Monozygotic twins discordant for congenital adrenal hyperplasia due to mosaicism

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Abstract

Introduction: Genotype-phenotype discordance occurs occasionally in congenital adrenal hyperplasia (CAH). Its causes are largely unknown. We describe a case of monochorionic, diamniotic twins with discordant clinical presentations of CAH, and show evidence for this being due to mosaicism resulting from a postzygotic full gene deletion of *CYP21A2* prior to twinning.

Case description: A 7-day-old 36-week gestation female infant (Twin A) presented to the emergency department with elevated 17-hydroxyprogesterone (17-OHP). Her identical twin (Twin B) had normal 17-OHP on newborn screening. Both twins showed signs of virilization, more pronounced in Twin B. Molecular genetic testing of both twins and their parents showed a WT paternally-inherited *CYP21A2* and a maternally-inherited copy containing the c.293-13C>G mutation. Both twins were also found to have a 5'-*CYP21A1P/CYP21A2*-3' hybrid (product of the common 30-kb deletion), derived from the deletion of the paternally-inherited *CYP21A2*. Neither mother nor father carried the deletion.

Conclusions: The genetic findings are consistent with mosaicism for two *CYP21A2* cell lines in both twins. The first cell line is expected, based on parental results, while the second line is due to a postzygotic full gene deletion of the paternally-inherited WT *CYP21A2*. The resultant genotype, compound heterozygosity for c.293-13C>G and a *CYP21A2* full gene deletion, is consistent with a salt-wasting CAH phenotype. Differential distribution of the second cell line between the twins is most likely the cause for their discrepant phenotypes. We believe this is the first report of somatic *CYP21A2* mosaicism, and represents a novel cause for discrepant CAH phenotypes in monozygotic twins.

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Introduction

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is an autosomal recessive condition of variable severity, which results from pathogenic *CYP21A2* mutations. The underlying genetic defects can be complex, and unequal crossover between *CYP21A2* and its highly homologous pseudogene *CYP21A1P* may result in deletion (or duplication) of the *CYP21A2* gene or a gene conversion event, where a section of *CYP21A1P* is copied onto *CYP21A2* (1). Indeed, gene deletions, gene conversion events (hybrid alleles)

and pseudogene-derived mutations (mutations present on the pseudogene that are transposed onto *CYP21A2* by a gene conversion) account for approximately 90–95% of *CYP21A2* mutant alleles in Western Europeans (1, 2). In addition, approximately 1% of *CYP21A2* mutations are *de novo* and, therefore, not found in either parent (1). Nonetheless, when appropriate genetic testing methods and expertise are employed, there is a strong genotype-phenotype correlation (1, 2, 3, 4, 5, 6, 7). When discordance is observed, it frequently involves the

common pathogenic mutations p.P31L, c.293-13C>G and p.I173N (legacy nomenclature: p.P30L; g.655A/C>G, IVS2-13A/C>G or I2G; and p.I172N) (2, 3, 4, 7). Genotype-phenotype discordance has also occasionally been noted in siblings with identical genotypes, a phenomenon that is poorly understood (5, 6).

In this report, we describe a case of monochorionic, diamniotic twins with discordant clinical presentations of 21-hydroxylase deficient CAH. We hypothesize that their discrepant phenotypes are most likely due to mosaicism resulting from a postzygotic full gene deletion of their paternally-inherited copy of *CYP21A2* (8, 9). Although Marino *et al.* reports of a suspected case of germline mosaicism in the unaffected father of two affected siblings sharing the same *de novo* mutation, the authors are unaware of any previous reports of mosaicism as a cause of discrepant CAH presentation in monozygotic twins (6). Nor are the authors aware of a previous report of somatic mosaicism for a mutation in *CYP21A2* giving rise to 21-hydroxylase deficient CAH. Postzygotic mutations resulting in mosaicism and discordant phenotypes in monozygotic twins have been reported in other hereditary conditions (8, 9).

Case

Clinical and biochemical presentation

A 7-day-old ex-36 week female infant (Twin A) presented to the emergency department with elevated 17-hydroxyprogesterone (17-OHP) by newborn screening (NBS; 86.4 ng/mL, normal: <30) drawn at 34 h of life. Her identical twin (Twin B) had normal 17-OHP by NBS (0.8 ng/mL).

