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Replication of clinical associations with 17-hydroxyprogesterone in preterm newborns

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Abstract

Nationally newborn screening programs use 17-hydroxyprogesterone (17-OHP) as the biomarker to detect the rare but potentially fatal inherited disease, congenital adrenal hyperplasia (CAH). However, this biomarker is highly variable with a high false positive rate of detection, particularly in neonates born preterm. Several studies have examined various clinical and genetic factors to explain the variability of 17-OHP in preterm infants. The purpose of this study was to replicate previous clinical and genetic associations with 17-OHP in a well-characterized cohort of 762 preterm infants. We replicated previous findings that respiratory distress syndrome ($P = 2 \times 10^{-3}$) is associated with higher 17-OHP. Higher 17-OHP and false positives were significantly associated with lower gestational age and birth weight, as previously reported. Incorporating gestational age and birth weight together decreases the false positive rate.

Introduction

Congenital adrenal hyperplasia (CAH; OMIM 201910) is an inherited autosomal recessive disorder of cortisol biosynthesis occurring in approximately 1 out of every 10,000 to 15,000 live births (1, 2). 17-hydroxyprogesterone (17-OHP) is the biomarker used in newborn screening programs nation-wide to screen for CAH and elevated levels of 17-OHP identify infants that need further testing. However, 17-OHP is highly variable and has a higher false positive rate, particularly in preterm and low birth weight neonates (2, 3). It is well known that preterm and sick term infants often have higher 17-OHP levels than healthy term infants even though they are not affected by CAH (2, 4). The underlying mechanism for this observation is unclear and has often been largely attributed to fetal stress (4). Several studies have examined neonatal illnesses such as respiratory distress syndrome (RDS) and maternal conditions such as pre-eclampsia and observed higher levels of 17-OHP in these infants (5). However, these associations have not replicated in several studies and healthy preterm infants still have much higher 17-OHP than their term counterparts (4, 6). Some explanations include immature adrenal function, immature kidney function and higher levels of adrenocorticotrophic hormone (7–9).

Genetic factors may also influence the variability of 17-OHP concentrations. Of particular interest are genes involved in the steroidogenic pathway, in particular cytochrome P450

genes *CYP21A2*, *CYP11B1* and *CYP17A1* that harbor rare variants responsible for different forms of CAH. Several studies have identified polymorphisms in *CYP21* that influence 17-OHP levels in patients with non-classical CAH (10, 11). Another study observed an association between the glucocorticoid receptor gene (*NR3C1*) and 17-OHP levels; however this was not replicated in a study of 1,000 random samples from routine newborn screening (12). It is feasible, but has not been previously investigated, that common polymorphisms in *CYP21* and related genes influence the variability of 17-OHP in preterm infants that do not have CAH.

The variability of 17-OHP in preterm infants leads to a significantly increased false positive rate in this population (3). Many states have taken measures, such as having birth weight or gestational age specific thresholds, to reduce the false positive rate in preterm infants. However, it is observed that there is still a high false positive rate in preterm and low birth weight infants even with this adjustment (3). Second-tier methods such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been proposed to lower the false positive rate of CAH testing (13); however, due to the analytical time required to run this method it is not feasible as a first-tier screen. In 2009 the Clinical and Laboratory Standards Institute (CLSI) recommended that all preterm and low birth weight infants be screened at birth, 48–72 hours after birth and at 28 days of life (14). This delays final reporting and significantly increases the cost of screening. Examining clinical and genetic influences on variation in 17-OHP will lead to a greater understanding of steroidogenesis in preterm newborns and result in better screening methods for CAH. We investigated this in preterm infants utilizing data from the Iowa Neonatal Metabolic Screening Program (INMSP).

