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Comprehensive Analysis of RCCX Heterogeneity and CAH-X Syndrome in Congenital Adrenal Hyperplasia Using Long-Read Sequencing

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Purpose: 21-hydroxylase, encoded by *CYP21A2* within the RCCX module, is a multiallelic and highly complex copy number variation locus. Defects in *CYP21A2* cause congenital adrenal hyperplasia (CAH). Due to the high degree of homology between active and pseudogenes, the RCCX module is highly susceptible to genetic rearrangements, which can result in a contiguous gene deletion syndrome involving *CYP21A2* and the adjacent *TNXB* gene, known as CAH-X syndrome. This study aimed to analyze the structure of RCCX alleles and identify CAH-X syndrome using long-read sequencing (LRS). **Methods:** This study included 13 patients with CAH, consisting of 11 with the salt-wasting type and 2 with the simple virilizing type, all of whom had at least one allele deletion of *CYP21A2* confirmed by conventional methods. After obtaining informed consent, LRS was performed using the Revio platform from Pacific Biosciences (PacBio, California, USA). The analysis was conducted in a genome assembly format to accurately characterize genetic rearrangements within the RCCX locus. This approach included phasing the RCCX locus for each individual, categorizing *CYP21A2* and *CYP21A1P* genes using pseudogene-specific markers. Additionally, generalized joint hypermobility was assessed to evaluate symptoms and signs of hypermobile Ehlers-Danlos Syndrome (EDS) using Beighton score. **Results:** In the analysis of 26 genome assemblies obtained from 13 patients, 7 different haplotypes within the RCCX region were identified: 77% (20/26) were bimodular, 12% (3/26) were monomodular, and 12% (3/26) were trimodular. Among the bimodular alleles, 50% (10/20) contained one pseudogene and one *CYP21A2* gene, while 20% (4/20) had

both a pseudogene and a *CYP21A1P/CYP21A2* fusion gene. Additionally, 15% (3/20) exhibited two pseudogenes, and the remaining alleles contained more complex variations. Of the 3 monomodular alleles, two containted a *CYP21A1P/CYP21A2* fusion gene, and one contained only a pseudogene. Among the trimodular alleles, two had two pseudogenes and one *CYP21A2* gene, while one included two *CYP21A1P* copies and one *CYP21A1P/CYP21A2* fusion gene. Additionally, 4 alleles were identified with *TNXA/TNXB* fusions: two were classified as CAH-X CH1 alleles, featuring a 120 bp deletion in *TNXB* exon 35, and two were classified as CAH-X CH2 alleles, characterized by the c.12174C>G variant in *TNXB* exon 40. The two patients carrying CAH-X CH1 alleles exhibited joint hypermobility (Beighton score 4/9) and easy bruising, consistent with features of CAH-X syndrome. **Conclusion:** The LRS characterized the variable haplotypes of the RCCX allele and classified the types of *CYP21A1P/CYP21A2* and *TNXA/TNXB* chimeras. Additionally, LRS identified CAH-X syndrome in four patients (31%), including asymptomatic individuals.

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