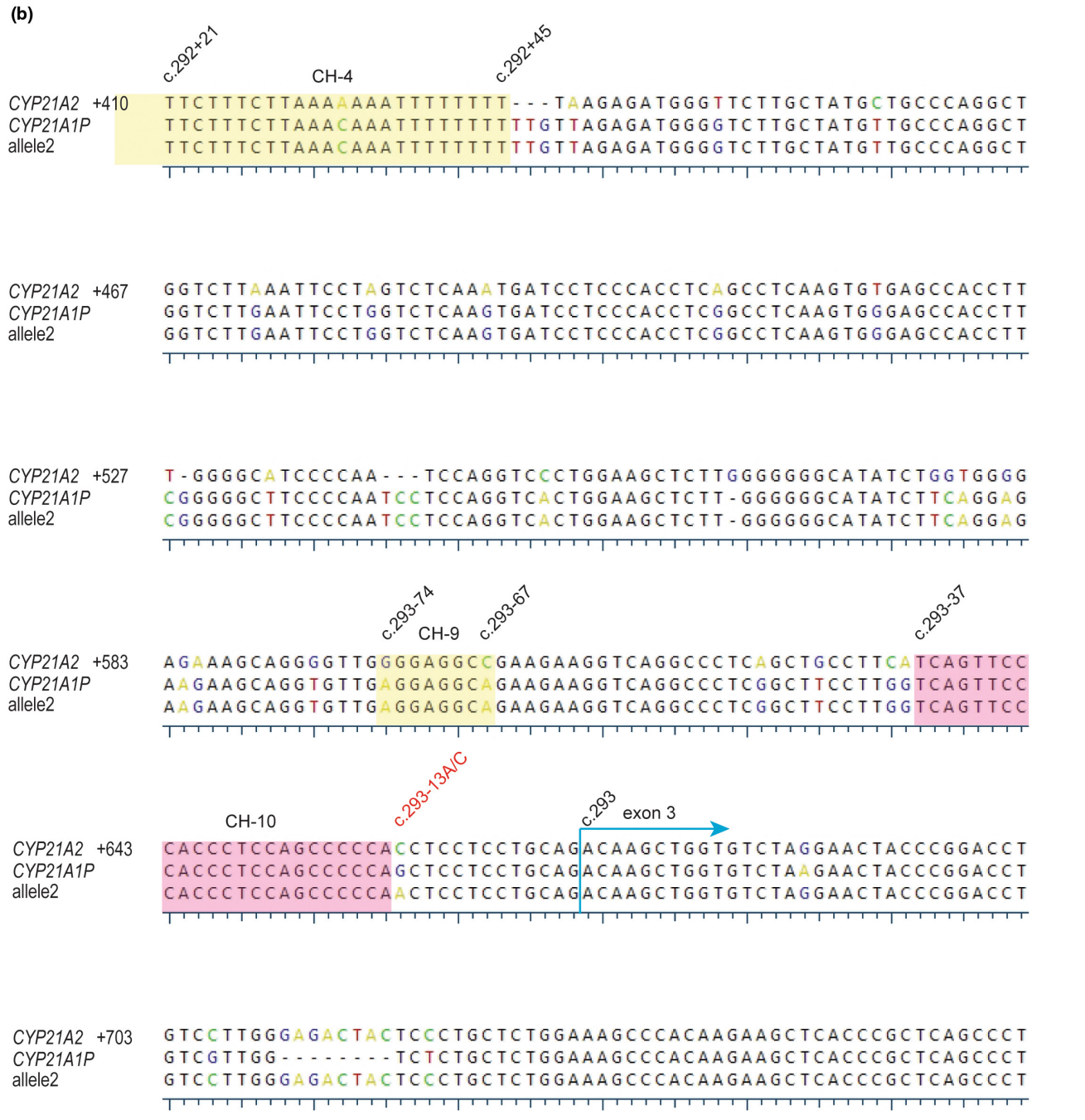
We describe a case of 21-OHD CAH due to two rare *CYP21A1P/CYP21A2* chimeras including a classic CAH CH-1 chimera without the usual P30L variant, and a novel attenuated chimera termed here CAH CH-10 with a junction site between c.293-37 and c.293-14 upstream of In2G. The subject's unexpected SV phenotype was therefore explained by her genotype. SLE was not entirely unexpected, as 21-OHD has been associated with an increased prevalence of autoimmune disorders (Falhammar et al., 2019). Our findings further document the complex nature of the *RCCX* module (Carrozza et al., 2021; Miller, 2020; Pignatelli et al., 2019; Tolba et al., 2022) and highlight that not all *CYP21A1P/CYP21A2* chimera severely impair 21OH activity.