Materials and Methods

Study Population

This is a retrospective analysis of data collected as part of a prospective cohort for studying the epidemiology and genetics of preterm birth (15, 16). Study samples were collected at the University of Iowa Hospitals and Clinics in Iowa City, IA. Signed informed consent (IRB200506792) was obtained from all families for enrollment. DNA was extracted from cord blood or buccal swabs collected from infants and venous blood, saliva samples, or buccal swabs collected from parents and other relatives between 2000 and 2009. Existing data collected by an interview with the mother, medical chart review or both was mined for fifty-four clinical and demographic factors; completeness of the data varied across subjects (Supplemental Table 1). Gestational age and birth weight were obtained from the medical record, if available; otherwise information reported on the newborn screen card was used. 17-OHP measurements were obtained from the State Hygienic Laboratory using the Clinical Laboratory Improvement Amendments. Quantification of 17-OHP was determined with a solid phase, time-resolved fluoroimmunoassay from dried newborn blood spots using PerkinElmer's DELFIA® or AutoDELFIATM platform (Waltham, MA, USA). Inclusion criteria included specimen collection between 24–72 hours after birth, no blood product transfusion and birth before 37 weeks gestation. There were genotype and phenotype data on 148 sets of multiples (twins or triplets) available for this study; the infant with the most complete information with respect to clinical and genetic data was chosen from each set for inclusion in this analysis. A total of 762 preterm infants met the inclusion criteria for this study.

Single nucleotide polymorphisms (SNPs) from candidate genes within the steroidogenic pathway and related CYP P450 genes were previously genotyped as part of a study examining genetic association with preterm birth and complications (unpublished work). CYP P450 genes encode for a diverse group of enzymes that have function in lipid and

steroid metabolism, vitamin D regulation and drug metabolism. Additional genotyping was performed in *CYP11B1*, *CYP17A1*, *CYP21A2* and *NR3C1* to saturate genes previously shown to associate with CAH or 17-OHP. All genotyping was completed using the Applied Biosystems (Foster City, CA, USA) TaqMan® chemistry under standard conditions. A total of forty-four SNPs were examined in this study (Supplemental Table 2). Genetic analysis was limited to Caucasian infants without congenital or chromosomal abnormalities to avoid potential confounding caused by population stratification and structural or chromosomal aberrations. A total of 393 infants had genotype data on some or all of the SNPs. Parental and/or sibling genotypes were available in most cases and were included in the family-based analysis.

Statistical Analysis

Analysis of variance (ANOVA) was performed to identify clinical and genetic associations with 17-OHP levels. 17-OHP was transformed using the Box Cox method of transformation to normalize measurement values. Standardized residuals were examined for outliers and measurements that were < -3.5 or > 3.5 were removed. Statistical modeling was performed to determine the most appropriate method of analysis and to identify potentially confounding covariates. Birth weight, gestational age, year of sample collection and major change in the assay lot of 17-OHP were included in models presented; differences in results between models and methods are indicated in Supplemental Table 3. A Bonferroni significance threshold of $P < 9 \times 10^{-4}$ (0.05/54 tests) was used to correct for multiple testing. Stata version 10.1 (Stata Corporation, College Station, Tx) was used for all analyses. All infants with borderline (N=44) and presumptive positive (N=30) CAH test results were normal on subsequent screens. We did not have the ability in our population to identify missed cases of CAH nor were we able to propose hypothetical thresholds based on our data as we had no confirmed cases of CAH in our study. Borderline and presumptive positive results are determined by the threshold of 17-OHP concentration with higher concentrations being diagnosed as presumptive positive; however, clinically borderline and presumptive positive test results were followed up in the same manner with each infant receiving a second screening test. Therefore, we combined presumptive positive and borderline test results into one false positive test result category. We compared false positive test results to negative results for factors associated with 17-OHP concentrations using Fisher's Exact tests. Analysis broken down into subgroups is provided in Supplemental Table 4.

Genotypes were tested for deviations from Hardy-Weinberg equilibrium (HWE) using Fisher Exact tests. Genotype data was analyzed using ANOVA adjusting for year of sample collection, major change in the 17-OHP assay, gestational age and birth weight. Additive, dominant, and recessive models were applied. A Bonferroni significance threshold of $P < 3.8 \times 10^{-4}$ (0.05/132 tests) was used to correct for multiple testing. Global haplotype analysis using permutation testing to obtain P values was performed within each gene for blocks of 2, 3 and 4 SNPs using PLINK software (17). Quantitative transmission disequilibrium tests (QTDT), adjusting for assay change, year, gestational age and birth weight were also performed on parent-infant trios including any genotyped siblings, to determine if the transmission of parental alleles to the offspring was associated with transformed 17-OHP concentrations (18). Permutation tests were used to correct for the non-linear distribution of 17-OHP.

