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Polymorphisms in *CYP2C9* are associated with response to indomethacin among neonates with patent ductus arteriosus

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BACKGROUND: Patent ductus arteriosus (PDA) is a common complication seen in preterm infants. Indomethacin is routinely used to treat PDA. Evidence suggests that the response of indomethacin is highly heritable. This study investigated the association between single-nucleotide polymorphisms (SNPs) in *CYP2C9* and the closure of PDA in response to indomethacin.

METHODS: Six SNPs in *CYP2C9* were analyzed for association with indomethacin response. A case–control analysis was performed among neonates who responded to indomethacin (responders) and among those who required surgical ligation (non-responders). Independent transmission disequilibrium tests were performed among parent–child trios of responders and non-responders.

RESULTS: The G allele of rs2153628 was associated with increased odds of response to indomethacin in the case–control analysis (odds ratios (OR): 1.918, 95% confidence interval (CI): 1.056, 3.483). Among indomethacin responders, the G allele of rs2153628 and the T allele of rs1799853 were overtransmitted from the parents to their child (OR: 2.667, 95% CI: 1.374, 5.177 and OR: 2.375, 95% CI: 1.040, 5.425, respectively), consistent with the case–control analysis.

CONCLUSION: We identified an association between two SNPs in *CYP2C9*, rs2153628 and rs1799853, and indomethacin response for the treatment of PDA. These findings suggest that response to indomethacin in the closure of PDA may be influenced by polymorphisms associated with altered indomethacin metabolism.

The ductus arteriosus is a vital fetal structure connecting the aorta and the pulmonary artery, shunting blood from the pulmonary circulation (1). Patent ductus arteriosus (PDA) is a neonatal complication in which the ductus arteriosus fails to close, resulting in continued postnatal shunting of systemic and pulmonary circulations. It commonly affects very preterm infants, although it can also affect term infants (2). Although many PDAs close over time without treatment, active treatment for PDA ranges from non-steroidal anti-inflammatory drugs (NSAIDs), including ibuprofen and indomethacin, to surgical ligation (3). Only two-thirds of neonates experience closure of PDA in response to indomethacin (4). Importantly, post-treatment plasma concentrations and interpatient pharmacokinetic differences have been shown to affect closure rates. Pharmacokinetic studies demonstrate considerable variability in indomethacin clearing among preterm infants with PDA (5,6). Furthermore, PDA requiring indomethacin therapy has been reported as highly heritable, as has failure to respond to indomethacin therapy (7).

The cytochrome P450 gene family encodes a variety of enzymes that are critical for drug metabolism. The importance of these enzymes in drug metabolism, and their altered efficiency by genetic variation, has been known for decades (8). Cytochrome P450 enzymes are largely responsible for the metabolism of NSAIDs. In particular, the enzyme encoded by the *CYP2C9* gene is known to metabolize many NSAIDs (9). *In vitro* studies have demonstrated that CYP2C9 is the major catalyst for metabolism of indomethacin to a product that accounts for 40–55% of total drug elimination in the urine (10). *CYP2C9* is highly polymorphic with many functional variants of pharmacological importance. Most of the described polymorphisms are associated with reduced enzyme activity and therefore with decreased drug metabolism (9). Although the effects of these variants on drugs with narrow therapeutic indexes, such as phenytoin and warfarin, make up a majority of the pharmacogenetic literature, the effect of variations in the gene on NSAID metabolism and therapy has also been described (11).

Given that response to indomethacin has been shown to be heritable, and polymorphisms in *CYP2C9* are known to affect NSAID metabolism, we investigated the association between specific polymorphisms in *CYP2C9* and its response to indomethacin, as defined by the closure of PDA without surgery.

METHODS

Study Population

This study population was chosen from an existing cohort of infants admitted to the Neonatal Intensive Care Unit at the University of Iowa Children's Hospital between 1999 and 2015. Infants in the

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initial cohort were recruited as part of an initiative to create a research biobank of biologic material (blood and/or buccal swabs) from infants for subsequent use in investigations into the genetic contributions to neonatal conditions. Parental informed consent was obtained for inclusion in this biobank. Current study participants were parent–child trios of preterm singleton neonates (<32 weeks' gestation) with PDA. All neonates in these analyses received indomethacin for the treatment of PDA following diagnosis by standard echocardiography around 1 week of life. Response to indomethacin was determined based on requiring surgery, in which surgical ligation was considered a non-response to indomethacin and no surgical ligation was considered a response to indomethacin. All study protocols and procedures were approved by the University of Iowa Institutional Review Board.