Follow-up serum studies on the sixth postnatal day revealed 17-OHP concentrations of 7840 ng/dL and <40 ng/dL, respectively (normal: <630 ng/dL). Serum

sodium was low in Twin A and normal in Twin B (131 mEq/L versus 141 mEq/L, normal: 134–144), while serum potassium was substantially elevated in Twin A (8.7 mEq/L, normal 3.5–5.2), but almost normal in Twin B (5.6 mEq/L).

Androstenedione, renin activity, and testosterone for Twin A are shown in Table 1. Androstenedione, renin activity and testosterone were not tested for Twin B. An ACTH (adrenocorticotrophic hormone) stimulation test was not performed for either twin. All hormonal measurements were performed by liquid chromatography-tandem mass spectrometry.

Twin A was started on hydrocortisone, fludrocortisone, and sodium chloride supplementation with gradual resolution of hyperkalemia, hyponatremia, and a decline in 17-OHP serum concentrations.

Initial physical examination of both twins at age 10 days revealed mild to moderate clitoromegaly. Twin A was noted to have a phallus length of 1.5 cm, and Twin B was noted to have a phallus length of 1.0 cm, width of 4.0 mm, and an anogenital distance of 2.5 cm (normal: 9.1 ± 2.8 mm in full term infant). Hyperpigmentation of the labia majora was present in both twins, along with posterior labial fusion, which was noted to be slight in Twin A and more pronounced in Twin B. Both twins also appeared to have a urogenital sinus. No gonads were palpable in the labia majora or inguinal canals. Subsequent evaluation showed that Twin A had normal vaginal and urethral opening, while Twin B was confirmed to have a urogenital sinus.

Twin A is being treated with hydrocortisone and fludrocortisone, and has required gradual increases in hydrocortisone with age. Her growth and development have been normal, and no surgical intervention has been required. Twin B has not required any hormonal treatment; urethroplasty and vaginoplasty were performed for distal urogenital sinus at 17 months of age. No other follow-up has occurred for Twin B.

Table 1 17-OHP, androstenedione, renin activity, and testosterone measurements for Twin A over a period of 35 months.

Age at collection	17-OHP (ng/dL)	Androstenedione (ng/dL)	Renin Activity (ng/mL/h)	Testosterone (ng/dL)
6 days	7840			
17 months	2630	58	23*	
20 months	11 600	82	55*	
23 months	4280	71	61*	9.6
27 months	338	<16	33**	<2.5
32 months	2440	32	16**	4.7
35 months	1270	23	12**	3.8

Bold values indicate elevated results. Normal ranges: 17-OHP: 0–28 days: <630 ng/dL, prepubertal females: <100 ng/dL; Androstenedione: <69 ng/dL; Renin activity: *0–2 years: 4.6 ng/mL/h (mean), range: 1.4–7.8 ng/mL/h; **3–5 years: 2.5 ng/mL/h (mean), range: 1.5–3.5 ng/mL/h; Testosterone: <2.5–10 ng/dL.

Genetic testing

Zygoty testing [Mayo Clinic Laboratories (MCL), Rochester, MN] revealed a >99.999% likelihood of monozygoty and ruled out non-paternity. Genetic testing by mini-sequencing for *CYP21A2* variants (Esoterix Laboratory, Calabasas Hills, CA) on blood-derived DNA from both twins failed to clarify their clinical phenotypes, finding the same heterozygous pseudogene-derived salt-wasting c.293-13C>G variant in both twins.

Further *CYP21A2* genetic testing was performed (MCL). A multi-method approach involving PCR amplification, bi-directional sequencing analysis, and multiplex ligation-dependent probe amplification (MLPA) was used to detect sequence variants and copy number [GenBank accession

number NM_000500.7; build GRCh37 (hg19)] (Fig. 1 and Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article).

