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Genes and Environment in Neonatal Intraventricular Hemorrhage

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Abstract

Emerging data suggest intraventricular hemorrhage (IVH) of the preterm neonate is a complex disorder with contributions from both the environment and the genome. Environmental analyses suggest factors mediating both cerebral blood flow and angiogenesis contribute to IVH, while candidate gene studies report variants in angiogenesis, inflammation and vascular pathways. Gene-by-environment interactions demonstrate the interaction between the environment and the genome, and a non-replicated genome-wide association study suggests that both environmental and genetic factors contribute to the risk for severe IVH in very low birth weight preterm neonates.

Introduction

Preterm birth affects an estimated 13 million newborns worldwide annually,^{1,2} and sophisticated advances in perinatal care have improved survival for the prematurely-born.³ In contrast, the incidence of neurodevelopmental handicap in the prematurely-born has changed little during the last two decades,^{4–6} mandating a more complete assessment of injury to the developing preterm brain. Intraventricular hemorrhage (IVH), or hemorrhage into the germinal matrix tissues of the developing brain with ventricular enlargement and parenchymal involvement, is one of the major causes of morbidity in the prematurely-born, often resulting in cerebral palsy and cognitive handicap.^{7,8} Notably, there are over 2,800 new cases of mental retardation attributable to IVH in the US each year, and the lifetime care costs for these children exceed \$4 billion (2010) annually.^{9,10} Emerging data suggest that IVH is a complex developmental disorder with contributions from both the environment and the genome.¹¹

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attributed to changes in cerebral blood flow to the immature germinal matrix microvasculature.¹² Inflammation, coagulation and vascular factors may also play a role.^{8,12} Severe IVH is characterized by the acute hemorrhagic distension of the cerebral ventricular system (Grade 3) and parenchymal venous infarction (Grade 4). The cascade of adverse events following IVH includes destructive, inflammatory and maturational disturbances and is characterized by white matter injury, delayed oligodendroglial maturation, loss of gamma-aminobutyric acid (GABA) interneurons and impaired thalamo-cortical connectivity.⁸ All may contribute to developmental disability.

The purpose of this report is to review both environmental and genetic data supporting the hypothesis that IVH is a complex disorder. Candidate genes, gene-by-environment interaction studies reviewed and a recent genome wide association study (GWAS) will be reported.

The etiology of IVH is multifactorial

Multiple sources of data support the hypothesis that, similar to most other morbidities in preterm neonates,^{13,14} the etiology of IVH is multifactorial. Maternal transport, antenatal steroid (ANS) administration for fetal lung maturation and improved resuscitation techniques have become standard of care for women in preterm labor and premature infants worldwide,^{15–18} but the incidence of severe IVH has remained 12–15% for the past 10–15 years.^{15,19–23} In addition, both gender and twin studies support the hypothesis that IVH is a complex disorder. Preterm males are more likely than females to experience IVH,²⁴ and data from a twin study suggests that environmental and familial factors contributed 43% of risk for IVH.²⁵ Furthermore, although the incidence of IVH is inversely related to gestational age (GA), the risk period for IVH is independent of this key variable, suggesting that either the transition to extra-uterine life and/or the environmental variables to which the neonates are exposed contribute to injury to the prematurely-born. Finally, recent data suggest that both candidate genes and gene-by-environment interactions may also play a role.^{11,26–28}

Environmental factors and health care disparities are permissive for hemorrhage

IVH occurs against the backdrop of preterm birth in which both risk and protective factors have been well described.¹⁵ Lower GA and birth weight (BW), male gender, white race, chorioamnionitis, Apgar < 3 at 5 minutes, delivery room resuscitation, surfactant administration, neonatal transport, illness severity, assisted ventilation, disturbances of partial pressure of CO₂, respiratory distress syndrome and high frequency ventilation have all been reported to increase risk for IVH, while a complete course of ANS, cesarean delivery and preeclampsia decrease the risk for IVH (for review please see Shankaran²⁹).

