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Candidate gene analysis: Severe intraventricular hemorrhage in inborn preterm neonates

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Abstract

Intraventricular hemorrhage 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 intraventricular hemorrhage and 389 matched controls. Only methylenetetrahydrofolate reductase was more prevalent in cases of intraventricular hemorrhage, emphasizing the need for more comprehensive genetic strategies.

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 are characterized by acute distension of the ventricular system (Grade 3) and parenchymal venous infarction (Grade 4). Nationally, ~15% of all very low birth weight infants have grade (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 two decades.² Recent studies have

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*List of members of the Gene Targets for IVH Study Group is available at www.jpeds.com (Appendix)

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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 birth weights (BW) 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 ELGAN,¹⁰ Iowa Prematurity⁶ and Oulu University¹¹ cohorts. The protocol was approved by the institutional review board of each institution.

Candidate genes

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

Eleven polymorphic genetic variants in 9 genes were identified. dbSNP ID numbers follow: *COL4A1*(rs113994114⁸); *ESR1*(rs2234693¹²); *F2*(rs1799963^{7,13}); *F5*(rs60256^{7,13}); *IL1B*(rs1143627⁶, rs16944^{6,14}); *IL6*(rs1800795¹⁵); *MTHFR*(rs1801133⁷; 1801131⁷); *TNFR*(rs1800629¹⁶); and *TNFB2*(rs138435669¹⁷).

Description of cases and controls

Cases had Gr 3-4 IVH based upon blinded cUS review and met the following criteria: inborn; ANS exposure; BW 500-1250 g; AGA; no congenital malformations, infections or chromosomal disorder; no family history of coagulopathy; and not a sibling of an enrolled subject. Controls met 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 as previously described.¹⁸ For SNPs not on the arrays, proxy SNPs selected secondary to strong linkage disequilibrium with the candidate were employed. The D' between the candidate and proxy¹⁹ was determined based on the 1000 genomes European data.²⁰

dbSNP ID numbers for the variants follow: *COL4A1*(rs3825481); *ESR1* (rs1643821); *F2*(rs5898); *F5* (rs6015); *IL1B*(rs16944, rs1143627); *IL6*(rs1800797); *MTHFR*(rs1801133; rs1801131); *TNFR*(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 ($p < 0.01$) from Hardy-Weinberg equilibrium. Each was pre-specified based on published reports in the primary analysis, and the frequencies of risk-associated variants and non-risk-associated variants were compared in cases and controls (Table I). In the secondary analysis, the distribution of all three genotypes was compared at each locus (Table II). Fisher exact test was applied to both.

Continuous variables were compared using Student t test and categorical variables using Fisher's exact test. P-value < 0.01 was considered statistically significant without adjustment for multiple comparisons. Analyses were performed with SAS (version 9.3).

RESULTS

Cases had lower BW and GA than controls (Table III). Case mothers had less preeclampsia and cesarean deliveries but more chorioamnionitis and multiple gestation pregnancies than control mothers. Similarly, there were more cases with five minute Apgar scores < 3 and more receiving intubation for resuscitation.

The overall genotype call rate for the variants was 99.8% (range, 98.2-100%). 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 ($p > 0.01$).

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's exact (Table I). The *MTHFR* 1298A>C polymorphism did not attain significance, but nearly so (Table II).

DISCUSSION

Although recent data have implicated genes contributing to the pathogenesis of IVH, we were unable to confirm as common risk factors for severe IVH 8 of the 9 published variants, and the 1298A>C variant for *MTHFR* gave equivocal results. Hartman reported that 4/16 preterm neonates with atypical Gr 4 IVH had this variant,⁷ and our cases were significantly more likely than controls to have the C allele. *MTHFR* catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is necessary for the conversion of homocysteine to methionine. Hyperhomocysteinemia is associated with polymorphisms at -677 and -1298, especially the 677 TT variant, and results in endothelial cell injury and alterations in coagulation.²¹⁻²³

We report a large reported cohort of inborn AGA preterm neonates of European ancestry with exposure to ANS, Gr 3-4 IVH and genotyping. Limitations include the small sample size, failure to date onset of hemorrhage and lack of homocysteine levels for study participants. This study was not able to evaluate rare variants, and, although we have previously reported a *COL4A1* duplication in twins with severe IVH,²⁴ the technology we employed was not designed to identify short insertions. The strengths include prospective collection of preterm neonates with tertiary neonatal care, centrally read ultrasounds and plans for state-of-the-art genetic analyses.

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ABBREVIATIONS

AGA	Appropriate for gestational age
ANS	Antenatal steroid
BW	Birth weight
COL4A1	Collagen 4A1
cUS	Cranial ultrasonography
GA	Gestational age
Gr	Grade
IL	Interleukin
IVH	Intraventricular hemorrhage
MTHFR	Methylenetetrahydrofolate reductase
TNF	Tumor necrosis factor