Marker Selection

Six SNPs located in *CYP2C9* were selected for genotyping using the Tagger function in Haploview (rs4918758, rs1799853, rs2253635, rs4086116, rs1505, and rs2153628) (12,13). SNPs were selected to provide >85% gene coverage. Six SNPs were selected with a minimum minor allele frequency of 0.20. These SNPs provided an estimated coverage of 86% of the alleles of 22 SNPs located within *CYP2C9*.

Genotyping

Subjects were genotyped for six SNPs using TaqMan assays (Applied Biosystems, Foster City, CA) using the EP1 SNP Genotyping System and GT 192.24 Dynamic Array Integrated Fluidic Circuits (Fluidigm, San Francisco, CA). All genotyping reactions were performed according to the standard protocol provided by Fluidigm. Three CEPH-CEU individuals (1000 Genomes Project) served as positive controls and double-distilled water served as a negative control.

Statistical Analysis

Statistical analyses were performed using Plink software (Broad Institute, Cambridge, MA) and SAS version 9.4 (Cary, NC). SNP markers were assessed for Hardy–Weinberg equilibrium and were excluded from subsequent analyses with $P<0.01$. Individual SNPs and subjects were examined for genotyping efficiency and were excluded from subsequent analyses for genotyping efficiency <95%.

SNPs were analyzed for association with response to indomethacin in several ways. First, a case–control analysis was performed, in which cases were defined as neonates who received indomethacin and did not require surgery (responders) and controls were defined as neonates who received indomethacin and required surgery (non-responders). Second, a transmission disequilibrium test (TDT) (14) was run among parent–child trios of neonates who did not respond to indomethacin and required surgery ("Non-responders TDT"). Third, a TDT was run among parent–child trios of neonates who received indomethacin and did not require surgery ("Responders TDT"). Odds ratios (ORs) and 95% confidence intervals (95% CIs) are reported for allelic tests. Haplotypes were tested for association in all three analyses.

RESULTS

All SNPs met Hardy–Weinberg equilibrium. All SNPs met a genotyping efficiency of >95%. Nineteen subjects were excluded for having a genotyping efficiency of <95%. Demographic characteristics are presented in **Table 1**. Responders and non-responders differed by gestational age, birth weight, and bronchopulmonary dysplasia status. Responders and non-responders did not differ by race, ethnicity, blood culture-positive sepsis within the first 21 days of life, intraventricular hemorrhage, necrotizing enterocolitis, respiratory distress syndrome, or retinopathy of prematurity.

Table 1. Demographic characteristics of responders and non-responders

Variable	Responders ($N=96$)	Non-responders ($N=52$)	*P* value
Gestational age (weeks)[a]	26.9 (2.29)	25.3 (1.75)	<0.0001
Birth weight (g)[a]	1,012.9 (355.8)	782.5 (201.9)	<0.0001
Gender			0.702
Female	43 (45%)	25 (48%)	
Male	53 (55%)	27 (52%)	
Race			0.164
Black/AA	7 (7%)	8 (15%)	
White	80 (83%)	43 (83%)	
Other	9 (10%)	1 (2%)	
Ethnicity			0.436
Hispanic/Latino	3 (3%)	3 (6%)	
Non-Hispanic/Latino	93 (97%)	49 (94%)	
Sepsis			0.114
Blood culture-positive	11 (11%)	11 (21%)	
No	85 (89%)	41 (79%)	
BPD			0.023
Yes	68 (79%)	46 (94%)	
No	18 (21%)	3 (6%)	
IVH			0.579
Grade I	5 (5%)	2 (4%)	
Grade II	6 (6%)	2 (4%)	
Grade III	6 (6%)	2 (4%)	
Grade IV	3 (3%)	4 (8%)	
None	75 (78%)	42 (80%)	
RDS			0.136
Yes	92 (96%)	52 (100%)	
No	4 (4%)	0 (0%)	
NEC			0.465
Yes	1 (1%)	0 (0%)	
No	95 (99%)	51 (100%)	
ROP			0.050
Stage I	19 (20%)	15 (29%)	
Stage II	18 (19%)	13 (25%)	
Stage III	7 (7%)	8 (15%)	
No	50 (52%)	15 (29%)	

AA, African American, BPD, bronchopulmonary dysplasia; IVH, intraventricular hemorrhage; NEC, necrotizing enterocolitis; RDS, respiratory distress syndrome; ROP, retinopathy of prematurity.
[a]Differences between responders and non-responders were analyzed by *t*-test. All other comparisons were made using χ^2-test.