Recent data suggest, however, that advances in neonatal and perinatal care and the increasing survival of extremely low birth weight infants may have altered these associations. In addition, the recognition of the importance of health care disparities in the etiology of IVH has recently been reported. Women of African ancestry are at greater risk for preterm labor and delivery than white women, and a higher percentage of very low birth

weight infants are born to African ancestry mothers.^{30–32} Further, infants of African ancestry mothers are less likely to receive surfactant or assisted ventilation,³³ and IVH-related mortality is two times higher in African ancestry neonates when compared to white preterm infants.³⁴ A recent large prospective analysis demonstrated that white race decreased risk for grades 2–4 IVH in preterm neonates of 500–1250 g;²⁹ notably, among white infants but not black neonates, multiple gestation was associated with increased risk of IVH, while higher maternal education was associated with a decreased incidence of hemorrhage. When compared to white neonates, infants of African ancestry less often received ANS exposure and required more vigorous delivery room resuscitation. For the infants of African ancestry mothers, having more than one maternal prenatal visit significantly decreased the risk for IVH, suggesting that initiating care prior to labor and delivery provides a distinct protective advantage.

Preclinical studies: Mutations in microvascular proteins confer vulnerability to IVH

The germinal matrix is a region of active angiogenesis, and IVH begins in this region. The microvessels of the germinal matrix lack the traditional components of the blood brain barrier, endothelial tight junctions, basement membrane proteins, glial endfeet and perivascular pericytes.¹² IVH is thought to be a critical period disorder, and previous work has suggested that it is the developmental stage of the germinal matrix micro-vessels that are permissive to hemorrhage.³⁵

Preclinical data suggest, however, that variants in one or more microvascular proteins confer vulnerability to the environmental factors discussed above. As a clinically-relevant model, mice with mutations in the basement membrane protein collagen 4A1 experience IVH mimicking human hemorrhage.^{36,37} Notably, IVH is prevented by surgical delivery of the pups, suggesting an interaction between an environmental trigger associated with murine labor and delivery and the genome.

Variants in genes subserving angiogenesis have also been associated with hemorrhage in preclinical models. A particularly important candidate is transforming growth factor- β (*TGF- β*). *TGF- β* activation and signaling is essential for angiogenesis in developing brain, and $\alpha v\beta 8$ integrin mediates its activation.³⁸ Fetal mice genetically null for integrin $\beta 8$ develop severe intracerebral hemorrhage; likewise, murine fetuses with the *TGF- β* endothelial cell receptor variants *Tgfb2* and *Alk5* also experience lethal IVH.^{39,40} Mutations in both receptors are associated with Loeys-Dietz syndrome in humans, an arteriopathy characterized by both aneurysms and arterial dissections.⁴¹

Variants in genes subserving cerebral blood flow may also result in hemorrhage. Annexin 7 (*Anx7*) is a gene encoding vascular Ca^{++} activity; in the presence of *Anx7* deficiency and hypoxia-ischemia, there is increased adhesion of erythrocytes to the vascular wall and secondary alterations in microcirculation.⁴² Of note, mice with mutations in *Anx7*, a gene encoding vascular Ca^{++} channel activity, experience hemorrhage,⁴³ suggesting an interaction of environmental perturbations such as hypoxia and ischemia and the genome.

Inflammation has been implicated in the pathogenesis of intracranial aneurysm formation and rupture, and variants in the inflammatory factors *IL-1 β* and *TNF- α* contribute to aneurysmal rupture and hemorrhage in preclinical models.^{44,45} In preclinical studies, in response to either hypoxic-ischemic injury or perinatal bacterial infection, *IL-1 β* differentially increases across the brain. Similar to *TNF- α* , it may result in alterations in both endothelial and smooth muscle cells. Finally, *IL-6* is an upstream cytokine responsible for initiating the angiogenic cascade. *IL-6* induces *MMP-3* and *MMP-6* expression and activity in murine brain and increases proliferation and migration of cerebral endothelial cells, and the *IL-6-174 G>C* promoter polymorphism is associated with intracranial hemorrhage in brain arteriovenous malformation patients.⁴⁶

Clinical studies suggest coagulation, inflammation and vascular pathways

IVH has been attributed to alterations in cerebral blood flow to the immature germinal matrix microvasculature, and risk factor studies across the past two decades have suggested the role of genes contributing to coagulation and vascular pathways. Similarly, the risk for injury is higher in preterm neonates exposed to prenatal inflammation, and candidate genes subserving infection and inflammation have also been interrogated.