Genetic testing results

Utilizing blood-derived DNA, PCR indicated the presence of *CYP21A2*, *CYP21A1P* and a 5'-*CYP21A1P/CYP21A2*-3' hybrid in both twins. The 5'-*CYP21A1P/CYP21A2*-3' hybrid is a byproduct of the common 30-kb *CYP21A2* deletion. Copy number determination by MLPA was ambiguous, yielding 1–2 copies of *CYP21A2* and 2–3 copies of *CYP21A1P* in both twins. This unusual result was confirmed by droplet digital PCR (ddPCR), which yielded 1.5 copies of the *CYP21A2* promoter and

Figure 1

Schematic depiction of *CYP21* locus and of the assay methodology used for diagram of the locus containing *CYP21A1P* and *CYP21A2*. (A) Relative location of *CYP21A1P* and *CYP21A2* long range PCR amplification primers, MLPA probes, and ddPCR probes. For detailed sequences, see Supplementary Table 1. (B) Possible results based on long range PCR and MLPA probes.

Figure 2

Sequencing of the *CYP21A2* gene and the 5'-*CYP21A1P/CYP21A2-3'* hybrid of Twin A. (A) Position c.293-13 in *CYP21A2* (black box); sequencing demonstrates that Twin A is heterozygous for both the wildtype cytosine (C) in blue and the mutant guanine (G) in black. Note the peak intensity for the WT C is reduced compared to the peak intensity for the mutant G. (B) Position c.293-13 in the 5'-*CYP21A1P/CYP21A2-3'* hybrid (black box); the mutant guanine is the only nucleotide present at the c.293-13 position. This is expected since the c.293-13C>G mutation is pseudogene-derived.

2.5 copies of the *CYP21A1P* promoter when compared to a known normal sample (2 copies of *CYP21A2* and 2 copies of *CYP21A1P*). Sanger sequencing utilizing primers specific for the *CYP21A2* gene confirmed, in both twins, the presence of the heterozygous c.293-13C>G mutation previously detected. However, the peak intensity of c.293-13C>G was unbalanced, with the WT peak (c.293-13C) being reproducibly smaller than the mutant peak (c.293-13G) (Fig. 2A). This phenomenon of unbalanced peak intensity was also noted in four common benign variants (c.-4C>T, c.293-67C>T, c.*368T>C, c.*373G>A) detected in the twins. Sanger sequencing of the 5'-*CYP21A1P/CYP21A2-3'* hybrid confirmed its presence in both twins and revealed pseudogene material [including the expected c.293-13G (Fig. 2B)] up through at least the end of exon 8.

PCR and MLPA utilizing blood-derived DNA from both parents revealed each parent has 2 copies of *CYP21A2* and *CYP21A1P*, therefore neither parent carries the 5'-*CYP21A1P/CYP21A2-3'* hybrid detected in the twins. Sanger sequencing of the *CYP21A2* gene of the mother detected heterozygosity for the c.293-13C>G mutation. However, the father has two copies of WT *CYP21A2*. The *CYP21A2* gene is known to be highly polymorphic, and the father was found to carry multiple heterozygous benign

Figure 3

Pedigree chart: Circles represent females, and squares represent males. Diagnosis of congenital adrenal hyperplasia is indicated by filled-in symbols. Carrier status is denoted by a dot. The zygote prior to twinning is represented by a circle with 'zygote' printed inside. The bar between Twin A (left) and Twin B (right) reflects monozygosity. The genotype of an individual is denoted next to their symbol. Maternal and paternal *CYP21A2* alleles are differentiated by gray shading and black diagonal lines respectively. The genotypes for cell lines 1 and 2 in Twin A and Twin B were determined based on the combined results of MLPA and ddPCR.

variants; these benign variants allowed for comparison with the WT copy of *CYP21A2* found in the twins. One of the paternal copies of the gene (carrying five benign variants) was found to match exactly with the WT copy of *CYP21A2* detected in both twins, suggesting that it was paternally inherited.

The four heterozygous benign variants with unbalanced peaks in the twins were investigated in the parents. It was noted that, similar to the twins' c.293-13C>G variant, the maternally-inherited nucleotide signals were reproducibly higher than the peaks corresponding to the paternally-inherited nucleotides (data not shown). Based on these results, the twins inherited one copy of *CYP21A2* carrying the c.293-13C>G salt-wasting mutation from their mother and one WT copy of *CYP21A2* from their father (Fig. 3). The 5'-*CYP21A1P/CYP21A2-3'* hybrid does not appear to have been inherited from either parent.

