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Genetic Variants Associated with Patent Ductus Arteriosus in Extremely Preterm Infants

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Abstract

Objective.—Patent ductus arteriosus (PDA) is a commonly observed condition in preterm infants. Prior studies have suggested a role for genetics in determining spontaneous ductal closure. Using samples from a large neonatal cohort we tested the hypothesis that common genetic variations are associated with PDA in extremely preterm infants.

Study Design.—Preterm infants (n=1013) enrolled at NICHD Neonatal Research Network sites were phenotyped for PDA. DNA was genotyped for 1634 single nucleotide polymorphisms (SNPs) from candidate genes. Analyses were adjusted for ancestral eigenvalues and significant epidemiologic variables.

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Result.—SNPs in several genes were associated with the clinical diagnosis of PDA and with surgical ligation in extremely preterm neonates diagnosed with PDA ($p<0.01$). None of the associations were significant after correction for multiple comparisons.

Conclusion.—We identified several common genetic variants associated with PDA. These findings may inform further studies on genetic risk factors for PDA in preterm infants.

Introduction

The ductus arteriosus (DA) is a vital conduit that maintains a fetal shunt, preventing pulmonary overcirculation *in utero*. In infants born at term, this vessel typically constricts in the first days after birth, an important step in the complex process of separating the systemic and pulmonary circulations. Persistent patency of the DA (PDA) is commonly seen in extremely preterm infants suggesting a developmental or environmental risk factor for this condition. Chorioamnionitis, for example, is reported to be an environmental risk factor for PDA [1]. In addition, gentamicin exposure in the face of sepsis appears to encourage relaxation of the DA [2]. PDA has been associated with subsequent morbidities seen in preterm infants, such as bronchopulmonary dysplasia; however, it is unclear whether this association is causative [3, 4].

The current clinical approach to management of the PDA varies widely [5, 6]. Among the more common approaches is treatment with a nonsteroidal anti-inflammatory drug when a clinician considers the PDA to be “hemodynamically significant” (a term with no standard definition). If the ductus remains patent after medical treatment and is still considered significant, clinicians may then opt for definitive surgical closure. While this is a common approach, thresholds for initiation of medical treatment, the medication used and its route of administration, and the risks and benefits of both medical and surgical closure are the subjects of ongoing investigation [7, 8].

There is growing evidence that genetic risk factors may play a role in PDA in preterm infants. Studies have clearly demonstrated increased concordance of PDA in monozygotic twins compared to dizygotic twins [9, 10]. More recently, single nucleotide polymorphisms (SNPs) in several genes, including *TFAP2B*, *PTGIS*, and *AGTR2*, have been reported to be associated with PDA in preterm infants [11, 12]. Several studies have also reported genetic risk factors for isolated PDA in term infants [13, 14]. Prior studies investigating the role of genetic factors in preterm PDA have been limited by relatively small sample sizes.

We used anonymized DNA samples, collected as part of a prior study [15], to address the hypothesis that common genetic variants are associated with PDA in extremely preterm infants. We performed a secondary analysis to determine whether common genetic variants play a role in response to medical treatment. We utilized a candidate gene approach and a case-control study design, as parental DNA was not collected in the primary study. Identifying genes and pathways involved in PDA closure may help direct further research and inform therapeutic decisions.

Methods

Samples.

Our study population included 1,013 preterm infants born at less than 1,000 grams who had been enrolled by the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Neonatal Research Network (NRN) between 1998 and 2001 in a study to examine the associations between cytokine levels from dried blood spots and neurodevelopment [15]. Genomic DNA was extracted from infant blood spot samples stored in an anonymized DNA biorepository by the NRN. This DNA underwent whole-genome amplification prior to genotyping. Studies of the role of genetic factors in retinopathy of prematurity [16], intraventricular hemorrhage [17], and bronchopulmonary dysplasia [18] using these samples have been reported previously. Infants who died prior to three days of age and those with multiple congenital anomalies were excluded from analysis.

