Candidate Gene Analysis: Severe Intraventricular Hemorrhage in Inborn Preterm Neonates

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Intraventricular hemorrhage (IVH) is a disorder of complex etiology. We analyzed genotypes for 7 genes from 224 inborn preterm neonates treated with antenatal steroids and grade 3-4 IVH and 389 matched controls. Only methylenetetrahydrofolate reductase was more prevalent in cases of IVH, emphasizing the need for more comprehensive genetic strategies. *(J Pediatr 2013;163:1503-6).*

Converging data suggest that intraventricular hemorrhage (IVH) of the preterm neonate is a disorder of complex etiology. IVH has been attributed to changes in cerebral blood flow to the immature germinal matrix microvasculature,[1] and the more severe grades (Grs) are characterized by acute distension of the ventricular system (Gr 3) and parenchymal venous infarction (Gr 4). Nationally, ~15% of all very low birth weight (BW) infants have Gr 3-4 IVH,[2] and one-half to three-quarters of survivors develop cognitive impairment and/or cerebral palsy.[3-5] Despite advances in neonatal intensive care, the incidence of Gr 3-4 IVH has changed little over the past 2 decades.[2] Recent studies have tested the hypothesis that IVH may be secondary to variability in risk genes, and candidate gene studies have implicated the coagulation, inflammatory, and vascular pathways.[6-8] However, few have been replicated in independent populations. Factors contributing to these findings include the wide range of ancestries of infants studied, small sample sizes, and lack of appropriate controls.

If a major focus of perinatal care is to prevent brain injury and abnormal development,[9] then physicians and scientists must better understand those factors that contribute to severe IVH. The objective of this report is to interrogate previously published genetic risk factors for Gr 3-4 IVH in a cohort of inborn appropriate for gestational age (AGA) preterm neonates.

Methods

Inborn infants with BWs 500-1250 g and Gr 3-4 IVH and neonates with normal cranial ultrasounds were enrolled prospectively at 24 universities; additional samples were provided from Extremely Low Gestational Age Newborn,[10] Iowa Prematurity,[6] and Oulu University[11] cohorts (**Table I**; available at www.jpeds.com). The protocol was approved by the institutional review board of each institution.

AGA	Appropriate for gestational age
BW	Birth weight
COL4A1	Collagen 4A1
Gr	Grade
IL	Interleukin
IVH	Intraventricular hemorrhage
MTHFR	Methylenetetrahydrofolate reductase
TNF	Tumor necrosis factor

Candidate Genes

PubMed searches were performed of original and review articles published prior to January 1, 2013, that reported significant associations between specific genotypes and Gr 3-4 IVH. PubMed terms included "candidate," "gene," "genetic," "Gr 3-4," "infant," "IVH," "neonate," "polymorphism," "premature," "preterm," and "risk factor".

Eleven polymorphic genetic variants in 9 genes were identified. Single Nucleotide Polymorphism Database identification numbers are as follows: collagen 4A1 (*COL4A1*) (rs113994114[8]); *estrogen receptor 1* (rs2234693[12]); *F2* (rs1799963[7,13]); *F5* (rs6025[6,7,13]); interleukin (*IL*)*1B* (rs1143627,[6] rs16944[6,14]); *IL6* (rs1800795[15]); methylenetetrahydrofolate reductase (*MTHFR*) (rs1801133[7]; 1801131[7]); tumor necrosis factor (*TNF*) (rs1800629[16]); and *TNFB2* (rs138435669[17]).

Description of Cases and Controls

Cases had Gr 3-4 IVH based upon blinded cranial ultrasonography review and met the following criteria: (1) inborn; (2) antenatal steroid exposure; (3) BW 500-1250 g; (4) AGA; (5) no congenital malformations, infections, or chromosomal disorder; (6) no family history of coagulopathy; and (7) not a sibling of an enrolled subject. Controls met

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Table II. Validation of predefined risk genotype comparisons in cases vs controls

Gene	Variant*	Genotype comparison	SNP	Proxy (D' value with original SNP)†	OR (95% CI)	*P* value
COL4A1	p.Gly1580Arg[8]	CG vs GG	rs113994114	rs3825481 (na‡)	0.89 (0.52-1.49)	.712
F2	c.97G > A[7,13]	AG vs GG	rs1799963	rs5898 (1.000)	1.40 (0.88-2.22)	.139
F5	c.1601G > A[6,7,13]	AG vs GG	rs6025	rs6015 (1.000)	1.07 (0.66-1.72)	.817
IL1B	c.-87-511T > C[6,14]	T vs C	rs16944	-	1.02 (0.79-1.30)	.902
	c.-87-31C > T[14]	C vs T	rs1143627	-	1.03 (0.80-1.31)	.853
IL6	c.-116-121C > G[15]	CC vs CG or GG	rs1800795	rs1800797 (1.000)	1.08 (0.76-1.56)	.662
MTHFR	c.677C > T[7]	TT or CT vs CC	rs1801133	-	0.95 (0.67-1.34)	.799
	c.1298A > C[7]	CC or CA vs AA	rs1801131	-	1.56 (1.10-2.20)	.009
TNF	c.-169-319G > A[16]	AA or AG vs GG	rs1800629	-	0.90 (0.62-1.31)	.587

