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Single Nucleotide Polymorphism in Toll-Like Receptor 6 is Associated with a Decreased Risk for *Ureaplasma* Respiratory Tract Colonization and Bronchopulmonary Dysplasia in Preterm Infants

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Abstract

Background—*Ureaplasma* spp. respiratory tract colonization is a risk factor for broncho-pulmonary dysplasia (BPD) in preterm infants, but differences in host susceptibility have not been elucidated. We hypothesized that variants in genes regulating the innate immune response are associated with altered risk for *Ureaplasma* spp. respiratory colonization and BPD in preterm infants.

Methods—Twenty-four tag SNPs from *TLR1*, *TLR2*, *TLR4*, and *TLR6* were assayed in 298 infants <33 weeks gestation who had serial respiratory cultures for *Ureaplasma* species and were evaluated for BPD.

Results—The majority of subjects (N=205 [70%]) were African-American. One hundred and ten (37%) were *Ureaplasma*-positive. Four SNPs in *TLR2* and *TLR6* were significantly associated with *Ureaplasma* respiratory tract colonization. Single SNPs in *TLR2*, *TLR4*, and *TLR6* were associated with BPD. *TLR6* SNP rs5743827 was associated with both a decreased risk for *Ureaplasma* respiratory tract colonization and decreased risk for BPD (OR=0.54 [0.34-0.86] and OR=0.54 [0.31-0.95], respectively). There was a significant additive interaction between *Ureaplasma* colonization and genotype at *TLR6* SNP rs5743827 ($P_{\text{additive}} = 0.023$), with an attributable proportion due to interaction of 0.542.

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Conclusions—Polymorphisms in host defense genes may alter susceptibility to *Ureaplasma* infection and severity of the inflammatory response contributing to BPD. These observations implicate host genetic susceptibility as a major factor in BPD pathogenesis in *Ureaplasma*-infected preterms.

Keywords

Ureaplasma parvum, *Ureaplasma urealyticum*, TLR6; single nucleotide polymorphism

Introduction

Approximately 90% of preterm births prior to 28 weeks gestation are associated with intrauterine infection and/or inflammation.¹ Despite recent advances in neonatal care to minimize postnatal lung injury from mechanical ventilation, exposure to antenatal infection and inflammation is associated with increased risk for bronchopulmonary dysplasia (BPD) in extremely low gestational age newborns (ELGANs, <28 weeks gestation).^{2, 3} Recently, research has focused on identifying the genetic contribution to risk of intrauterine infection, preterm birth, and BPD. Twin concordance studies have suggested that the contribution of genetic risk to BPD is high, accounting for 35-65% risk for the outcome.⁴

The genital mycoplasma species *Ureaplasma parvum* (4 serovars) and *U. urealyticum* (10 serovars) have been implicated in complications of human pregnancy⁵ and adverse neonatal outcomes including bronchopulmonary dysplasia (BPD)^{6, 7} and necrotizing enterocolitis (NEC).⁸ The organisms are vertically transmitted from mother to infant early during pregnancy as the result of an intrauterine infection or just prior to or at the time of preterm birth as the result of an ascending infection from the lower genital tract. The organism's virulence and host immunity factors that contribute to the establishment of an intrauterine infection, vertical fetal transmission, and neonatal lung injury are poorly understood.

Toll like receptors (TLRs) are important components of the innate immune system that respond to a broad range of pathogen-associated molecular patterns (PAMPs).^{9, 10} Engagement of TLR proteins and subsequent signaling activates the transcription factor NF- κ B and upregulation of gene expression of pro-inflammatory mediators by macrophages, neutrophils, dendritic cells, B cells, endothelial cells, and epithelial cells. Recent studies by Peltier et al.¹¹ and Shimizu et al.¹² demonstrated that lipoproteins from *U. urealyticum* serovar 4 and *U. parvum* serovar 3 are responsible for NF- κ B activation by these species. The serovar 3 extract activated NF- κ B through TLR2 cooperatively with TLR1 and TLR6,¹² while serovar 4 extract activated both TLR2 and TLR4.¹¹ Although SNPs in specific TLRs have been identified in association with increased susceptibility for many inflammatory diseases, the relationship of polymorphisms in relevant TLRs to susceptibility to neonatal *Ureaplasma* respiratory colonization and BPD is unknown.