Results

Clinical Results

Gestational age and birth weight were the strongest contributors to variation in 17-OHP concentration (Figure 1A and B). Significantly more positive test results ($P=1.1 \times 10^{-4}$) were

observed in infants at the upper (>2,500 grams) and lower (<1,250 grams) extremes of birth weight (Table 1) while no significant ($P=0.16$) differences were observed by gestational age. There was also a strong association between weight for gestational age (WGA) and 17-OHP ($P=8.3\times 10^{-4}$) (Figure 1C). Subsequent analysis identified that this effect was driven by the large for gestational age group (LGA; $P=9.1\times 10^{-4}$) not the small for gestational age group (SGA; $P=0.11$). LGA and SGA infants had fewer false positive CAH test results than AGA infants ($P=2.3\times 10^{-3}$). No other clinical or demographic variable was associated with 17-OHP concentrations in our study after correction for multiple testing ($P<9\times 10^{-4}$). We observed marginally higher concentrations of 17-OHP associated with respiratory distress syndrome (RDS) and this concentration difference resulted in more false positive test results in infants with RDS ($p=0.01$) (Figure 2 and Table 1).

Genetic Results

CYP24A1 (rs2248359 and rs927650), *CYP27A1* (rs4674338) and *CYP21A2* (rs12525076) were associated with 17-OHP concentrations; however, none reached significance after correction for multiple testing ($P < 3.8\times 10^{-4}$). Infants with either homozygous genotype (TT or CC) at rs2248359 in *CYP24A1* had higher 17-OHP levels compared to those with the heterozygous genotype ($P = 0.01$) and this correlated with significantly more false positive CAH test results (Figure 3A and Table 2). Infants with the CC genotype at rs927650 in *CYP24A1* had higher 17-OHP compared to those with the CT or TT genotypes ($P = 0.03$) and this correlated with more false positive CAH test results (Figure 3B and Table 2). Genotypes at rs4674338 in *CYP27A1* and rs12525076 in *CYP21A2* associated with 17-OHP concentrations; however, these were not significantly associated with false positive CAH test results (Table 2 and Supplemental Table 2). There were no significant haplotype associations with 17-OHP nor were any SNPs significantly associated with 17-OHP in the family-based analyses (Supplemental Table 2 and 5).

Discussion

17-OHP is highly variable, particularly in preterm infants which results in a high false positive rate for CAH in this group. However, the variability of this biomarker in preterm infants has been poorly characterized. Many studies attribute elevated 17-OHP in preterm infants to fetal stress; however, very little is known about the impact of maternal conditions, environmental influence, infant outcomes and genetic factors on the steroid profiles of premature neonates. We evaluated an extensive list of maternal and fetal conditions and characteristics for associations with 17-OHP to determine if these factors were not only important in understanding steroidogenesis in preterm infants but also for identifying factors that could potentially improve the specificity of CAH testing. This is of particular importance in the preterm population, as these infants and their parents are already under a great deal of stress due to the complications and treatments of prematurity.

Gestational age and birth weight were major contributors to the variation in 17-OHP concentrations in our preterm population. In Iowa, four birth weight categories (<1,250g, 1,250–1,749.9g, 1,750–2,249.9g and $\geq 2,250$ g) are used to adjust levels of 17-OHP when determining CAH test results. We observed that even with these adjusted thresholds preterm infants at the high (>2,250g) and low (<1,250g) extremes of birth weight are more likely to receive false positive results. This indicates that in preterm infants adjusting for birth weight with the current thresholds is not adequately controlling the false positive rate and additional thresholds should be evaluated, particularly at the extremes of birth weight in preterm infants. We found that incorporating gestational age and birth weight into three categories; small (SGA), large (LGA) and average (AGA) for gestational age resulted in fewer false positives at the extremes (SGA and LGA). This is in line with other studies that have suggested that including gestational age in addition to birth weight when determining the

diagnostic thresholds for 17-OHP is important (19). Smaller studies have also demonstrated that small for gestational age (SGA) infants have lower 17-OHP than term infants likely due to a decreased capacity for steroidogenesis; none of these studies evaluated the effect on newborn screening (5, 20). We found that SGA newborns had no false positives reported; however, we were unable to examine the impact of this on the false negative rate because we were unable to determine if cases of CAH were missed in our population. We were also not able to propose hypothetical thresholds based on our data as we had no confirmed cases of CAH in our study. Further investigation in larger studies that have true CAH cases and potentially missed CAH cases is needed to determine if thresholds based on weight for gestational age more adequately control for the false positive rate of 17-OHP testing while not increasing the false negative rate.