Table 1

Validation of predefined risk genotype comparisons in cases vs controls

Gene	Variant ^a	Genotype comparison	SNP	Proxy (D' value with original SNP) ^b	Odds Ratio (95% CI)	P value
<i>COL4A1</i>	p.Gly1580Arg ⁸	CG vs GG	rs113994114	rs3825481 (na ^c)	0.89 (0.52-1.49)	0.712
<i>F2</i>	c.97G>A ^{7,13}	AG vs GG	rs1799963	rs5898 (1.000)	1.40 (0.88-2.22)	0.139
<i>F5</i>	c.1601G>A ^{6,7,13}	AG vs GG	rs6025	rs6015 (1.000)	1.07 (0.66-1.72)	0.817
<i>IL1B</i>	c.-87-511T>C ^{6,14}	T vs C	rs16944	-	1.02 (0.79-1.30)	0.902
	c.-87-31C>T ¹⁴	C vs T	rs1143627	-	1.03 (0.80-1.31)	0.853
<i>IL6</i>	c.-116-121C>G ¹⁵	CC vs CG or GG	rs1800795	rs1800797 (1.000)	1.08 (0.76-1.56)	0.662
<i>MTHFR</i>	c.677C>T ⁷	TT or CT vs CC	rs1801133	-	0.95 (0.67-1.34)	0.799
	c.1298A>C ⁷	CC or CA vs AA	rs1801131	-	1.56 (1.10-2.20)	0.009
<i>TNF</i>	c.-169-319G>A ¹⁶	AA or AG vs GG	rs1800629	-	0.90 (0.62-1.31)	0.587

^a c - cDNA; p - protein

^b 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

^c Allele frequency 0 for the original SNP from 1000 genomes database

COL4A1 - collagen 4A1; *F2* - prothrombin; *F5* - factor V Leiden; *IL* - interleukin; *MTHFR* - Methylene tetrahydrofolate reductase; *TNF* - tumor necrosis factor

Table 2

Genotype frequencies and p values in cases with Grade 3 – 4 IVH and controls

Gene	Variant	Genotype	Number (%)		P value
			Controls	Cases	
<i>COL4A1</i>	p.Gly1580Arg ⁸ rs3825481 [*]	AA	85.8	87.4	0.817
		AG	13.9	12.6	
		GG	0.3	0.0	
<i>F2</i>	c.97G>A ^{7,13} rs5898 [*]	GG	84.5	79.9	0.306
		GA	14.5	19.2	
		AA	1.0	0.9	
<i>F5</i>	c.1601G>A ^{6,7,13} rs6015 [*]	CC	84.3	83.5	0.919
		CT	15.2	16.1	
		TT	0.5	0.5	
<i>IL1B</i>	c.-87-511T>C ^{6,14} rs16944	GG	41.4	37.5	0.206
		AG	44.7	51.8	
		AA	13.9	10.7	
	c.-87-31C>T ¹⁴ rs1143627	TT	41.4	37.1	0.184
		CT	44.7	52.2	
		CC	13.9	10.7	
<i>IL6</i>	c.-116-121C>G ¹⁵ rs1800797 [*]	GG	35.7	33.9	0.873
		AG	46.8	47.3	
		AA	17.5	18.8	
<i>MTHFR</i>	c.677C>T ⁷ rs1801133	CC	42.4	43.8	0.782
		CT	44.5	45.1	
		TT	13.1	11.2	
	c.1298A>C ⁷ rs1801131	AA	54.8	43.8	0.021
		AC	37.0	48.2	
		CC	8.2	8.0	

Gene	Variant	Genotype	Number (%)		P value
			Controls	Cases	
<i>TNF</i>	c.-169-319G>A ¹⁶ rs1800629	GG	68.4	70.5	0.843
		AG	29.3	27.7	
		AA	2.3	1.8	

* Proxy SNP

Table 3

Study Subjects

	Gr 3 - 4 IVH	Controls	p value
Number of subjects	224	389	
Birth weight grams (N, SD)	810.5 (224, 181.5)	864.3 (389, 174.0)	<0.0001
GA wks (N, SD)	25.6 (224, 1.5)	26.5 (389, 1.5)	<0.0001
Male subjects (%)	128/224 (57.1%)	231/389 (59.4%)	0.61
IVH status			
Grade 3	95		
Grade 4	129		
Maternal variables			
Maternal prenatal visit (%)	216/222 (97.3%)	384/389 (98.7%)	0.2193
Preeclampsia (%)	23/222 (10.4%)	91/388 (23.5%)	<0.0001
Clinical chorioamnionitis (%)	69/223 (30.9%)	69/389 (17.7%)	0.0003
Any ANS w/in 7 d prior to delivery (%)	195/223 (87.4%)	336/388 (86.6%)	0.8043
Complete ANS within 7 d (%)	118/224 (52.7%)	260/389 (66.8%)	0.0006
Multiple gestation (%)	81/224 (36.2%)	90/386 (23.3%)	0.0008
Cesarean delivery	133/224 (59.4%)	281/388 (72.4%)	0.0012
Delivery room variables			
Apgar 1 minute < 3 (%)	70/222 (31.5%)	89/388 (22.9%)	0.0216
Apgar 5 minutes < 3 (%)	19/223 (8.5%)	14/388 (3.6%)	0.0145
Intubation for resuscitation	166/203 (81.8%)	243/351 (69.2%)	0.0013
Epinephrine for resuscitation	8/162 (4.9%)	5/215 (2.3%)	0.2535

Table IV

Site	Cases	Controls	Total
Gene Targets for IVH	162	215	377
ELGAN	24	90	114
Iowa	17	46	63
Oulu	21	38	59
Total	224	389	613

* Central blinded ultrasound readings were performed for all subjects from the Gene Targets for IVH, ELGAN and Iowa cohorts (554/613, 90%). Ultrasounds for Oulu subjects were performed by blinded readers but were not centrally read.