We hypothesized that variants in genes that are activated by *Ureaplasma* are associated with altered susceptibility to *Ureaplasma* respiratory colonization and consequent risk for BPD in preterm infants. To test our hypothesis, we determined whether polymorphisms in *TLR1*, *TLR2*, *TLR4*, and *TLR6* are associated with *Ureaplasma* respiratory tract colonization and/or BPD in infants born <33 weeks gestation who had serial respiratory samples analyzed for *Ureaplasma* by culture and PCR.

Methods

Study Population

Subjects were recruited from two cohorts (Figure 1). The first cohort included infants with gestational age <33 weeks and birthweight <1501 g admitted to the neonatal intensive care units at the University of Maryland Medical Center and Mercy Medical Center (Baltimore, MD, USA) between May 1999 and December 2002.¹³ Serum-derived DNA was available from 195 infants; however only 174 had known *Ureaplasma* respiratory culture status in the first week of life. The second was a prospective cohort with infants <33 weeks gestation and <72 h of age admitted to the University of Maryland Medical Center neonatal intensive care unit recruited between March 2007 and March 2010. Of the 382 infants who were <33 weeks gestation, 329 were eligible and 53 infants were excluded due to non-viability assessment or major congenital malformations. Consent was not sought from parents of 112 eligible infants due to unavailability of parent (N=44); non-English speaking parent (N=6); mother <18 years of age (N=2); infant enrolled in another study (N=16); infant transferred to another facility or died <72 h of age (N=5); or missed due to unavailability of study investigators (N=39). Of the remaining 217 eligible infants, 124 (57.1 %) were enrolled after parental consent was obtained. For the second cohort, there were no significant differences between eligible, enrolled infants (N=124) and eligible, non-enrolled infants (N=205) for birthweight (1213 ± 404 vs. 1191 ± 420 g, $p = 0.632$), gestational age (29.2 ± 2.7 vs. 28.9 ± 2.6 week; $p = 0.248$) or severity of illness ($p=0.484$) assessed by mortality and need for respiratory support (0% vs. 1.2% death, 40% vs. 45.5% mechanical ventilation, 31.2% vs. 24.5% nasal continuous positive pressure, 28.8% vs. 28.8% no positive pressure support ($p=0.484$)), respectively. For the second cohort, buccal swabs were collected for genomic DNA and serial tracheal aspirates and/or nasopharyngeal specimens were obtained at 0-3 d, 7-10 d and 28-30 d of age for *Ureaplasma* culture and species-specific PCR.¹⁴ For both cohorts, demographic information including self-reported race and ethnicity, obstetric, and neonatal data were recorded. Self-reported race is highly correlated with geographic ancestry.¹⁵ The University of Maryland School of Medicine and Mercy Medical Center Institutional Review Boards approved the protocols.

Clinical Outcomes

An infant was considered *Ureaplasma*-positive if one or more respiratory sample from the nose or endotracheal tube was culture-confirmed and/or PCR-positive. An infant was assigned the diagnosis of BPD if the infant received supplemental oxygen and/or positive pressure support at 36 weeks post-menstrual age.

Genotyping Assays

DNA was extracted from serum using the QiAmp DNA Blood Mini kit or buccal swabs using the PureGene Buccal cell core kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. As part of phase II of the HapMap Project, TLR1, TLR2, TLR4 and TLR6 were resequenced from DNA obtained from 30 trios from the CEPH population (Utah residents with northern or western European ancestry) and 30 trios from the Yoruba population (residents of Ibadan, Nigeria).¹⁶ A haplotype-tagging strategy using publicly available software and single-nucleotide polymorphisms (SNPs) identified in the intronic sequence, 6 kb of 5' genomic DNA and 2 kb of 3' genomic DNA, was implemented to reduce the number of SNPs analyzed and to capture the polymorphic structure of the gene.¹⁷ The algorithm was based on polymorphic sites that exceeded a 10% minor allele frequency (MAF) for CEPH trios, a 20% MAF for Yoruba trios, and a within-bin linkage disequilibrium exceeding an r^2 value of 0.7. This algorithm resulted in several bins, often with multiple SNPs in each bin. A single SNP was selected for analysis from each bin. From bins containing multiple SNPs, we preferentially selected polymorphisms located in the

coding sequence. In addition, missense mutations with a minor allele frequency greater than or equal to 5% were included for genotyping. Twenty-four SNPs from four *TLR* genes were assayed.