Numerous variants in the coagulation pathway have been investigated, but those most frequently studied include the Factor V Leiden (F5) variant, polymorphisms of the methylenetetrahydrofolate reductase gene (MTHFR) and the prothrombin 20210G>A variant (F2). The first, the F5 variant, has been investigated in different populations with different results. The F5 variant presents with hypercoagulability secondary to a point mutation in an activated protein C cleavage site. In unaffected subjects, activated protein C cleaves activated F5, resulting in inhibition of the coagulation pathway. This polymorphism has been reported variously to (1) increase low grade IVH; (2) protect against severe hemorrhage; or (3) to not contribute to IVH.²⁷ In addition, the F5 variant has been associated with the atypical occurrence of IVH (i.e., that occurring more than 96 hours after birth).²⁶

MTHFR is another candidate gene for IVH. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyl-tetrahydrofolate, necessary for the conversion of homocysteine to methionine. Polymorphisms at -677 and -1298 result in hyperhomocysteinemia and result in endothelial damage and secondary alterations in coagulation including stroke, thrombosis and vascular disorders.⁴⁷

Low levels of folate increase hyperhomocysteinemia and secondarily increase the risk for cerebral injury. Both recent preclinical studies and clinical data of term infants with hypoxic-ischemic encephalopathy suggest the interaction between MTHFR polymorphism, folate and hypoxic-ischemic injury to developing brain.^{48,49} In a prospective study of 705 inborn European ancestry preterm neonates of 500–1250 g BW and ANS exposure, MTHFR -1298 CC or CA genotypes were associated with Grade 2–4 IVH. In addition, the MTHFR variant and the interaction term (Apgar₅ < 3-by-MTHFR allele) were independent and important predictors of hemorrhage.¹¹ Finally, coinheritance of more than one thrombophilia variant has been associated with greater risk of perinatal stroke, and both

–677 and –1298 polymorphisms have been reported in preterm infants with F5 mutations and hemorrhage; however, these data have not as yet been replicated.²⁶

Another common coagulation variant tested in large populations is F2; it results in increased thrombin and secondary thrombosis but has not been associated with risk for IVH.²⁷ Finally, the role of vitamin K in IVH has been investigated across the past 20 years, and a recent unreplicated study of polymorphisms in genes encoding vitamin D epoxide reductase complex 1 (VKORC1 – 1639G>A) and coagulation factor 7 (F7-323Ins10) suggests that genetic variants in the vitamin K-dependent coagulation system influence IVH risk in preterm infants, although further studies are necessary.⁵⁰

Inflammatory factors including *IL-1β*, *IL-6* and *TNF-α* have also been implicated in IVH of the preterm neonate. *IL-1β* is the major cytokine responsible for activation of the hypothalamic-pituitary-adrenal axis and has been implicated in the progression of injury in preterm brain. Notably, *IL-1β* is both developmentally and regionally regulated in the brains of typically developing fetuses and neonates (for review please see Ment¹¹). In two separate large prospective studies, both the *IL-1β* 511C>T and *IL-1β*-31 C allele were associated with a risk for hemorrhage.^{27,28} The C allele of *IL-1β*-31 is in strong linkage disequilibrium with the former variant, and both increase production of *IL-1β*. In addition, although an early report suggested that the CC genotype of *IL-6*-174 increased the risk for IVH, this finding has not been replicated in more recent large investigations. Similarly, data for the *TNF-α* -308A allele has yielded mixed results, suggesting that in many clinical studies of risk for hemorrhage, numbers of subjects, patient population and definition of injury may all play a role in varying results.

Finally, vascular genes including *COL4A1* and those mediating cerebral blood flow are candidates for hemorrhage. Variants in *COL4A1* have been associated with not only preterm IVH but also fetal intracerebral hemorrhage and white matter disease in adults.^{51,52} Likewise, the endothelial nitric oxide synthase gene promoter polymorphism 785T>C has been associated with increased risk for IVH,⁵³ while the rs8192287 superoxide dismutase 3 polymorphism has been reported to an independent protective factor for hemorrhage in preterm neonates.⁵⁴

Genome wide association study for IVH: An unbiased discovery strategy

Although environmental risk factors have been identified and candidate genes investigated, IVH remains a significant problem for the prematurely-born. To investigate potential genetic etiologies of severe IVH in preterm infants, employing a cohort of 458 inborn appropriate for gestational age neonates with severe IVH and 866 infants without IVH, we conducted a GWAS among cases and controls across the US and Scandinavia to identify disease-susceptibility or protection genes and generate neurobiological pathway hypotheses.