Table 2. Results of SNP–outcome association analyses

Analysis type	SNP	Effect allele	OR (95% CI)[a]	*P*
Case–control (N = *96 cases, 52 controls)*				
	rs4918758	C	0.843 (0.510, 1.395)	0.507
	rs2253635	A	0.898 (0.548, 1.469)	0.667
	rs1799853	T	1.727 (0.780, 3.827)	0.174
	rs4086116	T	1.179 (0.634, 2.191)	0.602
	rs2153628	**G**	**1.918 (1.056, 3.483)**	**0.031**
	rs1505	C	0.823 (0.497, 1.363)	0.449
Non-responders TDT (N = *46 families)*				
	rs4918758	C	0.867 (0.412, 1.821)	0.706
	rs2253635	G	1.083 (0.494, 2.374)	0.842
	rs1799853	T	0.500 (0.188, 1.332)	0.157
	rs4086116	T	0.692 (0.296, 1.62)	0.394
	rs2153628	G	1.800 (0.603, 5.371)	0.285
	rs1505	C	0.867 (0.412, 1.821)	0.706
Responders TDT (N = *98 families)*				
	rs4918758	C	0.741 (0.416, 1.321)	0.307
	rs2253635	G	0.750 (0.442, 1.273)	0.285
	rs1799853	**T**	**2.375 (1.040, 5.425)**	**0.034**
	rs4086116	T	1.385 (0.678, 2.826)	0.369
	rs2153628	**G**	**2.667 (1.374, 5.177)**	**0.003**
	rs1505	C	0.778 (0.440, 1.376)	0.387

CI, confidence interval; OR, odds ratio; P, *P* value; SNP, single-nucleotide polymorphism; TDT, transmission disequilibrium test.
[a]Allelic test.
Bold indicates $P<0.05$.

Results of the statistical analyses are shown in **Table 2**. In all, 148 subjects were included in the case–control analysis (96 cases/responders and 52 controls/non-responders), where cases were defined as neonates who received indomethacin and did not require surgery (responders) and controls were defined as neonates who were treated with indomethacin and required surgery (non-responders). The G allele of rs2153628 was associated with increased odds of response to indomethacin (OR: 1.918, 95% CI: 1.056, 3.483, $P=0.031$). The genotype frequencies of rs2153628 among responders were 47.9%, 46.9%, and 5.2% for AA, AG, and GG, respectively. The genotype frequencies of rs2153628 among non-responders were 69.2%, 27.0%, and 3.8% for AA, AG, and GG, respectively. Among the 96 responders, 53 were males and 43 were females. Among the 52 non-responders, 27 were males and 25 were females. Sex was not significantly associated with response to indomethacin (data not shown). Among the 96 responders, 79 were Non-Hispanic White, six were Non-Hispanic Black, and 11 were of another race/ethnicity. Among the 52 non-responders, 41 were Non-Hispanic White, seven were Non-Hispanic Black, and four were of another race/ethnicity. The mean gestational age was 26.9 weeks among responders and 25.4 weeks among non-responders. Gestational age was significantly higher among responders compared with that among non-responders ($P<0.0001$, data not shown). Adjusting for gestational age, the OR for rs2153628 remained similar at 1.834 (95% CI: 0.951, 0.3.535), albeit not statistically significant ($P=0.070$). The OR for rs1799853 was not significant after adjusting for GA ($P=0.272$).

In all, 46 families (14 parent–child dyads, 32 parent–child trios, total $N=124$) were included in the "Non-responders TDT" analysis of neonates who did not respond to indomethacin, requiring surgery to ligate the ductus. No SNPs were significantly associated with requiring surgery ($P>0.05$). Among the 46 neonates, 24 were males and 22 were females. Among the 46 neonates, 37 were Non-Hispanic White, six were Non-Hispanic Black, and three were of another race/ethnicity. The mean gestational age was 25.5 weeks.

In all, 98 families (39 parent–child dyads, 59 parent–child trios, total $N=255$) were included in the "Responders TDT" analysis of neonates who responded to indomethacin and thus did not require surgery. The G allele of rs2153628, located in *CYP2C9*, was significantly overtransmitted from parents to a child who responded to indomethacin with the closure of PDA (OR: 2.667, 95% CI: 1.374, 5.177, $P=0.003$). The genotype frequencies of rs2153628 among probands were 47.0%, 49.0%, and 4.0% for AA, AG, and GG, respectively. The genotype frequencies of rs2153628 among parents were 62.7%, 33.5%, and 3.7% for AA, AG, and GG, respectively. The T allele of rs1799853, located in *CYP2C9*, was also significantly overtransmitted from parents to a child who responded to indomethacin with the closure of PDA (OR: 2.375, 95% CI: 1.040, 5.425, $P=0.034$). The genotype frequencies of rs1799853 among probands were 74.5%, 23.5%, and 2.0% for CC, CT, and TT, respectively. The genotype frequencies of rs1799853 among parents were 77.0%, 22.4%, and 0.6% for CC, CT, and TT, respectively. Among the 98 neonates, 57 were males and 41 were females. Among the 98 neonates, 82 were Non-Hispanic White, five were Non-Hispanic Black, and 11 were of another race/ethnicity. The mean gestational age was 26.9 weeks.