DNA samples were genotyped using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom Inc., San Diego, CA). Multiplex PCR assays and associated extension reactions were designed using the SpectroDESIGNER software (Sequenom Inc.). Primer extension products were loaded onto a 384-element chip with a nanoliter pipetting system (Sequenom Inc.) and analyzed by a MassARRAY mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The resulting mass spectra were analyzed for peak identification by using SpectroTYPER RT 4.0 software (Sequenom Inc.). For genotyping quality control, Hardy-Weinberg calculations were performed to ensure that each marker was within the expected allelic population equilibrium. Assays not in Hardy-Weinberg equilibrium were excluded from our analyses ($n = 2$). Genotype information was obtained for 298 individuals and 24 tagSNPs.

Statistics

Comparison of demographics, obstetric and neonatal variables, and clinical outcomes (BPD and death) between *Ureaplasma*-positive and negative infants for the combined cohorts were analyzed by two-sample Student t-tests and chi-square tests. For analyses stratified by race, one individual from each multiple birth ($n = 23$ excluded) was randomly chosen, infants of Hispanic ethnicity were excluded due to the small numbers ($n = 8$), and all those not of singularly non-Hispanic Caucasian or African-American self-reported race ($n = 13$) were excluded. Mode of delivery-, cohort-, and sex-adjusted logistic regression analyses was performed for *Ureaplasma* colonization and BPD as outcomes. For the total sample, race was included as a covariate and parameterized as either African-American or not. Odds ratios (ORs) were calculated based on an additive model, where gene dosage was parameterized as 0, 1, or 2 copies of allele 2. For the *TLR6* rs5743827 polymorphism, we tested for both multiplicative and additive interaction. Multiplicative interaction was quantified by modeling the polymorphism-*Ureaplasma* status product terms in the regression models. The attributable proportion due to interaction (AP) and corresponding *P*-value served as the primary measure of additive interaction, in which an AP equal to 0 corresponds to no interaction and an AP of 1.0 corresponds to complete additive interaction.¹⁸ The 95% CIs were calculated for the AP using the method of Hosmer and Lemeshow.¹⁹ These analyses were performed using the software package SAS version 9.2. Because this was a hypothesis-driven study with a specific candidate gene list, correction for multiple testing was not done.

Results

The characteristics of the subjects in the two groups, *Ureaplasma*-positive ($n = 110$; 37%) and *Ureaplasma*-negative ($n = 188$), are shown in Table 1. The population was evenly balanced with regard to sex, with 156 (52%) males and 142 (48%) females. The majority of subjects ($n=205$ (70%); $n=191$ after exclusion of all but one infant from multiple births) were African-American, while the remaining subjects were non-Hispanic Caucasian ($n=72$ (24%); $n=63$ after exclusion of all but one infant from multiple births), Asian ($n=4$; 1%), Native Hawaiian/Pacific Islander ($N=1$; 0.3%), mixed ($n=7$; 2%), or unknown race ($n=1$; 0.3%). Eight (3%) individuals reported Hispanic ethnicity. As expected in a study of premature birth, 71 (24%) were twins or triplets.