Methods

Inborn infants with BW 500–1250 g and either severe (Grade 3–4) IVH or normal cranial ultrasounds were enrolled prospectively at 24 universities; additional samples were provided from the ELGAN (Extremely Low Gestational Age Newborns),⁵⁵ Iowa Prematurity²⁷ and

Oulu University⁵⁶ studies. The protocol was approved by institutional review boards at each institution and all subjects have appropriate informed consent.

Description of cases and controls

Cases had Grade 3–4 IVH based upon blinded cranial ultrasonography review and met the following criteria: inborn; ANS exposure; BW 500–1250 g; appropriate for gestational age;⁵⁷ no congenital malformations, infections or chromosomal disorders; no family history of coagulopathy; and not a sibling of an enrolled subject. Enrollment was limited to one sibling of a multiple birth set as previously described. Control infants met these criteria, had two normal cranial ultrasounds within the required time intervals described below and were matched to cases based on the following criteria: 1) site; 2) gender; and 3) BW range (i.e., 500–749 g; 750–999 g; 1000–1250 g). In addition, every effort was made to match controls based on self-reported maternal race and ethnicity. Cranial ultrasounds were centrally read by blinded reviewers, and perinatal data were prospectively collected and entered into a secure online database housed at Yale University.

Genotyping and quality control

Genomic DNA was isolated from buccal swabs, umbilical cords or blood from 1473 Grade 3–4 IVH subjects and controls, and genotyped on Illumina HumanOmni1-Quad v1.0 and HumanOmniExpress-12 v1.0 and v1.1 arrays as previously described.⁵⁸ There were 681,612 single-nucleotide polymorphisms (SNP) common to all three chips. We initially performed quality control (QC) on each chip then on combined data from the three chips. Samples were excluded if the overall genotype call rate was < 95%, the imputed gender and the reported gender did not agree, or they showed high relatedness to another sample by identity-by-descent. We excluded SNPs with an individual genotype call rate < 95%, minor allele frequency < 1% or a significant departure from Hardy-Weinberg Equilibrium (HWE) among controls by ancestry or among all controls ($p < 1 \times 10^{-4}$). In addition, SNPs with a significant difference in genotype missing rates between cases and controls ($p < 5 \times 10^{-7}$) were excluded. The ancestry of the subject was identified using STRUCTURE^{59,60} based on the genotypes of 5,000 randomly selected SNPs from our data and Hapmap Phase 3 data.⁶¹ The genetic ancestry we identified accounted for the ethnic differences among the subjects as suggested by the multidimensional scaling plot (not shown). A total of 1,324 subjects and 583,626 SNPs passed QC. The QC procedures were performed using PLINK v1.07.⁶²

Statistical analysis for environmental risk factors

Continuous variables were compared using Student's t test and categorical variables using Fisher's exact test. A generalized linear mixed model was employed to assess the relative contributions of the significant environment variables and top SNPs on IVH. P-value < 0.05 was considered statistically significant. Analyses were performed with SAS (version 9.3).

SNP association analysis

A GWAS was performed using PLINK v1.07⁶² to determine risk variants for IVH. We tested for association between IVH and single SNP by using logistic regression with gender, ancestry and BW as covariates. Since multiple SNPs in the intergenic region between a non-

coding RNA *GM140* (uncharacterized LOC100287948) and *CACNA1E* (calcium channel, voltage-dependent, R type, alpha 1E subunit) showed association with IVH Grade 3–4 in the single-SNP based association analysis, a logistic regression model incorporating gender, ancestry and BW as covariates was applied to carry out haplotype-based association analysis. The sliding-window framework^{62–64} was used to specify all haplotypes consisting of SNPs with odds ratio (OR) > 1 or those with OR < 1 in that region. Following the identification of a significant 10-SNP haplotype in the region between *GM140* and *CACNA1E*, we repeated the analysis for all 10-SNP haplotypes across the whole genome, and a Bonferroni correction was applied to adjust for multiple testing for haplotype-based tests.