Phenotyping.

In the original study cohort, subjects were classified as having a PDA if clinical evidence of left to right PDA shunt was documented by continuous murmur, hyperdynamic precordium, bounding pulses, wide pulse pressure, congestive heart failure, increased pulmonary vasculature or cardiomegaly by CXR, and/or increased oxygen requirement or echocardiographic evidence of PDA with documentation of left to right ductal shunting. Standard scoring systems considering variables such as ductal geometry or shunt volume were not used at that time and thus those data were not available. Clinicians treated the PDA at their discretion at each of the individual participating NRN study sites. PDA was diagnosed in 511 infants. Medical treatment with non-steroidal medications was recorded in the research record. Surgical closure of the PDA was assumed to reflect failure of medical management and was performed in 163 infants who had received prior medical therapy.

Genotyping.

Investigators from several individual NRN sites (each interested in different conditions associated with prematurity) participated in the organization and genotyping effort of this cohort of preterm infants. Genotyping was performed from whole-genome amplified DNA using the Illumina GoldenGate platform for 1,634 SNPs from 145 candidate genes, each selected on the basis of biologic plausibility or associations with morbidities of prematurity in prior cohort studies. Statistical analyses for the PDA outcomes of interest included all genotyping data generated, regardless of whether the SNP was initially requested specifically for the study of PDA (n=51 SNPs). The majority of the study subjects (n=961; 94.9%) were successfully genotyped (SNP call rate >90%), indicating that high quality DNA was obtained from the stored blood spots. SNPs that did not achieve a sample call rate of >90% were not included in our analyses (21.8% removed).

Statistical analysis.

Characteristics of the study population were compared using chi-square tests for categorical variables and t-tests for continuous variable. Multivariate logistic regression was used to estimate the associations between each genetic variant and PDA status. Covariates examined

for inclusion in the models were: gestational age, birth weight, sex, ancestry principal components (represented by the first 10 eigenvectors generated by the study sample), small-for-gestational age status, multiple birth, and antenatal steroid use. A secondary analysis tested the associations between genetic variants and surgical closure of the PDA following diagnosis and medical treatment among infants with PDA. Because 1278 SNPs were evaluated in 2 analyses, 2556 independent tests were performed in this study. The Bonferroni correction for multiple testing suggests a significance p-value threshold of 1.9×10^{-5}. Although applying this stringent correction would certainly reduce false positive signals, it can essentially eliminate true positive signals in genetic studies. This is especially true if any individual genetic effect is small, as has typically been found in most GWA studies. Since our investigation was an exploratory, hypothesis-generating study, we opted to present SNPs with a p-value <0.01 as evidence of association. All analyses were performed using R and PLINK 1.9 [19].

Results

Demographic characterization of the study cohort is shown in Table 1. As expected, infants with a PDA were of lower birth weight and gestational age, and less likely to have been exposed to antenatal corticosteroids. Unexpectedly, SGA status was seen less frequently in preterm neonates with a PDA compared to those without a PDA (P< 0.0001). This finding is consistent with two other recent reports focused on outcomes for SGA infants, but may reflect a higher mortality seen in SGA infants before the diagnosis of PDA [20, 21]. Hispanic ethnicity was more common in preterm infants with a PDA compared to those without (P=0.036). The racial distribution of the current study population, approximately 50% Caucasian, differed significantly from that of our prior single-center genetic study of PDA, which was approximately 90% Caucasian [11].

The results of a multivariable analysis comparing preterm infants with and without a PDA are shown in Table 2. The adjusted odds ratios presented in the table are greater than one if the major allele of a given variant is overrepresented in PDA and less than one if it is underrepresented in PDA. Three of the top 12 results (p-value < 0.01) were from SNPs within *EPAS1* (previously known as *HIF2A*), a gene encoding endothelial PAS domain protein 1. Because of the likely role of oxygen signaling in a number of common morbidities seen in preterm infants, 60 SNPs throughout the *EPAS1* gene were genotyped in this study. In contrast to our prior reports [11, 22], variants in *TFAP2B* (Transcription Factor AP-2 Beta) were not identified in this study as associated with PDA. The current study identified a genetic variant (rs1502245) in the gene *PTGIS*, which encodes prostaglandin I_2 (prostacyclin) synthase, associated with PDA in preterm infants.

