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Neonatal Genetic Variation in Steroid Metabolism and Key Respiratory Function Genes and Perinatal Outcomes in Single and Multiple Courses of Corticosteroids

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Abstract

OBJECTIVE—To evaluate the association of steroid metabolism and respiratory gene polymorphisms in neonates exposed to antenatal corticosteroids (ACS) with respiratory outcomes, small for gestational age (SGA) and response to repeat ACS.

STUDY DESIGN—This candidate gene study is a secondary analysis of women enrolled in a randomized controlled trial of single versus weekly courses of ACS. Nineteen single nucleotide polymorphisms (SNPs) in 13 steroid metabolism and respiratory function genes were evaluated. DNA was extracted from placenta or fetal cord serum and analyzed with TaqMan genotyping. Each SNP was evaluated for association via logistic regression with respiratory distress syndrome (RDS), CPAP/ventilator use (CPV) and SGA.

RESULTS—*CRHBP*, *CRH* and *CRHR1* minor alleles were associated with an increased risk of SGA. *HSD11B1* and *SCNN1B* minor alleles were associated with an increased likelihood of RDS.

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Carriage of minor alleles in *SerpinA6* was associated with an increased risk of CPV. *CRH* and *CRHR1* minor alleles were associated with a decreased likelihood of CPV.

CONCLUSION—Steroid metabolism and respiratory gene SNPs are associated with respiratory outcomes and SGA in patients exposed to ACS. Risks for respiratory outcomes are affected by minor allele carriage as well as by treatment with multiple ACS.

Keywords

antenatal corticosteroids; candidate gene; respiratory distress syndrome

Introduction

Antenatal corticosteroids have long been shown to decrease the rates of neonatal respiratory distress syndrome and overall mortality in premature infants.[1] Although several randomized trials have found benefits in neonatal respiratory outcomes with multiple corticosteroid courses, increased risks of intraventricular hemorrhage and impaired fetal growth, particularly head circumference, have also been reported.[2,3,4] Corticosteroids are widely used in medicine and impact a variety of physiologic systems including the immunologic response, metabolism and the stress response. Genes involved in corticosteroid metabolism and respiratory function have been evaluated in other disease states. The impact of genetic variation in Corticotropin Releasing Hormone Receptor 1 (*CRHR1*) and lung function has been evaluated in asthmatics by looking at the response to inhaled corticosteroids. Evidence of variation in response to corticosteroids related to the genotype was identified.[5,6] Functional studies of 11 Beta Hydroxysteroid Dehydrogenase, Type 2 (*HSD11B2*) have shown corticosteroid mediated regulation of surfactant protein A altered by *HSD11B2* activity.[7] Given these examples of clinical outcome associations with gene polymorphisms in the corticosteroid metabolism pathway, we hypothesized that genetic variation in corticosteroid metabolism and key respiratory function genes may play a role in adverse neonatal outcomes and anthropometric measurements through altered response to antenatal corticosteroid administration.

Evidence suggesting a genetic contribution to RDS come from both human and mouse studies. Lethal forms of respiratory distress syndrome (RDS) can be caused by mutations in pulmonary genes including: Surfactant Protein B (*SFTPB*), Surfactant Protein C (*SFTPC*) and ATP-Binding Cassette, Subfamily A, Member 3 (*ABCA3*).[8,9,10] Additionally, *Sftpb* knockout mice die immediately after birth of respiratory failure.[11] An intronic polymorphism in *SFTPB* has been associated with RDS in premature neonates.[12] These studies suggest that genetic variation in key pulmonary genes may contribute to RDS diagnosed in the preterm infant.

Materials and Methods

This is a secondary analysis of a cohort of pregnant women enrolled at 23 0/7–31 6/7 weeks of gestation from a previously reported, randomized, placebo-controlled, multi-center trial of single versus multiple courses of antenatal corticosteroids (2 doses of Betamethasone 12mg IM administered 24 hours apart), conducted between March 2000 and April 2003 at

participating centers of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Maternal-Fetal Medicine Units (MFMU) Network.[3] A subset of enrolled subjects had placental samples and fetal cord serum collected per study protocol. Following IRB review, this study was determined to be exempt from IRB approval due to de-identification of study samples and data prior to this analysis.

