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Case Report

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Nonvirilized Genitalia in 3 Female Newborns With the Salt-Wasting Congenital Adrenal Hyperplasia Phenotype

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Abbreviations: 11β-HSD-2, 11β hydroxysteroid dehydrogenase type 2; 17OHP, 17-hydroxyprogesterone; ACTH, adrenocorticotropin; AR, androgen receptor; CAH, congenital adrenal hyperplasia; D4A, androstenedione; DOL, day of life; HPA, hypothalamic-pituitary-adrenal; NBS, newborn screening; NC, nonclassic; PRA, plasma renin activity; SV, simple-virilizing; SW salt-wasting; wpc, weeks post conception.

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Abstract

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency, a form of primary adrenal insufficiency characterized by impaired cortisol secretion and elevated androgen production, is the leading cause of atypical genitalia in the female newborn. Females with classic CAH, either salt-wasting or simple-virilizing form, usually present at birth with atypical genitalia ranging from clitoromegaly to male-appearing genitalia, due to in utero to elevated androgens (androstenedione and testosterone). Females with mild nonclassic CAH usually present with typical genitalia. Proving the importance of always keeping an open mind for exceptions to the rule, we report on 3 female newborns who presented with the nonvirilized genitalia, salt-wasting CAH phenotype and genotype most consistent with simple-virilizing CAH. It is only through a positive newborn screen identifying the females with CAH that they were diagnosed before developing adrenal and/or salt-wasting crisis.

Key Words: congenital adrenal hyperplasia, newborn screening, genitalia, virilization, genotype/phenotype

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Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is a form of primary adrenal insufficiency characterized by impaired cortisol secretion and elevated androgen production. Depending on the pathogenic variants in the *CYP21A2* gene and degree of residual enzymatic activity, patients are classified in 1 of the 2 classic (severe) forms, salt-wasting (SW) and simple-virilizing (SV), or the milder nonclassic (NC) form. Severe *CYP21A2* genotypes composed of pathogenic variants causing null or less than 1% enzyme activity show an excellent correlation with the SW-CAH phenotype. However, there is variability associated with less severe *CYP21A2* pathogenic variants causing 1% to 2% enzyme residual activity (SV-CAH phenotype) [1-6].

Classic CAH is the leading cause of atypical genitalia in the female newborn, with presentation ranging from mild clitoromegaly (Prader stage 1) to male-appearing genitalia (Prader stage 5) that can lead to incorrect sex assignment. Virilization of the external genitalia of female newborns with classic CAH starts in utero due to excess production of adrenal androgens, such as androstenedione (D4A) and testosterone, during the critical period of sex differentiation, 8 to 12 weeks post conception (8-wpc). Depending on the degree of virilization, females with classic CAH may undergo multiple genital surgeries.

It is generally accepted that females with the classic form of CAH will present at birth with some degree of atypical genitalia that triggers evaluation and diagnosis of CAH, most often even before the newborn screening (NBS) for CAH results are made available. We report on 3 cases of female newborns with the SW-CAH phenotype whose genitalia at birth were not virilized and whose positive NBS for CAH led to their diagnosis. These 3 cases highlight the importance of NBS for CAH, as they would have been missed if it were not for NBS for CAH. It also underscores the importance of immediate referral of females with positive NBS for CAH for further evaluation of possible classic CAH even in the absence of atypical genitalia.

Case 1

A female infant was born full term via normal spontaneous vaginal delivery with no complications. She had normal female external genitalia and no episodes of hypoglycemia in the neonatal period. Family history was negative for CAH or early infant death.

On day of life 5 (DOL 5) her family was notified that her NBS, collected between 24 to 36 hours after birth, was positive for CAH (second-tier 17-hydroxyprogesterone (17OHP) was elevated at 31.5 ng/mL). Based on the positive NBS, she was evaluated at the pediatric emergency department, where she had no signs of adrenal and/or SW crisis. Her sodium was 142 mmol/L (range, 133-146 mmol/L), her potassium was 4.8 mmol/L (range, 3.2-6.0 mmol/L), aldosterone was 23.4 ng/dL (range, 6-179 ng/dL), and plasma renin activity (PRA) was 23 ng/mL/hour (range, 2-35 ng/mL/hour). Her adrenal steroid profile obtained prior to initiating hydrocortisone therapy showed mildly elevated 17OHP at 974 ng/dL (< 110 ng/dL) and D4A at 127 ng/dL (< 51 ng/dL). Remaining adrenal steroids were within the acceptable range: 17-hydroxypregnenolone at 1140 ng/dL (range, 229-3104 ng/dL), testosterone at 12 ng/dL (< 7-20 ng/dL), and deoxycortisol at 97 ng/dL (< 344 ng/dL). Her external genitalia examination at the emergency department reported 2 separate openings, vaginal and urethral, and edema of the clitoral hood. She was started on hydrocortisone (daily dose 16 mg/m^2/day), salt supplementation (4 mEq/kg/day) and fludrocortisone (0.1 mg/day).