A secondary analysis was performed to investigate genetic variation associated with surgical closure of a PDA, which was used as a surrogate for failed medical management (i.e., the PDA failed to permanently close after treatment with indomethacin or ibuprofen). Nine SNPs were associated with surgical closure of the PDA in extremely preterm infants, as shown in Table 3. Three SNPs in the *STAT1* gene were identified with a p-value of <0.01. A total of 10 *STAT1* SNPs were genotyped in the study. None of the findings presented here met statistical significance when corrected for multiple comparisons.

Discussion

After correcting for confounding variables, we identified three SNPs in *EPAS1* and one SNP in *EDN1* (gene encoding Endothelin-1) as associated with PDA (using a threshold of P<0.01), as shown in Table 2. Both *EPAS1* and *EDN1* encode proteins present in the mouse ductus arteriosus [23]. Additionally, the expression of both genes is positively regulated by *TFAP2B,* the gene mutated in Char syndrome, which presents with multiple anomalies, including PDA in term infants [24]. Although *TFAP2B* knockout mice die from the effects of a PDA shortly after birth and genetic variations in *TFAP2B* have been associated with PDA in other studies of preterm infants, our current analysis failed to replicate this finding. It is possible the study lacked power to identify any *TFAB2B* variants or that the significant racial/ethnic diversity of the current study population played a role in the absence of this association. This latter hypothesis is supported by the fact that our prior study utilized a family-based approach, which is less susceptible to the effects of population stratification compared to a case-control design, and the prior cohort was more racially homogeneous [11].

We also identified a variant (rs6505469) in *NOS2*, which encodes inducible nitric oxide, as being associated with PDA. In a mouse model, *NOS2* is regulated by indomethacin, the most common pharmacologic agent used in the treatment of PDA [25]. It is likely that risk for PDA is influenced by several common pathways affected by cyclooxygenase activity and the resulting alterations in bioactive lipid mediators. Our finding of a genetic variant in *PTGIS* associated with PDA further supports the critical role of lipid mediators in PDA physiology. Prostaglandin I_2 (PGI_2) is a vasodilatory prostanoid and levels of this compound are likely to be important in maintaining ductal patency [26]. Additionally, both PGI_2 synthase and the PGI_2 receptor are expressed in the rodent ductus arteriosus [27]. The *PTGIS* variant identified in this study, rs1502245, was not included in the haplotype found previously to be associated with PDA [11].

Vascular endothelial growth factor (VEGF) has been reported to play a possible role in a number of common complications of prematurity including bronchopulmonary dysplasia [28], retinopathy of prematurity [29] and PDA [30]. VEGF plays an important role in the formation of ductal neointimal mounds, a critical step in permanent anatomical closure of the PDA. While none of the *VEGF* variants tested was associated with PDA, we identified two genes in the VEGF pathway associated with PDA: *FLT1* (Fms Related Tyrosine Kinase 1), which encodes a VEGF receptor; and *NRG1* (Neuregulin 1), which encodes a protein that upregulates VEGF expression in an animal model [31] and alters vascular neointimal growth [32].