The 2 cohorts differed for several variables. Compared to cohort 2 recruited from 2007-2010, cohort 1 recruited from 1999-2002 was less mature (GA 27.1 ± 2.3 vs. 28.9 ± 2.8 wk, mean $\pm$ SD; $p<0.0001$), lower birthweight (BW 936 ± 265 vs. 1206 ± 415 g,

$p < 0.0001$), greater frequency of African-American race (75% vs 60%, $p = 0.009$) and experienced longer duration of mechanical ventilation (18.2 ± 19.2 vs. 9.3 ± 16.7 d, $p < 0.0001$). There was no difference in antenatal steroid use between cohorts 1 and 2 (cohort 1, 83.33%; cohort 2, 82.26%, $p = 0.808$). There was a reduced exogenous surfactant use in the second cohort (45% vs 76%, $p < 0.001$) reflecting the increase use of non-invasive respiratory support during the later recruitment period. The difference in BPD rates between the cohorts was not statistically significant (cohort 1, 26.7%; cohort 2, 19.7%, $p = 0.168$). Because of differences in clinical practice and characteristics of the 2 cohorts, cohort was included in all regression models as well as race, sex, and mode of delivery.

The *Ureaplasma* colonization rate was slightly higher in cohort 2 than cohort 1, but the difference in colonization rate was not statistically significant (Cohort 1, 34.5%; Cohort 2, 40.3%, $p = 0.303$). Only 2 of 50 (4%) infants in cohort 2 were detected by PCR alone, suggesting that few colonized infants were missed in cohort 1 with culture methodology alone. Seventy-three percent of *Ureaplasma*-positive infants were colonized with *U. parvum*, 25% with *U. urealyticum*, and 2% with both species. *Ureaplasma* respiratory tract colonization was associated with gestational age ≥ 28 weeks, birthweight ≥ 1000 grams, preterm labor, preterm premature rupture of membranes (pPROM), vaginal delivery, histologic chorioamnionitis, fetal vasculitis, and prolonged mechanical ventilation (Table 1). *Ureaplasma* respiratory colonization was also associated with a 1.94-fold increased risk for BPD (95% CI = 1.11-3.39; $p = 0.018$).

Minor allele frequencies for all tested tagSNPs are summarized in Table 2 for *Ureaplasma* cases and controls in the total sample and stratified by African-American race and Table 3 for BPD cases and controls in the total sample and stratified by African-American race. Tables, Supplemental Digital Content 1 and 2, <http://links.lww.com/INF/B537> and <http://links.lww.com/INF/B538> provide the summary of minor allele frequencies of tested tagSNPs in non-Hispanic Caucasians for *Ureaplasma* cases and controls and BPD cases and controls, respectively. The minor allele frequencies for all polymorphisms were consistent with frequencies reported from the HapMap project (<http://www.ncbi.nlm.nih.gov/SNP/index.html>).

To address our hypothesis, we tested the association of 24 SNPs in *TLR1*, *TLR2*, *TLR4* and *TLR6* with *Ureaplasma* respiratory tract colonization or BPD. For both outcomes, there was a consistent association with *TLR6* (rs5743827). The A-allele for rs5743827 was associated with decreased odds of *Ureaplasma* colonization ($p_{\text{adj}} < 0.01$; $p_{\text{adj}} = 0.03$) and BPD ($p_{\text{adj}} = 0.03$; $p_{\text{adj}} = 0.03$) in the total sample and African-Americans respectively, suggesting a protective effect for this polymorphism (Tables 2 and 3). Compared to infants homozygous for the major allele G at rs5743827, infants with one or 2 copies of the A allele were less likely to have been delivered following preterm labor (83% GG vs. 70% AA/AG, $p = 0.008$), preterm premature rupture of membranes (49% GG vs. 34% AA/AG, $p = 0.0123$), or rupture of membranes > 24 h (43% GG vs. 24% AA/AG, $p = 0.008$). While this association was not statistically significant in the non-Hispanic Caucasians, the magnitude and direction of the point estimate is nearly the same (OR = 0.55 per “A” allele in African Americans and 0.48 per “A” allele in non-Hispanic Caucasians) (Table, Supplemental Digital Content 1, <http://links.lww.com/INF/B537>). Also, adding non-Hispanic Caucasian to African-Americans in the total sample strengthens the association.