Replication study

Following analysis of the discovery GWAS data, we selected 603,183 usable common SNPs for replication in an independent replication data set of 64 severe IVH cases and 226 controls who were genotyped using the Illumina Omni-1 Quad platform. The replication data set was provided by the Genome Subcommittee of the NICHD Neonatal Research Network (NRN). Patients included in the NRN validation cohort were a subset of patients enrolled in the Eunice Kennedy Shriver NICHD Neonatal Research Network's (NRN) Cytokines study.⁶⁵ A full description of the process of development of the Network's Anonymized DNA bank that houses the samples and data has been previously described.⁶⁶ The Cytokines study enrolled newborns from 1999–2002. Infants weighing 401–1000 g at birth, < 72 hours of age and free of major congenital anomalies were eligible. Replication study data were analyzed both individually and in combination with the discovery set, the latter being termed joint analysis.

Results

Patient characteristics

Neonates with Gr 3–4 IVH had lower BWs and GAs than controls ($p < 0.001$ for both; Table 1). Mothers of cases were less likely to have at least one prenatal visit ($p = 0.044$), preeclampsia ($p < 0.001$), or a complete course of ANS ($p < 0.001$) than control mothers. In contrast, they more often had chorioamnionitis ($p < 0.001$).

Cases were less likely than controls to have Cesarean section delivery ($p < 0.001$), and more often had low 5-minute Apgar scores (Apgar₅ < 3; $p < 0.001$), intubation for resuscitation ($p < 0.001$) and epinephrine for resuscitation in the delivery room ($p = 0.005$). A generalized linear mixed model for the environmental data alone demonstrated that the following factors were associated with an increased risk for Gr 3–4 IVH: lower GA, failure to be exposed to a complete course of ANS, maternal preeclampsia, 5-minute Apgar score < 3, intubation for resuscitation and pneumothorax (data not shown).

Discovery GWAS

GWAS candidates ($p < 1.0E-4$, Table 2) that did not reach GWAS significance ($p < 5.0E-8$) include *CLIC4* (chloride intracellular channel 4), *CACNA1E* (calcium channel, voltage-dependent, R type, alpha 1E subunit), *ANK1* (ankyrin 1), *PFKFB3* (phosphofructokinase-2/

fructose-2,6-biphosphatase 3), *MMP21* (matrix metalloproteinase 21), *FLT1* (fms-related tyrosine kinase 1), *AQP9* (aquaporin 9) and *NCAM2* (neural cell adhesion molecule 2).

A generalized linear mixed model for the environmental and genetic data is shown in Table 3. In addition to environmental factors and single SNPs, we noted a significant interaction between preeclampsia and the *CACNA1E* T variant. This demonstrated that among infants with mothers with preeclampsia, the T variant was associated with a decreased risk for IVH [*CACNA1E*: OR = 1.72, $p = 4.53\text{E-}07$; *CACNA1E*-x-preeclampsia: OR = 0.40, $p = 9.09\text{E-}06$].

Finally, a haplotype analysis with a 10-SNP moving window resulted in a total of 8,731,791 haplotypes across the whole genome. The 10-SNP haplotype (Table 4, Figure 1) ranging from the intergenic region of *GM140* and *CACNA1E* to the intron region of *CACNA1E* (OR = 2.3) was the most significant predictor of IVH; this had a p-value of $7.16\text{E-}10$ before correction and $p = 0.0063$ after Bonferroni correction. *CACNA1E* is a calcium channel that is mutated in a Mendelian form of hemiplegic migraine, a known vascular phenotype.⁶⁷ Finally, preeclampsia protected neonates with this variant (*CACNA1E*: OR = 1.72, $p = 4.53\text{E-}07$; *CACNA1E*-x-preeclampsia: OR = 0.40, $p = 9.09\text{E-}06$).