Our secondary analysis examined genetic variants associated with surgical closure in extremely preterm infants following PDA diagnosis. Surgical ligation was performed in 31% (163/511) of the study infants. Although the incidence of PDA ligation has dropped considerably since the initial study (1998 to 2001), ligation was commonly performed at that time if the PDA remained open after medical therapy. Thus, the clinical decision to ligate is a valid marker of a failure to respond to indomethacin or ibuprofen administration. Three variations in the *STAT1* (Signal Transducer and Activator of Transcription 1) gene were identified, as shown in Table 3. *STAT1* encodes a transcriptional activator that responds to a

growing number of growth factors and cytokines. Although *STAT1* has been implicated in both preterm rupture of membranes [33] and in altered immune response in preterm infants [34], there have been no prior reports of association with PDA. Subsequent efforts to replicate this finding in an independent population will be important. One SNP in *MTHFR*, which encodes methyltetrahydrofolate reductase, was associated with surgical closure of the PDA. Although the mechanism through which *MTHFR* variants may act on the preterm PDA is unknown, this finding is interesting given a recent link reported between genetic variations in *MTHFR* and PDA in term infants in Taiwan [13]. Our identification of a genetic variation in *SOD3* (Superoxide Dismutase 3) is consistent with a prior report demonstrating decreased plasma superoxide dismutase (SOD) levels in preterm infants who developed a hemodynamically significant PDA compared to those who did not [35]. The findings of our candidate gene study support several prior studies of PDA mediators in preterm infants, term infants, and animal models.

Because subjects undergoing surgical closure of a PDA are a smaller subset of all infants with a PDA one might expect overlap between the SNP's listed in Table 2 and Table 3. One simple explanation for the lack of common SNPs between the two groups is the sample size which influences significance of association. It is also possible that genes in pathways that are influenced more by prostaglandin-related mechanisms are present in Table 2 and that those in Table 3 participate in more prostaglandin-independent pathways since they are associated with PDA after prostaglandin inhibition. Finally, a large number of random false positive signals may be present in both groups.

This is one of the largest studies to date examining genetic associations with PDA in extremely preterm infants. However, this strength in a study of preterm infants must be considered in comparison to reported genetic studies of common adult diseases, which often include hundreds of thousands of individuals [36]. The limitation in sample size underscores the critical need for generating more neonatal biorepositories (both nationally and internationally) along with improved collaboration and sharing of neonatal samples and data among investigators to prevent underpowered studies involving preterm infants. Combining genotyping data from all NRN investigators studying several distinct conditions, as was done in this study, can also be viewed as a limitation of the study for statistical reasons. We intentionally evaluated all 1278 SNPs run on the platform rather than limiting the analysis to the 51 SNPs originally chosen exclusively for PDA analysis. A further limitation of the study was that diagnosis and treatment of PDA was at the treating clinicians' preference, likely showing wide variations based on clinician and site preference. We were also limited by the quality and amount of PDA-related data available in a retrospectively-collected database. The initial cohort studied was also generated over 15 years ago (when PDA diagnosis and management was significantly different compared to today [37]), which may limit extrapolation of our results to current preterm populations. From a statistical standpoint, we performed 2 analyses (PDA and surgical closure of a PDA) so the p-value considered statistically significant after correcting for multiple comparisons dropped to 1.9×10^{-5}. On the other hand, greatly increasing the pool of genes/SNPs possibly contributing to PDA (from 51 to 1278) can be considered a strength by allowing the consideration of many genes free from selection bias. Determining this balance between hypothesis testing and hypothesis generation is similar to the considerations involved with planning whole-genome

studies and was an important aspect of the study design. We arbitrarily chose a p-value of <0.01 to report as associations in this study to decrease the likelihood of missing true positive signals, knowing that false positive signals will inevitably be present. This approach is commonly used in preliminary genetic studies. Our finding of multiple SNPs in the same genes (e.g., *EPAS1* and *STAT1*), however, increases the confidence that these may actually represent true positive results. In our study, the probability of randomly identifying associations between PDA and 3 SNPs in *EPAS1* is 1:8000, and identifying 3 SNPs in *STAT1* is less than 1 in a million. These numbers represent the probability of positive findings given the total number of SNPs we evaluated in our study, not the probability of finding associations in a given individual. Thus, our goal of using this information as part of an overall genetic risk score to help in treatment decisions remains a possibility. Genetic risk scores have been useful in predicting a number of common complex diseases in older children and adults[38, 39, 40]. Finally, the heterogeneity in ancestry of the current study population can be considered a strength with respect to the generalization of these findings, but it is also a limitation in identifying any ancestry-specific etiologies for PDA. We attempted to overcome this limitation by using ten principal components to correct for ancestral differences. The sample size of our study was too small to determine whether genetic variation was a significant risk factor in persistent patency of the ductus arteriosus when comparing different races/ethnicities. In addition to the candidate genotyping presented in this study, genome-wide data has also became available on a limited subset of these samples and is currently being analyzed. One advantage of a candidate gene approach over a genome-wide association study is the ability to look at specific genetic polymorphisms previously associated with PDA, or other related neonatal morbidities, rather than SNPs that may or may not be functional but are chosen for their ability to capture a large amount of the variation across the genome.