No other tested SNP in the total sample or African-Americans was associated with *Ureaplasma* respiratory tract colonization. An additional SNP in *TLR4* (rs11536898) was associated with BPD, infants with the A-allele had an increased risk for BPD compared with those with the C-allele in the total sample and in the African-American population (OR = 1.9, $p = 0.01$ and OR = 1.83, $p = 0.03$, respectively) (Table 3). Among non-Hispanic

Caucasians, additional tagging SNPs in *TLR2* and *TLR6* were associated with *Ureaplasma* colonization and BPD, although the population was very small ($n = 63$) (Tables, Supplemental Digital Content 1 and 2, <http://links.lww.com/INF/B537> and <http://links.lww.com/INF/B538>).

Analyses were then conducted to examine the additive and multiplicative interaction between *TLR6* SNP rs5743827 and *Ureaplasma* colonization in relation to BPD among the total sample and among African-Americans. For the total sample, compared to infants without either risk factor, infants with both the risk genotype (GG) at rs5743827 and *Ureaplasma* colonization had greater odds of BPD (OR=3.46, CI=1.58-7.61) (Figure 2A, Table 4A). There was significant evidence for an additive interaction with *Ureaplasma* colonization and the risk genotype ($P_{\text{additive}} = 0.023$); however no interaction was seen using multiplicative modeling ($P_{\text{multiplicative}} = 0.25$). Since the sample size was small, the multiplicative test was underpowered. In the African-American population, compared to infants without either risk factor, infants with both the risk genotype (GG) at rs5743827 and *Ureaplasma* colonization had greater odds of BPD (OR=5.25, CI=1.82-15.11) (Figure 2B, Table 4B). There was evidence of an additive interaction, but the test was underpowered again due to small sample size.

Discussion

The present study evaluated the associations of common genetic variations in *TLR1*, *2*, *4*, and *6* to risk for *Ureaplasma* respiratory tract colonization and BPD. We demonstrated for the first time that tagSNPs in relevant TLRs are associated with either an increased or decreased risk for *Ureaplasma* respiratory colonization. Specifically, the A allele of *TLR6* rs5743827 reduced both the risk for *Ureaplasma* respiratory tract colonization and BPD outcome in *Ureaplasma*-colonized infants. We speculate that, by causing altered host recognition of ureaplasma lipoproteins, TLR6 mutations impact susceptibility to *Ureaplasma* respiratory tract colonization, pathogen clearance, and duration and severity of inflammatory response that contributes to BPD and other adverse outcomes.

The *TLR6* SNP rs5743827 located in the 3' untranslated region (UTR) was associated with decreased *Ureaplasma* colonization. This suggests that the mutation contributed to decreased susceptibility to *Ureaplasma* infection or enhanced clearance in affected infants. However, since infants with the minor A allele were less likely exposed to preterm labor, preterm premature rupture of membranes, and longer duration of ruptured membranes than infants homozygous for the major G allele, we speculate that an alternative explanation is less exposure to *Ureaplasma in utero* or lower rates of vertical transmission. This will need to be resolved by subsequent studies of paired maternal/neonatal cultures and genotyping.

TLR6 interacts with TLR2 to form distinct binding pockets to engage diacylated lipopeptides in Gram-positive, Gram-negative, and mycoplasma bacteria.^{20, 21} Studies in human embryonic kidney HEK293T cell transfected with naturally occurring *TLR1*, *2*, or *6* variants *in vitro*, demonstrated that many variants encoding amino acid alterations impair NF- κ B activation.²² This suggests that mutations in these TLRs will adversely affect immune responses, thereby contributing to increased susceptibility to infections and dysregulated inflammatory responses. As shown in Figure 3 and Tables 4a and 4b, TLR6 SNP rs5743827 has the greatest effect on BPD when an infant is colonized with *Ureaplasma*. This suggests that when *Ureaplasma* is present and TLR2/TLR6 signaling is normal (GG at rs5743827), then an inflammatory signaling cascade may occur, increasing the likelihood of BPD. The SNP's location in the 3' UTR suggests the potential to alter mRNA translation, localization, and stability.²³ A recent systematic analysis of 3' UTR mutations suggested that 0.2% of known disease-associated mutations reside in this region,

but the authors suggested that this is likely a conservative estimate.²⁴ However, since tagSNPs were assayed in this study, this SNP may not be the causal SNP; rather it may be in linkage disequilibrium (LD) with the causal SNP located elsewhere in the gene.