We replicated previous studies that observed RDS results in higher concentrations of 17-OHP (5). Previously it was concluded that this result was a marker for infant sickness and due solely to the association with birth weight (5). However, we observe that RDS is associated independent of birth weight and results in more false positive CAH test results. We also did not find any other associations between other infant illnesses and 17-OHP when controlling for birth weight and gestational age. Therefore, these data suggest that unlike fetal illnesses 17-OHP levels are particularly sensitive to RDS; further investigation on the mechanism of this phenomenon is warranted.

We identified novel genetic associations between *CYP21A2*, *CYP24A1* and *CYP27A1* with 17-OHP levels. Rare mutations in the *CYP21A2* gene cause the most common form of CAH. Our association was between a common intronic variant (allele frequency of 36%) in *CYP21A2*; however, the functional significance of this finding is unclear. *CYP24A1* and *CYP27A1* are vitamin D metabolism-related genes and studies show a link between delayed bone maturation and CAH due to treatment with glucocorticoids (21). However, the uncertain biological and weak statistical evidence for the involvement of genetic variants in the regulation of 17-OHP levels makes additional replication studies necessary. Identifying genetic associations in a population of healthy term infants is also important for determining the impact of gene-environment interactions on 17-OHP concentrations and is currently underway.

These findings are important for guiding future studies of the clinical impact on 17-OHP concentrations as well as determining factors that may potentially influence false positive CAH results. Understanding the interactions between key environmental and genetic factors on 17-OHP will allow for a more accurate newborn screening test. These findings provide important insights into the steroid profiles of preterm neonates; which is particularly important as this group of infants has a high rate of infant morbidity and mortality. Further investigation of genes within the steroidogenic pathway will deepen the understanding of steroidogenic pathway dynamics and contributors to 17-OHP variability in newborns, leading to improvements in the interpretation of screening data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Associations between 17-OHP and birth weight (A) gestational age (B) and weight for gestational age (C). The significance (P value) is presented adjusted for year of collection and change in the 17-OHP assay.

Figure 2.

Associations between 17-OHP and RDS. The significance (P value) is presented adjusted for birth weight, gestational age, year of collection and change in the 17-OHP assay.

Figure 3. Associations between 17-OHP and *CYP24A1* rs2248359 (A) and rs927650 (B). The significance (P value) is adjusted for gestational age, birth weight, year of collection and change in the 17-OHP assay.

Table 1

Associations between CAH test results and clinical variables.

	Test Result		P-value
	Normal (N = 688)	False Positive (N = 74)	
<u>Birth Weight</u>			1.1×10 ⁻⁴
<1,250 gm	174 (89.2%)	21 (10.8%)	
1,250–1,749.9 gm	182 (95.8%)	8 (4.2%)	
1,750–2,249.9 gm	181 (93.3%)	13 (6.7%)	
≥2,250 gm	151 (82.5%)	32 (17.5%)	
<u>Gestational Age</u>			0.16
23–28 weeks	140 (87.5%)	20 (12.5%)	
29–32 weeks	246 (92.8%)	19 (7.2%)	
33–36 weeks	301 (89.6%)	35 (10.4%)	
<u>WGA</u>			2.3×10 ⁻³
AGA	573 (89.1%)	70 (10.9%)	
LGA	36 (92.3%)	3 (7.7%)	
SGA	71 (100.0%)	0 (0%)	
<u>RDS</u>			0.01
No	270 (93.8%)	18 (6.3%)	
Yes	336 (88.0%)	46 (12.0%)	

Table 2

Associations between CAH test results and infant genotype.

	Test Result		P-value
	Normal (N = 356)	False Positive (N = 37)	
<i>CYP24A1</i> (rs2248359)			3.8×10^{-3}
TT	69 (84.2%)	13 (15.9%)	
TC	140 (96.6%)	5 (3.5%)	
CC	50 (90.9%)	5 (9.1%)	
<i>CYP24A1</i> (rs927650)			0.05
TT/TC	254 (92.4%)	21 (7.6%)	
CC	51 (83.6%)	10 (16.4%)	
<i>CYP27A1</i> (rs4674338)			0.07
AA	34 (82.9%)	7 (17.1%)	
AG/GG	159 (92.4%)	13 (7.6%)	
<i>CYP21A2</i> (rs12525076)			1.00
AA	37 (92.5%)	3 (7.5%)	
AG	111 (90.2%)	12 (9.8%)	
GG	105 (90.5%)	11 (9.5%)	