Genetic approaches are yielding valuable information regarding several adult diseases, including risk assessment and improved treatment strategies [41, 42, 43, 44]. Large sample sizes are critical to performing association studies, especially in complex conditions with both genetic and environmental components. We report one of the largest studies to date of preterm infants with PDA, with respect to sample size and the number of SNPs analyzed. We replicated an association of PDA with genes identified in prior studies and identified new potential candidates for future studies. This information may prove useful in designing future studies, determining a genetic risk score for stratification, or identifying biological pathways that can be manipulated for therapeutic purposes.

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Table 1.

Demographics of study cohort

Characteristic	PDA Absent (N=502)	PDA Present (N=511)
Gestational age	26.5 weeks	25.3 weeks
Birthweight	792 g	734 g
Sex (% male)	47.8%	48.7%
Race/Ethnicity		
–Caucasian	50%	48.9%
–African American	48%	48.9%
–Hispanic	16.3%	21.1%
SGA	20.7%	8.2%
Antenatal Steroids	79.1%	74.1%

Table 2.

Genetic variants associated with PDA

Gene	SNP ID	Major Allele	MAF*	N	aOR	p-value
EPAS1	rs3088359	C	T=0.46	961	1.64	0.00036
PTGIS	rs1502245	A	G=0.38	956	1.47	0.00054
PGPEP1L	rs1521484	C	G=0.44	940	1.45	0.0028
IGF1	rs5742627	G	A=0.009	934	0.616	0.0044
EDN1	rs5370	G	T=0.25	961	0.533	0.0045
EPAS1	rs17035091	C	G=0.09	960	2.317	0.0046
NRG1	rs16879773	G	A=0.13	961	0.513	0.0053
LRP4	rs2306036	T	C=0.33	959	0.69	0.0064
EPAS1	rs11690950	A	G=0.43	959	0.693	0.0076
NOS2	rs6505469	A	T=0.37	946	1.39	0.0082
FLT1	rs7332329	G	A=0.42	961	1.38	0.0084
ABCA4	rs1931566	G	C=0.17	960	1.84	0.0085

*Minor allele frequency from 1000 Genomes in European Samples

N= Sample size genotyped

aOR= Adjusted odds ratio

Table 3.

Genetic variants associated with surgical closure of PDA

Gene	SNP ID	Major Allele	MAF*	N	aOR	p-value
STAT 1	rs1914408	G	A=0.20	484	2.45	0.0025
MTHFR	rs4846049	G	T=0.37	483	1.64	0.0029
STAT1	rs2280234	T	C=0.41	484	0.622	0.0050
SOD3	rs699473	C	T=0.44	484	0.604	0.0051
STAT1	rs2280235	T	C=0.26	484	2.16	0.0054
NAA15	rs1901173	T	C=0.28	483	1.72	0.0054
F13A1	rs5985	G	A=0.15	482	1.81	0.0055
IGF1R	rs8034284	T	C=0.47	484	1.56	0.0072
IL1B	rs1143627	C	T=0.47	483	0.660	0.0094

*Minor allele frequency from 1000 Genomes in European Samples

N= Sample size genotyped

aOR= Adjusted odds ratio