Replication Study

The replication cohort of 64 cases and 226 controls was characterized by several significant differences when compared to the discovery cohort (Table 5). Replication infants had lower BW, fewer male subjects and differences in race and ethnicity when compared to the discovery cohort. In addition, fewer Replication mothers had had at least one prenatal visit and fewer Cesarean section deliveries but more ANS exposure within 7 days prior to delivery. In addition, although replication neonates had fewer 5 minute APGAR scores < 3 and less periventricular leukomalacia, replication neonates experienced more death prior to discharge when compared to discovery infants.

No SNP from the discovery stage replicated, and join analysis of discovery and replication cohorts did not yield any genome-wide significant SNPs.

Moving forward: Gene-by-environment interactions for the prevention of IVH

Severe IVH of the prematurely-born is a high-prevalence, high-impact disorder, but the genetic investigations of IVH have thus far been largely disappointing. Future studies supporting the rationale design of multicenter randomized clinical trials must therefore include not only large-scale GWAS, but also whole exome sequencing data and, ultimately, whole genome sequencing. Transcriptomics and epigenomics also merit examination, as IVH is a critical period disorder. Finally, causal relations of possible genetic targets and environment perturbations should ideally be confirmed in preclinical models.

Published reports of both preclinical and clinical candidate gene investigations suggest that IVH may be attributable to numerous genes with small effect sizes and the environmental triggers that interact with them. Because common variants have small-to-moderate effects, a

large-scale neonatal genomic medicine network must be developed with the infrastructural capacity to host an accessible database of sequence variants, their phenotypic associations and environmental risk factors. This network must also support a framework for defining and cataloging clinically actionable pathways and variants.

A major focus of perinatal care is to prevent brain injury in the prematurely-born. A better understanding of the genetic contributions to IVH will help us to achieve that goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ANS	Antenatal steroids
BW	Birth weight
GA	Gestational age
GWAS	Genome-wide association study
IVH	Intraventricular hemorrhage
NRN	Neonatal Research Network
OR	Odds ratio
QC	Quality control
SNP	Single nucleotide polymorphism

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Figure 1.

A haplotype-based association analysis for *CACNA1E* revealed that a 10-SNP haplotype including two haplotype blocks between *GMI40* and *CACNA1E* was a significant risk factor for IVH Grade 3–4 (OR = 2.3, $P = 7.16\text{E-}10$). Numbers in the blocks are D' values. Figure was generated using Haploview.⁶⁰

Table 1**Study Subjects**

	Grade 3–4 IVH	Controls	p value
Number of subjects	458	866	
Birth weight grams (N, SD)	799 (458, 181)	847 (866, 175)	<0.001
GA weeks (N, SD)	25.5 (458, 1.6)	26.4 (866, 1.5)	<0.001
Male subjects (%)	267/458 (58.3)	464/866 (53.6)	0.10
AA subjects (%)	151/427 (35.4)	255/807 (31.6)	0.18
White subjects (%)	276/427 (64.6)	552/807 (68.4)	
IVH status Grade 3 (%) Grade 4 (%)	181 (39.5) 277 (60.5)		
Maternal variables			
Maternal prenatal visit (%)	437/457 (95.6)	841/861 (97.7)	0.044
Preeclampsia (%)	42/456 (9.2)	206/865 (23.8)	<0.001
Clinical chorioamnionitis (%)	150/457 (32.8)	180/866 (20.8)	<0.001
Any ANS w/in 7 d prior to delivery (%)	391/457 (85.6)	756/864 (87.5)	0.35
Complete ANS within 7 d (%)	218/458 (47.6)	571/866 (65.9)	<0.001
Multiple gestation (%)	122/458 (26.6)	190/863 (22.0)	0.07
Cesarean section (%)	267/458 (58.3)	609/865 (70.4)	<0.001
Delivery room variables			
Apgar 1 minute < 3 (%)	181/456 (39.7)	203/865 (23.5)	<0.001
Apgar 5 minutes < 3 (%)	54/457 (11.8)	30/865 (3.5)	<0.001
Intubation for resuscitation (%)	378/437 (86.5)	582/824 (70.6)	<0.001
Epinephrine for resuscitation (%)	29/364 (8.0)	16/468 (3.4)	0.005
Neonatal variables			
High frequency ventilation (%)	237/413 (57.4)	164/770 (21.3)	<0.001
Pneumothorax (%)	44/413 (10.7)	37/770 (4.8)	0.0002