On DOL 12 she was seen at the University of Minnesota Masonic Children's Hospital multidisciplinary CAH clinic. External genitalia examination revealed no clitoromegaly and no hyperpigmentation or posterior fusion of the labia majora. Based on her slightly elevated 17OHP concentration obtained on DOL 5 and her normal genital exam on DOL 12, she was presumed to have NC-CAH and fludrocortisone and salt-supplementation were discontinued. Full sequencing of the *CYP21A2* gene was performed at Mayo Clinic (polymerase chain reaction amplification followed by DNA sequence analysis and gene dosage analysis by multiplex ligation-dependent probe amplification).

On DOL 19, results of her adrenal steroid concentrations measurements obtained on DOL 12 became available and were notable for elevated 17OHP at 12 000 ng/dL (< 110 ng/dL), D4A at 236 ng/dL (< 51 ng/dL), and PRA at 96 ng/mL/hour (range, 2-35 ng/mL/hour), consistent with SW-CAH. Her sodium of 138 mmol/L (range, 133-146 mmol/L) and potassium of 4.8 mmol/L (range, 3.4-5.3 mmol/L) were normal. However, her molecular testing results revealed that she was a compound heterozygote of 2 pathogenic variants (g. 655A/C > G aka intron 2 splice mutation and g. 999T > A aka p.I172N), which are consistent with SV-CAH. The parental studies showed the mother to be a carrier of g. 999T > A aka p.I172N and the father of the g. 655A/C > G aka intron 2 splice mutation. Owing to the patient's SW, she was restarted on fludrocortisone and salt supplementation.

Currently she is age 2 years and has no signs of external genitalia virilization including clitoromegaly.

Case 2

A female infant was born full term via normal spontaneous vaginal delivery with no complications. She had normal

female external genitalia and no episodes of hypoglycemia in the neonatal period. Her family history was negative for CAH or early infant death. Parents were notified on DOL 5 that the newborn screen (taken between 24 and 36 hours) was positive for CAH, and she was evaluated at an outside hospital emergency department. Her sodium level was normal at 140 mmol/L (range, 133-146 mmol/L) with elevated potassium at 6.6 mmol/L (range, 3.2-6.0 mmol/L). Repeat 17OHP was elevated at 3150 ng/dL (< 110 ng/dL).

On DOL 6, laboratory results showed sodium at 135 mmol/L (range, 133-146 mmol/L), potassium at 5.8 mmol/L (range, 3.2-6.0 mmol/L), and elevated PRA at 100 mg/mL/hour (range, 2-35 ng/mL/hour), consistent with SW-CAH. Electrolytes were repeated daily and on DOL 8 the patient's sodium decreased to 132 mmol/L (range, 133-146 mmol/L) and she was started on hydrocortisone (daily dose 18 mg/m^2/day), fludrocortisone (0.1 mg/day), and salt supplementation (3meq/kg/day). Molecular testing of the *CYP21A2* gene showed that she was compound heterozygote of 2 pathogenic variants (30 kb deletion and g. 999T > A aka p.I172N), consistent with SV-CAH. Full *CYP21A2* sequencing was performed at Mayo Clinic as described in case 1. Parental studies were not performed because of insurance. The genotype suggested SV-CAH phenotype; however, because of her SW, she was continued on hydrocortisone, fludrocortisone, and salt supplementation.

The patient was followed by a pediatric endocrinology provider at another hospital until she presented at the University of Minnesota Masonic Children's Hospital multidisciplinary CAH clinic at age 14 months for further evaluation. An external genitalia examination revealed no clitoromegaly and no hyperpigmentation or posterior fusion of the labia majora. At the time of this report she is age 6 years and has no signs of external genitalia virilization, including clitoromegaly.