The 5'-*CYP21A1P/CYP21A2-3'* hybrid is the typical by-product of the common 30-kb deletion that results from unequal crossover between *CYP21A2* and *CYP21A1P* (Fig. 4). Therefore, the 3'-UTR of the hybrid is expected

Figure 4

Unequal crossover resulting in *CYP21A2* deletion. Unequal crossover and gene conversion events may occur due to the high homology of not only the *CYP21A2* and *CYP21A1P* genes, but also other genes in this locus (*RP1-C4A-CYP21A1P-TNXA* and *RP2-C4B-CYP21A2-TNXB*). Unequal crossover between *CYP21A1P* and *CYP21A2* may result in the deletion of a 30-kb region and generation of a 5'-*CYP21A1P/CYP21A2-3'* hybrid. This image is a generic example of this event, multiple breakpoints generating these hybrids or chimeras have been detected.

to match the 3'-UTR of the copy of *CYP21A2* that was deleted. To investigate this the 3'-UTR of *CYP21A2* was sequenced in the twins and both parents and compared to the 3'-UTR of the twins' 5'-*CYP21A1P/CYP21A2-3'* hybrid. Sanger sequencing of *CYP21A2* revealed two heterozygous benign sequence variants in the mother: c.*368T>C and c.*373G>A, while the father was WT at these positions (c.*368T and c.*373G) (data not shown). Both twins were heterozygous for both variants, supporting that the c.*368T>C and c.*373G>A variants are in cis with the c.293-13C>G variant on the maternal allele. The 3'-UTR of the 5'-*CYP21A1P/CYP21A2-3'* hybrid revealed that both positions were WT (c.*368T and c.*373G), supporting that the paternally-inherited copy of *CYP21A2* was deleted.

The combination of these findings suggest that the apparently *de novo* 5'-*CYP21A1P/CYP21A2-3'* hybrid resulted from a postzygotic gene conversion that occurred prior to twinning. This gene conversion event resulted in the replacement of the 5' end (up through at least exon 8) of the paternally-inherited WT copy of *CYP21A2* with pseudogene material. Therefore, both twins are mosaic for two cell lines. Cell line 1 is the expected cell line based on the parental results: one maternally-inherited copy of *CYP21A2* that carries the c.293-13C>G mutation and one paternally-inherited WT copy of *CYP21A2*. Cell line 2 resulted from the gene conversion event: one maternally-inherited copy of the *CYP21A2* that carries the c.293-13C>G mutation and one *de novo* inactive 5'-*CYP21A1P/CYP21A2-3'* hybrid where the 3' end of the hybrid matches the paternally-inherited copy of *CYP21A2*.

Germline mosaicism for the 5'-*CYP21A1P/CYP21A2-3'* hybrid cannot be fully ruled out in the father of the twins; however, if the father was germline mosaic, the twins would have inherited the hybrid from their father and the

CYP21A2 allele carrying c.293-13C>G from their mother. A postzygotic somatic rescue event would have had to result, whereby the *CYP21A2* material was copied from the available maternal allele. At that point, to match the genotype detected in the WT copy of *CYP21A2* detected in the twins, five individual mutational events would have had to occur to convert the *CYP21A2* gene back to the genotype detected in the twins' father. Therefore, the authors consider a postzygotic gene conversion resulting in mosaicism in the twins to be by far the most likely explanation; it would only require one event (a gene conversion).

Discussion

The results of the combined methodologies suggest a postzygotic *de novo* gene conversion event that resulted in the deletion of the paternally-inherited WT copy of *CYP21A2* and led to somatic mosaicism for two cell lines in both twins. Due to this *de novo* event, the zygote carried two cell lines before twinning (Fig. 3). Cell line 1 would result in an individual being a carrier of CAH. Cell line 2 is typically associated with a salt-wasting CAH phenotype.

After twinning, both cell lines appear to have been passed to both twins, but resulted in discordant phenotypes. Twin A presented with a biochemical phenotype consistent with salt-wasting CAH, but she did not have physical manifestations requiring surgery. On the other hand, Twin B, presented with, and has maintained, a normal biochemical phenotype, but was found to have a distal urogenital sinus.