The objective of this secondary analysis, utilizing a candidate gene design, was to determine if genetic variation in fetal corticosteroid metabolism genes and key respiratory genes were associated with adverse neonatal outcomes and growth abnormalities evaluated by birthweight. The neonatal outcomes evaluated were respiratory distress syndrome (RDS), assisted ventilation, and small for gestational age (SGA), all of which have been associated with antenatal corticosteroid administration. RDS was defined as clinical features of RDS with the need for oxygen and respiratory support from 6 to 24 hours or more of age, an abnormal chest-x-ray, and either administration of a full course of surfactant or a fraction of inspired oxygen of at least 60%. Assisted ventilation included all neonates requiring either continuous positive airway pressure or intubation and ventilation (CPV) for greater than 1 day. SGA was defined as birth weight less than the tenth percentile using the Alexander nomogram.[13] All infants with a cord blood serum or placental sample available for analysis were included in this study. Exclusions from the original study were preterm premature rupture of the membranes, confirmed fetal lung maturity, chorioamnionitis, major fetal anomaly, nonreassuring fetal status, systemic corticosteroid used during the current pregnancy, or insulin-dependent diabetes.[3] As we described in our previous publication,[14] there were 272 infants with biologic samples for DNA analysis. In this study, 215 samples were genotyped successfully. Placental samples were used preferentially, when both cord serum and placental samples were available, secondary to higher quality DNA.[14] There were 43 twin pregnancies and samples were available on both twins in 21 of these multiple gestations. One infant of each twin pair was randomly selected for inclusion in this analysis, which removed 21 infants leaving a total of 194 samples analyzed in this study, out of 594 fetuses/infants enrolled in the original trial.[3]

DNA was extracted from placental samples and/or umbilical cord serum collected at the time of delivery. DNA was extracted from approximately 0.7 gm of fresh placental tissue or from 3–6, five micron sections obtained from the paraffin block using the PureGene DNA Purification System (Qiagen, Valencia, CA) as per the manufacturer's protocols. DNA was also extracted from umbilical cord serum samples by centrifuging 250–1000 μL of cord serum at 10,000 × g for 10 minutes to pellet any cells in the sera. The supernatant was discarded and DNA was extracted with the same procedure used for placental samples.[14]

Candidate genes were chosen based on pathway analysis, review of the current literature and biological plausibility. Single nucleotide polymorphisms (SNPs) within each gene were selected using data from the International HapMap project (www.hapmap.org) with a minor allele frequency of 0.1 or greater. Each gene was minimally tagged given limited sample quantity for the analysis. We evaluated 19 SNPs in 13 genes that are listed in Table 1.

The samples were diluted to 2ng/ul and 1ul transferred to a 384 well plate for genotype analysis. Positive and negative controls were included on each 384 well plate. Allelic

variation was performed using the TaqMan® genotyping system (Applied Biosystems, Foster City, CA, US), with PCR reactions performed utilizing an ABI GeneAmp9700 thermocycler. Allelic discrimination was performed using the Sequence Detection Systems 2.3 software (Applied Biosystems).

Hardy-Weinberg equilibrium (HWE) exact test was performed for all markers and those with a p-value<0.01 were not included in further analysis. Chi-square test or Wilcoxon rank sum test as appropriate was used to assess differences in maternal and neonatal characteristics. Genotypes were included as predictors in a series of regression models that assumed an additive, dominant or recessive genetic pattern. The additive model assumes that having 2 copies of the minor allele has twice the effect of having only 1 copy; the dominant model assumes that having at least 1 copy of the minor allele is sufficient for outcome effect and the recessive model assumes that 2 copies of the minor allele are needed for outcome effect. The genotype model reported for each identified SNP is the one with lowest p-value determined in logistic regression analyses. A categorical variable of race was included in our models as an adjustment term. An interaction effect of single versus multiple dose steroids and genotype was evaluated by the chi-square p-value in logistic regression model. A p-value of <0.05 was considered to be statistically significant and no adjustments were made for multiple comparisons, as this was an exploratory analysis. All statistical analysis was performed using R (http://www.r-project.org/) and SAS software (SAS Institute, Inc, Cary, NC).

Results

Demographic characteristics for subjects included in this analysis are shown in Table 2. As summarized in Supplemental Table 1, the samples included in this study are representative of the samples in the original trial.[3] The percentage of genotype call by marker varied from 98.5% to 81%. Two SNPs had HWE exact test with a p-value <0.01 (Surfactant protein B (*SFTPB* rs2040349) and Corticotropin-releasing hormone (*CRH* rs4613981)). Both of these SNPs had higher rates of genotyping failures at 13% and 19% respectively. This higher rate of genotyping failure may be the cause of Hardy Weinberg disequilibrium. Further analysis of these markers was not performed due to limited available DNA.

Only one significant genetic association (*CRHBP* rs10062367 and SGA) was observed among all the recessive models. However, a more significant result was observed when the additive model was used for this association. Significant genetic associations with SGA, RDS and CPV are shown in Table 3. They were detected by either the dominant or the additive genetic models. The gestational age variable was evaluated for each reported SNP and no significant difference was observed.