Case 3

This patient was born full term following normal spontaneous vaginal delivery and was discharged home on DOL 2. Her neonatal course was uncomplicated apart from mild jaundice that self-resolved, and newborn examination revealed normal-appearing external genitalia without evidence of hyperpigmentation or clitoromegaly. Her initial NBS for CAH showed elevated 17OHP (48.5 ng/mL), which remained elevated (89 ng/mL) on repeat screening on DOL 7. She was followed as an outpatient by her pediatrician and was noted to have regained her birth weight with normal feeding patterns. Following the repeat abnormal NBS result, her sodium was measured at 135 mmol/L (range, 132-144 mmol/L) and she was referred to a pediatric endocrinology clinic on DOL 15. There was no reported family history of CAH or early infant death. Her physical exam appeared grossly normal on initial visual inspection, without obvious clitoromegaly, and with a normal-appearing vaginal opening and urethral meatus. Further measurements revealed that the clitoral length was 1 cm, clitoral width was 0.8 cm, anus-fourchette distance was 2.1 cm and anus-clitoris distance was 4 cm. The anogenital ratio (anus-fourchette distance/anus-clitoris distance) was 0.525, suggesting possible mild posterior labial fusion because of the greater than 0.5 value [7]. Pelvic ultrasound demonstrated a uterus but ovaries were not visualized. Laboratory evaluation on DOL 15 to 16 showed a lower sodium concentration at 132 mmol/L (range, 132-144 mmol/L) and hyperkalemia (> 6 on heel stick and 6.4 mmol/L on repeat, range 3.4-6.2 mmol/L) and PRA of 67.65 ng/mL/hour (range, 0.2-5.82 ng/mL/hour), suggestive of SW. Testosterone was elevated at 26 ng/dL (range, 2-10 ng/dL). Cosyntropin stimulation test performed on DOL 16 (125 µg) showed a baseline cortisol of 1.5 µg/mL and peak cortisol of 6.5 µg/mL (≥ 18 µg/dL) at 60 minutes, and baseline 17OHP of 1135 ng/dL and peak 17OHP of 18 240 ng/dL (< 107 ng/dL), confirming classic CAH. The patient was started on hydrocortisone (18.75 mg/m^2/day), fludrocortisone (0.1 mg twice a day), and sodium chloride tablets (2 g total daily). Common mutation panel of the *CYP21A2* gene, which was performed at Quest Diagnostics by electrophoresis, minisequencing, and polymerase chain reaction, showed that she was a compound heterozygote of 2 pathogenic variants (g.655A/C > G aka intron 2 splice mutation, and g.999T > A aka I172N), a combination most often associated with SV-CAH [4]. *CYP21A1* gene sequencing and parental studies were not performed because of high out-of-pocket cost. At age 7 months, the patient was noted to have new finding of labial adhesions (managed conservatively without medication treatment), and her clitoral measurements had reduced (clitoral length 0.5 cm, clitoral width 0.5 cm) with an anogenital ratio of 0.5. She was last evaluated at age 1 year, at which time she was continued on treatment with hydrocortisone (12.5 mg/m^2/day), fludrocortisone (0.05 mg twice a day), and sodium chloride (0.5 g/day).

Discussion

We report on 3 females with nonvirilized external genitalia, genotypes most consistent with SV-CAH, and phenotypes consistent with SW-CAH. If not for NBS identifying them with classic CAH, they would not have been diagnosed until they developed symptoms of SW and/or adrenal crisis as occurred in an affected female infant from Saudi Arabia who did not undergo NBS [8]. Similarly to our 3 cases she had

typical external genitalia but in her case she was not diagnosed until age 6 weeks, when she presented in adrenal and SW crisis (sodium of 114 mmol/L, potassium of 7.8 mmol/L, and PRA > 550 µIU/mL, range 4.2-59.7 µIU/mL) [8].

Discordance between clinical phenotypes and underlying *CYP21A2* genotypes, as seen in our patients, can be caused by either cis mutations in the promoter *CYP21A2* gene regions or unnoticed pathogenic variants of the *CYP21A2* gene as part of minor gene conversions/chimeric genes [9-11], which can further decrease the 21-hydroxylase enzyme activity resulting in more severe clinical phenotype. All 3 of our cases had the *CYP21A2* gene pathogenic variant g.999T > A aka I172N usually associated with the SV-CAH phenotype, but their clinical phenotype was more consistent with the SW-CAH phenotype, a discordance seen in other studies [3].

A cardinal feature of classic CAH in affected female newborns is prenatal virilization of the external genitalia caused by excessive adrenal androgen production during the critical period of sex differentiation (8-12 wpc). Generally, there is a tendency toward higher degrees of genital virilization in females with SW-CAH compared to females with SV-CAH with some overlap between the 2 classic forms [2-6, 12]. Variation of genital virilization has been observed even among patients within the same group of *CYP21A2* pathogenic variants [13, 14] suggesting modifying factors independent of the *CYP21A2* genotypes. Increased glucocorticoid sensitivity, increased amounts of maternal cortisol in the fetal circulation, and decreased sensitivity to androgen may be some of the mechanisms that could explain the phenotypic variability.