The relative contribution of species or serovar-specific pathogen virulence and host factors to *Ureaplasma*-mediated lung injury has been debated. Abele-Horne et al.²⁵ reported that the BPD rate was 2-fold higher for *U. urealyticum*-colonized infants than the rate for *U. parvum*-colonized infants. Katz et al.²⁶ found no difference in BPD rates between infants colonized with either species, but a higher rate of BPD in infants positive for both species. In a previous analysis of *Ureaplasma* species and serovars from our cohort, we did not observe differences in BPD rates between the *Ureaplasma* species or differences in BPD severity among the serovars.¹⁴ This suggests that *Ureaplasma* virulence may be species and serovar-independent with regards to neonatal lung disease. The current study suggests that host factors such as genetic differences in host defense genes may be more important in determining the differences in susceptibility to *Ureaplasma* respiratory tract colonization and variable severity of lung injury and inflammation.

Arrested alveolar and vascular development is the hallmark of the “new BPD”.²⁷ Multiple clinical studies of human infants and experimental models confirm that an imbalance of pro- and anti-inflammatory factors in the lung during critical periods of lung development contribute to altered developmental signaling.²⁷⁻²⁹ Lung inflammation is often initiated *in utero* by subclinical intrauterine infections. Despite evidence from twin concordance studies that the genetic contribution to BPD is substantial, candidate-gene association studies have identified few specific mutations associated with BPD.³⁰ This may be due, in part, to small sample sizes and the imprecision of the clinical diagnosis of BPD. In contrast, in an analysis of 9 candidate SNPs in TLR pathway genes in a predominantly Caucasian population, Sampath et al.³¹ observed that a *TLR5* variant (rs5744168), but not select *TLR2* or *4* variants, was associated with BPD and BPD severity. There were no differences among IL-10 -1082 SNP genotypes for *Ureaplasma* respiratory colonization or BPD in a cohort of mechanically ventilated very low birth weight infants.³² In the current study, tagSNPs in *TLRs 2, 4, and 6* were significantly associated with BPD in the total sample and/or among African-Americans or non-Hispanic Caucasians. Our observations provide further support for a role of innate immune pathways in the pathogenesis of BPD, although this finding is still preliminary. Additional studies will be needed to identify functional SNPs within the significant tag regions.

This study had several limitations, including lack of maternal *Ureaplasma* colonization status and genetic information and a predominantly African-American population that limited power to detect associations in other ethnic or other racial groups. The sample size lacked power to detect interactions and excess risk. Because vertical transmission is the only known mode of *Ureaplasma* acquisition in the newborn, maternal *Ureaplasma* colonization status would allow analysis of transmission rates by genotype. Maternal genotype is also likely to influence maternal (and possibly fetal) *Ureaplasma* colonization. The interaction between maternal and fetal genotypes may influence fetal colonization, the intrauterine inflammatory response and clinical outcomes. Subsequent replication studies should include maternal culture and genotyping.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Characteristics of the two cohorts that comprise the total sample

The total sample includes infants from two cohorts. The two cohorts were recruited at different times and the clinical and genotype data was compiled for analysis. Cohort was included as a covariate in all analyses. TA = tracheal aspirate; NP = nasopharyngeal.

Figure 2. Relationship between *TLR6* rs5743827 genotype, *Ureaplasma* respiratory tract colonization, and BPD

There was a three-way relationship between *TLR6* SNP rs5743827, *Ureaplasma* colonization, and BPD. Infants homozygous for the major allele, G, at rs5743827 and positive for *Ureaplasma* colonization had the highest percentage of BPD (38% total sample; 39% African-Americans) as compared to those without *Ureaplasma* colonization (20% total sample; 21% African-Americans), without the risk genotype (19% total sample; 22% African-Americans), or without both *Ureaplasma* colonization and the risk genotype (18% total sample; 13% African-Americans). A. Total Population, B. African-American Population.