F2, prothrombin; *F5*, factor V Leiden; *SNP*, single nucleotide polymorphism.
*c = cDNA; p = protein.
†D′ = D/D_{max} where D = the deviation of the observed frequency from the expected; and D_{max} = the theoretical maximum for the observed allele frequencies.
‡Allele frequency 0 for the original SNP from 1000 genomes database.

these criteria and had normal ultrasounds. Analysis was performed on neonates of European ancestry to reduce confounding from racial admixture. Data were entered into a secure online database at Yale University.

Genotyping

Genomic DNA was isolated from buccal swabs, umbilical cords, and blood, and genotyping was performed using Illumina 1 Million Quad and HumanOmniExpress-12 v1.0 arrays (Illumina Inc, San Diego, California) as previously described.[18] For single nucleotide polymorphisms not on the arrays, proxy SNPs selected secondary to strong linkage disequilibrium with the candidate were employed. The D′ between the candidate and proxy[19] was determined based on the 1000 genomes European data.[20]

dbSNP ID numbers for the variants are as follow: *COL4A1* (rs3825481); *ESR1* (rs1643821); *F2* (rs5898); *F5* (rs6015); *IL1B* (rs16944, rs1143627); *IL6* (rs1800797); *MTHFR* (rs1801133; rs1801131); *TNF* (rs1800629). D′ values for the best proxies for *ESR1* and *TNFB2* were 0.496 and 0.732, and, thus, these were not evaluated, permitting evaluation of 9 variants in 7 genes.

Statistical Analyses

Distributions for the 9 variants in cases and controls were examined for significant deviation ($P < .01$) from Hardy-Weinberg equilibrium. Each was prespecified based on published reports in the primary analysis, and the frequencies of risk-associated variants and nonrisk-associated variants were compared in cases and controls (Table II). In the secondary analysis, the distribution of all 3 genotypes was compared at each locus (Table III). Fisher exact test was applied to both.

Continuous variables were compared using Student *t* test and categorical variables using Fisher exact test. *P* value of <.01 was considered statistically significant without adjustment for multiple comparisons. Analyses were performed with SAS v. 9.3 (SAS Institute, Cary, North Carolina).

Results

Cases had lower BW and gestational age than controls (Table IV). Case mothers had less pre-eclampsia and cesarean deliveries but more chorioamnionitis and multiple gestation pregnancies than control mothers. Similarly, there were more cases with 5-minute Apgar scores <3 and more receiving intubation for resuscitation.

The overall genotype call rate for the variants was 99.8% (range, 98.2%-00%). To validate the quality of the genotypes from the markers, deviation from Hardy-Weinberg equilibrium was calculated. All showed insignificant deviation from expected by Hardy-Weinberg equilibrium ($P > .01$).

None of the putative risk variants showed significant differences in frequency between cases and controls except the *MTHFR* 1298A > C polymorphism. To confirm these results, we analyzed the genotypes by 2 × 3 Fisher exact (Table II).

Table III. Genotype frequencies and *P* values in cases with Gr 3-4 IVH and controls

			Number (%)		
Gene	Variant	Genotype	Controls	Cases	*P* value
COL4A1	p.Gly1580Arg[8]	AA	85.8	87.4	.817
	rs3825481*	AG	13.9	12.6	
		GG	0.3	0.0	
F2	c.97G > A[7,13]	GG	84.5	79.9	.306
	rs5898*	GA	14.5	19.2	
		AA	1.0	0.9	
F5	c.1601G > A[6,7,13]	CC	84.3	83.5	.919
	rs6015*	CT	15.2	16.1	
		TT	0.5	0.5	
IL1B	c.-87-511T > C[6,14]	GG	41.4	37.5	.206
	rs16944	AG	44.7	51.8	
		AA	13.9	10.7	
	c.-87-31C > T[14]	TT	41.4	37.1	.184
	rs1143627	CT	44.7	52.2	
		CC	13.9	10.7	
IL6	c.-116-121C > G[15]	GG	35.7	33.9	.873
	rs1800797*	AG	46.8	47.3	
		AA	17.5	18.8	
MTHFR	c.677C > T[7]	CC	42.4	43.8	.782
	rs1801133	CT	44.5	45.1	
		TT	13.1	11.2	
	c.1298A > C[7]	AA	54.8	43.8	.021
	rs1801131	AC	37.0	48.2	
		CC	8.2	8.0	
TNF	c.-169-319G > A[16]	GG	68.4	70.5	.843
	rs1800629	AG	29.3	27.7	
		AA	2.3	1.8	

*Proxy single nucleotide polymorphism.