An interacting effect of single course antenatal corticosteroids versus multiple course steroids was evaluated and significant interactions are displayed in Table 4. The referent cases are single course corticosteroids without minor alleles. An interacting effect was seen (p=0.019) with peptidase inhibitor, clade A, member 6, also known as corticosteroid binding globulin or *SerpinA6* rs3748320 and RDS with a significantly decreased risk of RDS in the multiple course steroid treatment group without minor alleles (odds ratio 0.30, 95%

confidence interval [0.09, 0.95]) but no significant difference in risk of RDS in the multiple course steroid group with minor alleles (odds ratio 1.29, 95% confidence interval [0.43, 3.91]) compared to those without a minor allele in single dose steroid group. An interacting effect was also noted with the epithelial sodium channel (ENaC), nonvoltage-gated 1-β gene, *SCNN1B* rs239349 and CPV with a decreased risk of CPV in the multiple course steroid treatment group without minor alleles (odds ratio 0.08, 95% confidence interval [0.009, 0.66]) but no significant difference in risk in the multiple course steroid group with any minor allele (odds ratio 0.78, 95% confidence interval [0.28, 2.18]) compared to those without a minor allele in single dose steroid group.

Discussion

We performed a candidate gene analysis of steroid metabolism and respiratory function genes and found associations both with the genotype and respiratory outcomes as well as treatment effect with genotype. Previous studies have evaluated corticosteroids, genotype and clinical outcomes, such as effectiveness in relationship to asthma.[5,6] This study is the first step in evaluating a novel gene-environment interaction, key respiratory genes and antenatal corticosteroids, on perinatal outcomes.

HSD11B1, 11 Beta Hydroxysteroid Dehydrogenase, Type 1, mediates the reversible conversion of cortisol to the inactive cortisone. Glucocorticoid response may be altered by *HSD11B1* function. A role for *HSD11B1* in the inflammatory response has been identified,[15] and previous studies have suggested a role for prenatal inflammation and fetal/neonatal lung injury.[16] This may explain the relationship identified between *HSD11B1* genotype and increased risk for RDS. *SerpinA6*, also known as cortisol binding globulin or transcortin has been shown to alter cortisol binding and degradation when mutated.[17] This finding lends biologic plausibility to genetic variation in *SerpinA6* being associated with altered cortisol effectiveness and risk of RDS and respiratory impairment.

Low activity levels of the sodium channel, nonvoltage-gated1-β gene (*SCNN1B*) have been associated with impaired lung fluid clearance in the mouse.[18] Our study found a decreased risk of CPAP or ventilation with multiple steroids and no minor alleles in *SCNN1B* which may relate to expression levels and clearance of amniotic fluid from the neonatal lungs that are related to genotype and steroid dose.

Both *CRH* and *CRHR1* have been identified to play a role in glucocorticoid reactivity. A recent study of corticotropin releasing hormone knockout mice found reduced lung membrane glucocorticoid receptors in response to stress in the knockout mouse compared to wildtype.[19] This may explain how a genetic variant in *CRH* may effect respiratory status in the stressful neonatal transition. Genetic variation in *CRHR1* has also been associated with persistent pulmonary hypertension of the newborn suggesting a possible role with pulmonary disease in the newborn.[20] Prior studies also suggest a role for the corticotrophin releasing hormone system regulation of birthweight and SGA which we again identified in our study.[21,22,23]

Strengths of the study include that we assessed multiple genetic variants and their relationship to neonatal outcomes and antenatal corticosteroid dose and that this was done in the context of a large randomized, controlled trial to minimize biases of inclusion. Limitations to this study include that only neonates were evaluated for genetic variation and there may be maternal genetic variation that contributes to corticosteroid effectiveness and adverse perinatal outcomes. Another limitation is that samples from all neonates of enrolled subjects in the steroid trial were not available. It is possible there were differences between those who had samples available and those who did not with respect to genotypes. Likewise, the DNA extracted from cord blood serum and fixed placental sections provides lower quality and quantity DNA than that extracted from peripheral blood leukocytes. As a result, a lower genotype call rate was seen in this study. As documented by our generally wide confidence intervals, the relatively small sample size emphasizes the exploratory nature of this report.

The reported SNP associations may be the result of one of three findings: the reported SNP is itself associated with the outcome, the reported SNP is in linkage disequilibrium with the causal SNP which is associated with the outcome or the association reported is a false positive association. Association with prematurity or gestational age rather than neonatal outcomes is unlikely given the negative association of the SNPs with gestational age. Replication of these findings in a second population is important to validate these findings. Due to the exploratory nature of this study Bonferroni correction was not applied to the statistical results. If this was performed the p-value to suggest a significant association would be .05/17=0.003. None of our SNP associations reach this level of significance.