Haplotypes containing rs2153628 were associated with indomethacin response in the case–control analysis ($P<0.05$), although they did not meet statistical significance by Bonferroni correction (data not shown). Haplotypes were not significantly associated with indomethacin response in the TDT analyses (data not shown).

DISCUSSION

In a case–control analysis and a family-based TDT of neonates who responded to indomethacin, the G allele of rs2153628 was significantly associated with response to indomethacin ($P=0.031$ and $P=0.003$, respectively). The T

allele of rs1799853 was significantly associated with response to indomethacin in the TDT analysis ($P = 0.034$) and with the same direction of effect in the case–control analysis, albeit not statistically significant ($P = 0.174$). Although rs2153628 has not been described in association with NSAID metabolism, it has been identified as a predictor of warfarin dosage (15) and *CYP2C9* is known to be an important enzyme in NSAID metabolism (9). Its presence in the borderline significant haplotypes of the case–control population further supports its effect on indomethacin response.

The *CYP2C9*2* polymorphism, represented by rs1799853, encodes a change from arginine to cysteine and is associated with slower metabolism of warfarin, and is thus associated with a lower required dose (16). The same study demonstrated that rs1799853 is the main driving polymorphism determining warfarin dosage, although many polymorphisms in *CYP2C9* are in perfect linkage disequilibrium with rs1799853 (ref. 16). The SNPs rs1799853 and rs2153628 are not linked, with an R^2 of 0.03 (ref. 12), suggesting that these loci confer independent effects.

This study is strengthened by its utilization of both population-based and family-based analytical methods, the results of which suggest consistent direction of effect of the findings. In addition, the PDA phenotypes are carefully curated to prevent the inclusion of PDA diagnosed in the first few days of life, before the spontaneous closure is still likely. Although rs1799853 and rs2153628 were not statistically significant in the TDT of infants undergoing surgery for PDA, which may be due to the small sample size, the result of no association is consistent with the case–control findings because the effect alleles are associated with odds of response to indomethacin, rather than odds of requiring surgery. We were underpowered to detect an OR less than 3.5 in the case–control analysis. Although this may have caused us to miss some significant effects, it would not affect our ability to identify the positive associations described with CYP2C9. Adjusting for gestational age reduced the significance of our findings. This could be because gestational age is a confounder of the relationship between *CYP2C9* polymorphisms, or it could be due to the small sample size. A limitation of this study is the small sample size. Replication or meta-analysis in another population would help clarify the relationship between gestational age, *CYP2C9* polymorphisms, and response to indomethacin.

Population stratification is unlikely to be present in this study population, as most subjects were non-Hispanic White. A limitation of our study is the retrospective design that does not allow measurements of indomethacin levels; thus, we cannot determine whether genetic variation in *CYP2C9* actually alters the rate of indomethacin metabolism in association with the closure of PDA. Notably, *CYP2C9* is also involved with the metabolism of ibuprofen; thus, the results of this study are likely generalizable to infants whose PDAs are treated with ibuprofen (9).

We demonstrate that two genetic polymorphisms are associated with response to indomethacin for the treatment of PDA in a case–control analysis of neonates who required surgery compared with neonates who responded to indomethacin and in a family-based TDT of neonates who responded to indomethacin. Given that these polymorphisms are located in a gene that encodes an enzyme for indomethacin metabolism, these findings identify candidate polymorphisms for a genetic screening tool for indomethacin treatment of PDA. These findings are supported by pharmacokinetic studies, which report high variability in indomethacin clearing among preterm infants with PDA. Shaffer *et al.* concluded that indomethacin clearance is solely due to individual pharmacokinetic differences regardless of weight (5). Smyth *et al.* also reported high interindividual variability, although indomethacin metabolism did vary by weight (6). Validation studies to demonstrate the value of these polymorphisms in predicting response to indomethacin should be performed and translated into clinical practice to reduce the morbidity associated with prolonged PDA and indomethacin toxicity in which indomethacin treatment is likely to be futile.

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