Although there are limited studies of the *RCCX* pseudogenes, *CYP21A2*-like variants are commonly found in *CYP21A1P*, with those corresponding to P30, V281 accounting for 24% and 21% of total *CYP21A1P* alleles respectively (Tsai & Lee, 2012). Therefore, if *CYP21A2*-like *CYP21A1P* is involved in unequal crossovers, the resulting chimeras could sometimes be missing P30L and V281L (c.844G>T, p.Val282Leu). In fact, a common chimera CAH CH-5 which has all variants of pseudogene origin spanning from the pre-coding region to the L307fs (c.923dup, p.Leu308Phefs*6) variant is often missing V281L (Chen et al., 2012). However, CAH chimeras missing P30L have been rarely reported. Our large U.S. cohort of approximately 500 subjects has only one additional family carrying a CAH chimera without P30L (Finkielstain et al., 2011). A report from Brazil described an individual with 21-OHD carrying a CAH chimera without P30L, but the chimera type was not specified (Coeli et al., 2010).

Unlike classic CAH chimeras that null the corresponding *CYP21A2* allele, attenuated chimeras correspond mostly to a SV phenotype (Chen et al., 2012), or sometimes a phenotype with severity between NC and SV, such as a NC form of early onset (l'Allemand et al., 2000). Two attenuated chimeras, CH-4 and CH-9, have been reported with junction sites between c.138 and c.292+45 spanning from exon 1 to intron 2, and between c.293-74 and c.293-67 inside intron2, respectively. Both chimeras feature a pseudogene 5'-UTR and a P30L variant as the phenotype determinant. Since the *CYP21A1P* promoter is known to be weaker compared to that of *CYP21A2*, the usual phenotype associated with an attenuated chimera is classic SV CAH, more severe than the NC phenotype typically associated with

FIGURE 2 (a) Schematic of two rare *CYP21A1P/CYP21A2* chimeras: allele 1 is a classic CAH CH-1 but without P30L, allele 2 is a novel attenuated CAH chimera termed CH-10 with junction site between c.293-37 and c.293-14. Pink and blue colors represent *CYP21A1P* and *CYP21A2* sequence, respectively, shown in scale. (b) Alignment of *CYP21* genes at a locus corresponding to *CYP21A2* intron 2 and exon 3, *CYP21A2* served as the reference and its numbering is shown at the beginning of each row. Conserved DNA residues are shown in black while non-conserved are shown in an UGene physiochemical scheme. The junction sites of known attenuated chimeras CH-4 and CH-9 are in yellow, and the novel CH-10 is in pink. Dashed lines denote gaps. Remarkable DNA residues are marked on the top with their respective cDNA nomenclature numberings, including c.293 to start exon 3 and c.293-13A/C (in red) where In2G (c.293-13A/C>G) occurs.


(a)
5'-UTR cluster
P30
In2G
(c.293-13A/C>G)
G110Efs
allele 1
5'-UTR cluster
P30L
c.293-13A
allele 2
200 bp


(b)
c.292+21
CH-4
c.292+45
CYP21A2 +410
CYP21A1P
allele2
TTCTTTCTTAAAAAAATTTTTTTT---TAAGAGATGGGTTCTTGCTATGCTGCCCAGGCT
TTCTTTCTTAAACAAATTTTTTTTTTGTTAGAGATGGGGTCTTGCTATGTTGCCCAGGCT
TTCTTTCTTAAACAAATTTTTTTTTTGTTAGAGATGGGGTCTTGCTATGTTGCCCAGGCT
CYP21A2 +467
CYP21A1P
allele2
GGTCTTAAATTCCTAGTCTCAAATGATCCTCCCACCTCAGCCTCAAGTGTGAGCCACCTT
GGTCTTGAATTCCTGGTCTCAAGTGATCCTCCCACCTCGGCCTCAAGTGGGAGCCACCTT
GGTCTTGAATTCCTGGTCTCAAGTGATCCTCCCACCTCGGCCTCAAGTGGGAGCCACCTT
CYP21A2 +527
CYP21A1P
allele2
T-GGGGCATCCCCAA---TCCAGGTCCCTGGAAGCTCTTGGGGGGGCATATCTGGTGGGG
CGGGGGCTTCCCCAATCCTCCAGGTCACTGGAAGCTCTT-GGGGGGCATATCTTCAGGAG
CGGGGGCTTCCCCAATCCTCCAGGTCACTGGAAGCTCTT-GGGGGGCATATCTTCAGGAG
c.293-74
CH-9
c.293-67
c.293-37
CYP21A2 +583
CYP21A1P
allele2
AGAAAGCAGGGGTTGGGGAGGCCGAAGAAGGTCAGGCCCTCAGCTGCCTTCATCAGTTCC
AAGAAGCAGGTGTTGAGGAGGCAGAAGAAGGTCAGGCCCTCGGCTTCCTTGGTCAGTTCC
AAGAAGCAGGTGTTGAGGAGGCAGAAGAAGGTCAGGCCCTCGGCTTCCTTGGTCAGTTCC
CH-10
c.293-13A/C
c.293
exon 3
CYP21A2 +643
CYP21A1P
allele2
CACCCTCCAGCCCCCACCTCCTCCTGCAGACAAGCTGGTGTCTAGGAACTACCCGGACCT
CACCCTCCAGCCCCCAGCTCCTCCTGCAGACAAGCTGGTGTCTAAGAACTACCCGGACCT
CACCCTCCAGCCCCCAACTCCTCCTGCAGACAAGCTGGTGTCTAGGAACTACCCGGACCT
CYP21A2 +703
CYP21A1P
allele2
GTCCTTGGGAGACTACTCCCTGCTCTGGAAAGCCCACAAGAAGCTCACCCGCTCAGCCCT
GTCGTTGG--------TCTCTGCTCTGGAAAGCCCACAAGAAGCTCACCCGCTCAGCCCT
GTCCTTGGGAGACTACTCCCTGCTCTGGAAAGCCCACAAGAAGCTCACCCGCTCAGCCCT