These discordant biochemical phenotypes are likely due to the frequency and distribution of the mutant

cell line 2 in the twins' cells and tissues. Although the authors can only hypothesize, Twin A may have more cells derived from the *de novo* cell line, or Twin A's adrenal glands may contain more of cell line 2, while Twin B's may contain more of cell line 1. Only whole blood samples were available for testing; however, it has been suggested that, because 95% of monochorionic twins exchange blood *in utero* through vascular anastomoses in their shared placenta, blood mosaicism may result in monochorionic twins (8, 10, 11). Therefore, future testing of fibroblast cells, or another non-blood-derived sample type, may help to rule out blood-confined mosaicism in Twin B. However, even with testing of additional sample types, the state of mosaicism in the twins' adrenal glands cannot be assessed to test these hypotheses. In addition, one cannot rule out that other genetic factors, such as methylation or additional somatic changes in other genes, may have also played a role in the observed biochemical phenotypic differences observed between Twin A and B.

The cause of the virilization observed in Twin B remains unsolved. However, although she had no biochemical evidence of 21-hydroxylase deficiency, Twin B and Twin A shared a placenta *in utero*, and thus almost certainly shared circulation (10, 11). Due to this shared circulation, there is also a shared hormonal environment (10, 12). Twin A would have secreted elevated androgen levels *in utero*, exposing both twins, and through arteriovenous anastomoses in the placenta, Twin B may have been exposed to more androgens than Twin A (10). Further, even in siblings that share the same genotype, the level of virilization is not always equivalent. Tze *et al.* reported on a set of dizygotic female infants with 21-hydroxylase CAH where one presented with ambiguous genitalia and the other was thought to have normal genitalia until closer examination revealed an enlargement of the clitoris with no other anomalies. Unfortunately, the twins in Tze *et al.* were not genotyped, so the affected status of both twins was not confirmed genotypically; however, biochemical results suggested that both dizygotic twins were affected (13).

The results highlighted in this report have important implications for the future management of both Twin A and Twin B, as well as risk counseling for future offspring. Since germline mosaicism in the twins' parents is highly unlikely in this case, the recurrence risk in future pregnancies of the parents is low (approximately 1%). Twin A and Twin B should continue to be managed appropriately based on their phenotype; however, because both cell lines appear to be present in both twins, they are at risk of having affected children. Genetic counseling

should include a discussion that both twins' germline may be mosaic and contain all three *CYP21A2* genotypes (WT, c.293-13C>G, or the *CYP21A2* deletion). The risk for the twins to pass on a *CYP21A2* mutation might range anywhere from 50% (if their germline is composed only of cell line 1) to 100% (if their germline is composed only of cell line 2).

In summary, we feel the evidence is compelling that mosaicism resulted in, not only the discordant phenotypes between Twin A and Twin B, but also in the discordance between genotype and biochemical phenotype observed. Intrauterine transfer of sex steroids between the twins is the likely cause for virilization in both twins (with no evidence of glucocorticoid or mineralcorticoid deficiency in Twin B). This result is important for both the continued management of Twin A and Twin B, and for genetic counseling of this family.

This report also illustrates the importance of considering somatic mosaicism as a mechanism for disease in 21-hydroxylase deficient CAH. Indeed, it is interesting to speculate if some of the previously reported genotype-phenotype discordance in a minority of patients with the c.293-13C>G/full gene deletion genotype could represent somatic mosaicism (2, 5). To our knowledge, this is the first report of suspected somatic mosaicism in CAH, possibly due to the relatively low incidence (1–2%) of *de novo* genetic events in *CYP21A2* cases (1, 3, 4, 5, 6).

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-19-0249>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this case report.

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Informed consent

Written informed consent for publication of clinical details was obtained from the parent or guardian of the patients.

Author contribution statement

K F and E G clinically characterized and counseled the patients. H L S performed PCR, Sanger sequencing, and MLPA. D M performed droplet digital PCR. M L K, S K G, and L V H conducted analysis of the results. K W C conducted an additional phasing experiment. M L K prepared the draft manuscript. All authors contributed to the discussion of results, and edited and approved the final manuscript.

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