Debate about the use of multiple courses of antenatal corticosteroids is longstanding. While improved respiratory outcomes have been attributed to multiple courses of steroids, especially in early gestation, there continues to be concern about the effect of multiple doses on growth and development.[3,24] If genotype plays a role in determining risk for neonatal outcomes or complications of antenatal steroids, then this may provide a way to stratify risk of a repeat course of steroids in the future. It is currently unknown if using genotype to risk stratify for multiple doses of antenatal corticosteroids would decrease overall neonatal morbidity and mortality. This study provides preliminary data and genes of interest for further translational as well as functional studies. In the future, these novel gene-environment interactions may help to predict adverse perinatal outcomes and steroid treatment response. Ultimately, this genotype information may guide clinical care with regards to antenatal corticosteroid administration and safety.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

In addition to the authors, other members of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network are as follows:

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Table 1

Candidate Genes

Gene Name	Pathway	Rs number	Chromosome	Position	Alleles
HSD11B1	Steroid Metabolism	2235543	1	209860668	T/C
HSD11B1	Steroid Metabolism	2282739	1	209886001	C/T
SFTPB	Respiratory Function	2040349	2	85889525	T/G
SFTPB	Respiratory Function	1030862	2	85902569	G/A
CRHBP	Steroid Metabolism	1700678	5	76239396	C/A
CRHBP	Steroid Metabolism	10062367	5	76264354	A/G
NR3C1	Steroid Metabolism	33383	5	142709986	C/T
NR3C1	Steroid Metabolism	4128753	5	142826792	A/G
SFTPC	Respiratory Function	2070687	8	22021388	C/G
CRH	Steroid Metabolism	4613981	8	67114113	A/G
CRH	Steroid Metabolism	2446432	8	67118308	T/G
SFTPD	Respiratory Function	721917	10	81706324	T/C
SCNN1A	Respiratory Function	3759333	12	6491947	C/T
SERPINA6	Steroid Metabolism	3748320	14	94780608	T/C
SCNN1G	Respiratory Function	4486893	16	23219210	G/T
SCNN1B	Respiratory Function	239349	16	23352848	A/G
HSD11B2	Steroid Metabolism	5479	16	67469733	A/C
CRHR1	Steroid Metabolism	7225082	17	43848495	T/C
CRHR1	Steroid Metabolism	173365	17	43901074	T/C

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Table 2

Characteristics of subjects with single vs. multiple ACS

	Single ACS (97 total)	Multiple ACS (97 total)	P-value
Ethnicity			
African American	37 (38.1)	37 (38.2)	
Caucasian	35 (36.1)	36 (37.1)	0.983
Other	25 (25.8)	24 (24.7)	
Gender			
Male infant	52 (53.6)	50 (51.6)	0.774
Female infant	45 (46.4)	47 (48.4)	
Gestational age at birth (weeks)	34.9 ± 3.9	35.4 ± 3.9	0.271
Twin gestation	26 (26.8)	17 (17.5)	0.120
RDS	15 (15.5)	11 (11.3)	0.399
CPAP/Ventilation>1day	24 (24.7)	15 (15.5)	0.107
SGA	17 (17.5)	19 (19.6)	0.712
Birth weight (grams)	2347.3 ± 800.3	2405.1 ± 820.2	0.590

Data presented as mean ± SD or n (%).

Table 3

Significant genetic association with SGA, RDS and CPV

Gene rs number	Outcome	Odds Ratio with 95% confidence interval, (p-value)	Strongest Genetic Model
CRHBP rs10062367	SGA	2.04 [1.12, 3.71] (0.019)	Additive
CRH rs2446432	SGA	2.88 [1.03, 8.07] (0.044)	Dominant
CRHR1 rs7225082	SGA	3.42 [1.22, 9.58] (0.019)	Dominant
HSD11B1 rs2282739	RDS	2.09 [1.08, 4.05] (0.030)	Additive
SCNN1B rs239349	RDS	3.18 [1.08, 9.42] (0.036)	Dominant
CRH rs2446432	CPV	0.44 [0.20, 0.97] (0.041)	Dominant
SerpinA6 rs3748320	CPV	2.35 [1.02, 5.37] (0.044)	Dominant
CRHR1 rs7225082	CPV	0.39 [0.17, 0.91] (0.029)	Dominant

*The referent group is without minor alleles

Table 4

Interaction effect of single versus multiple dose steroids and genotype

Strongest Genetic Model	Gene rs number	Outcome (interaction p-value)	Single Course with minor alleles	Multiple Courses without minor alleles	Multiple Courses with minor alleles
Additive Model	**SerpinA6** rs3748320	RDS (0.019)	0.44 [0.11, 1.81]	0.30 [0.09, 0.95]	1.29 [0.43, 3.91]
Dominant Model	**SCNN1B** rs239349	CPV (0.040)	0.87 [0.32, 2.41]	0.08 [.009, 0.66]	0.78 [0.28, 2.18]

*The referent group is single course steroids without minor alleles. Results for comparison with single course and multiple courses are Odds ratios with 95% confidence intervals.