Specific glucocorticoid receptor haplotypes have been associated with varying glucocorticoid sensitivity, with certain haplotypes causing individuals to be more sensitive to glucocorticoids [15]. One study specifically looked at the N363S polymorphism in 200 patients with CAH [16]. Girls with CAH who were carriers of the N363S polymorphism were found to have milder genital virilization when matched to girls that shared the same *CYP21A2* genotype but did not carry the N363S polymorphism [16]. N363S polymorphism or any other glucocorticoid receptor gene polymorphisms that increase sensitivity would lead to decreased fetal hypothalamic-pituitary-adrenal (HPA) axis activity and adrenocorticotropin (ACTH) production in response to cortisol and thus lesser androgen production and variable genital virilization of the affected fetus.

During differentiation of the external genitalia, the functioning fetal HPA axis is regulated by an integrated maternal-placental-fetal steroidogenic unit [17]. Adrenal explants from 8-wpc fetuses have a robust capacity to produce cortisol, which is regulated by ACTH through a negative feedback loop.

High cortisol production at 8 to 9 wpc suppresses the fetal HPA axis, keeping ACTH-driven androgen production at relatively low levels, safeguarding this major period of female sexual development and preventing genital virilization. Most maternal cortisol cannot normally reach the fetus because it is oxidized by the placental enzyme 11β hydroxysteroid dehydrogenase type 2 (11β-HSD-2) to cortisone, which is the inactive form of cortisol [18, 19]. Downregulation of the *11β-HSD-2* gene may alter the enzyme activity and the amount of maternal cortisol that reaches the fetal circulation, allowing a greater proportion of maternal cortisol to cross into the placenta and then enter fetal circulation. In a fetus with underlying CAH, a decrease in 11β-HSD-2 activity would allow higher amounts of maternal cortisol into the fetal circulation, leading to decreased activity of the fetal HPA axis and decreased ACTH and androgen production. Decreased fetal HPA activity due to either increased glucocorticoid sensitivity or increased amounts of maternal cortisol crossing to the fetal circulation may also result in low 17OHP concentrations at birth, as was described in case 1, and could be the reason some cases of CAH are missed by the NBS process [20-22], especially when NBS is performed within the first 36 to 48 hours after birth. The decreased activity of the fetal HPA axis by the aforementioned mechanisms recovers after birth in the absence of the negative feedback of maternal cortisol, leading to the rise of 17OHP concentrations. The later the timing of the NBS or serum confirmation sample is taken in affected infants, the higher the 17OHP concentrations would be, as shown in our case 1 patient, in whom 17OHP rose from 974 ng/dL on DOL 5 to 12 000 ng/dL(< 110 ng/dL) on DOL 12. The opposite effect occurs in unaffected premature babies, in whom 17OHP levels are higher at birth and decrease over time [23]. In premature babies 17OHP levels can be elevated at birth because of illness, immaturity of the adrenal glands, and assay cross-reactivity with other 17-hydroxylated steroids and can lead to false-positive results.

Polymorphisms of the androgen receptor (AR) such as CAG and GGn repeat polymorphisms of the *AR* gene have been shown to have significant impact on AR function in vitro [24, 25]. For both CAG and GGN repeats, there appears to be an optimal length and a shift in either direction would potentially modify transcriptional activity and androgen sensitivity. Rocha et al evaluated the number of CAG repeats in 106 patients with classic CAH (52 SV, 49 SW) and 302 controls to determine whether the number of CAG repeats, which is inversely correlated to the transcriptional activity of the *AR* gene, could take part in the variability of genital virilization in females with classic CAH [26]. The authors reported that the median of nCAG repeat lengths and its expression when analyzed together with

the severity of the *CYP21A2* genotype may have an influence on the degree of external genitalia virilization (Prader stage I-V). No difference in skewed X-inactivation was noted among the girls with minimal and severe virilization. Welzel and colleagues did not find any significant correlation between CAG or GGn repeat lengths of the *AR* with the phenotype of external genitalia in 206 females with classic CAH and variable genital virilization (Prader stage 0-V) [12]. Giwercman et al studied 12 females with classic SV-CAH and found no significant difference in CAG repeat lengths between CAH girls with minimal or severe virilization and no evidence of skewed X-inactivation. Of note, an *AR* pathogenic variant (E653K) was found in 2 sisters with normal female genitalia and classic CAH, but in vivo studies were not able to demonstrate that the E653K *AR* gene mutation was the sole modifier of their genital phenotype. All the studies mentioned here indicate that normal genital development is most likely the result of multiple genetic and epigenetic modifiers [27].

Although the aforementioned mechanisms of the phenotypic variability in genital virilization remain theoretical, our cases demonstrate the importance of ruling out severe forms of CAH in female infants with abnormal NBS even in the absence of virilized genitalia. In addition, in those infants presenting with signs of SW or adrenal crisis, CAH should still be at the top of the differential even with a negative NBS.

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Additional Information

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