P30L. To date, chimeric *CYP21A1P/CYP21A2* genes are termed chronologically after determination of the junction sites (Chen et al., 2012). Here we define and name CH-10. It is commonly accepted that newly identified junction sites of *CYP21A1P/CYP21A2* chimeras based on an array of variants of pseudogene origin define a new chimeric gene, but it is possible that some of these chimeras are formed by minor conversion of a previously described chimera, rather than a deletion or major conversion event. However, the likelihood of this mechanism should be very low since there are no reports of minor conversion events between *CYP21A1P/CYP21A2* chimera types.

Our data suggest that the junction site of attenuated chimeras can be anywhere spanning from c.138 (found in CH-4) to c.293-14 (found in CH-10) just upstream of the In2G site. These junction sites might have been historically under-detected because sequencing analysis of TA clones is often needed to overcome the frequent insert-deletion interference inside intron 2 in a Sanger assay. Attenuated CAH chimeras, previously considered rare, might be under-detected by current *CYP21A2* testing which commonly excludes the 5'-UTR and thus can easily confuse an attenuated chimera with a P30L variant (Baumgartner-Parzer et al., 2020), which is sometimes unexplainably associated with a classic phenotype (Araujo et al., 2005; Finkielstain et al., 2011). A CAH genetic platform that includes the promoter might offer an explanation for these genotype–phenotype discrepancies. Given that all attenuated *CYP21A1P/CYP21A2* chimeras identified to date include a *CYP21A1P* 5'-UTR, P30L variant, and a junction site between P30L and c.293-13A/G (absent of In2G), a unified methodological approach should be incorporated into CAH genetic testing. Moreover, our results reinforce that the presence of P30L and the absence of In2G correlates with the attenuated phenotype. Finally, our study agrees with a previous conclusion that Sanger sequencing of CYP779f/Tena32f PCR products offers the most comprehensive genetic test of *CYP21A2* in terms of testing and profiling chimeras (Chen et al., 2012).

AUTHOR CONTRIBUTIONS

Qizong Lao and Deborah P. Merke: Conceptualization. **Qizong Lao and Deepika D. Burkardt**: Methodology. **Deborah P. Merke**: Resources. **Qizong Lao, Sarah Kollender, and Fabio R. Faucz**: Data curation. **Qizong Lao, Deepika D. Burkardt, and Sarah Kollender**: Writing-original draft. **Deborah P. Merke**: Writing-review & editing. **Qizong Lao**: Visualization. **Deborah P. Merke**: Supervision. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

D.P.M. received unrelated research funds from Diurnal Limited and Neurocrine Biosciences through the NIH Cooperative Research and Development Agreement. The authors declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available in the article.

ETHICS STATEMENT

All procedures were in accordance with good clinical practice and ethical standards. This study was approved by the National Institutes of Health IRB.

ORCID

Qizong Lao https://orcid.org/0000-0003-1816-134X
Deepika D. Burkardt https://orcid.org/0000-0002-1743-7503
Sarah Kollender https://orcid.org/0009-0001-6114-6885
Fabio R. Faucz https://orcid.org/0000-0001-7959-9842
Deborah P. Merke https://orcid.org/0000-0002-3746-0460

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