Table 2

Top Candidate SNPs

CHR	SNP	BP	A1	OR	MAF	P value	Gene Symbol	Gene Location
1	rs4649014	25015530	A	1.44	0.35	3.61E-05	CLIC4	Intergenic
1	rs7523745	181274415	T	1.48	0.41	3.78E-06	CACNA1E	Intergenic
8	rs11784487	41602668	A	1.49	0.22	9.16E-05	ANK1	Intron
10	rs17151529	6224566	T	1.53	0.24	1.49E-05	PFKFB3	Intron
10	rs7922546	127460753	A	1.43	0.31	6.12E-05	MMP21	Intron
13	rs664393	29071001	A	1.65	0.10	1.74E-04	FLT1	UTR
15	rs12593703	58498259	T	1.66	0.13	7.65E-05	AQP9 LIPC	Intergenic
21	rs1015020	24334028	G	0.62	0.22	9.60E-06	NCAM2 NCRNA00158	Intergenic

Table 3

Generalized Linear Mixed Model for IVH Grade 3–4 Risks

Effect	OR	95% Confidence Limits		P value
GA weeks	0.78	0.71	0.86	9.82E-07
Complete ANS	0.56	0.42	0.75	9.33E-05
Apgar at 5 minutes < 3	2.42	1.39	4.23	1.86E-03
Intubation	2.42	1.62	3.60	1.59E-05
Pneumothorax	2.12	1.24	3.63	6.14E-03
CACNA1E_rs7523745	1.72	1.39	2.12	4.53E-07
ANK1_rs11784487	1.60	1.26	2.03	1.38E-04
MMP21_rs7922546	1.41	1.14	1.74	1.76E-03
AQP9_rs12593703	1.48	1.10	1.98	1.01E-02
NCAM2_rs1015020	0.63	0.49	0.81	3.21E-04
Preeclampsia × CACNA1E	0.40	0.26	0.60	9.09E-06

Table 4

SNPs in the 10-SNP Haplotype

SNP	BP	A1	OR	P value	Gene Symbol	Gene Location
rs4651099	181227661	A	0.70	1.61E-02	GMI140 CACNA1E	INTERGENIC
rs12027236	181240106	G	0.79	7.93E-03	GMI140 CACNA1E	INTERGENIC
rs75444832	181240950	A	0.74	1.15E-02	GMI140 CACNA1E	INTERGENIC
rs6674511	181260215	T	0.72	2.38E-02	GMI140 CACNA1E	INTERGENIC
rs7525299	181296112	A	0.67	9.54E-06	GMI140 CACNA1E	INTERGENIC
rs7548242	181296409	G	0.67	7.83E-06	GMI140 CACNA1E	INTERGENIC
rs12756919	181310522	G	0.81	1.30E-02	GMI140 CACNA1E	INTERGENIC
rs3845428	181311555	C	0.78	3.20E-03	GMI140 CACNA1E	INTERGENIC
rs650635	181386651	C	0.79	4.92E-02	GMI140 CACNA1E	INTERGENIC
rs683927	181489802	T	0.80	9.28E-03	CACNA1E	INTRON

Table 5

Comparison of the NRN and GTI Study Subjects

	GTI	NRN	P value
Number of subjects	1324	290	
Birth weight grams (N, SD)	830 (1324, 179)	774 (290, 129)	<.0001
GA weeks (N, SD)	26.1 (1324, 1.6)	26.0 (290, 1.5)	0.33
Male subjects (%)	731/1324 (55.2)	128/290 (44.1)	0.0007
AA subjects (%)	407/1235 (33.0)	160/288 (55.6)	<.0001
White subjects (%)	828/1235 (67.0)	128/288 (44.4)	
HS race (%)	137/1324 (10.3)	51/290 (17.6)	0.0008
IVH status			
Grade 3 (%)	181 (39.5)	23(35.9)	0.68
Grade 4 (%)	277 (60.5)	41(64.1)	
Maternal variables			
Maternal prenatal visit (%)	1278/1314 (97.3)	266/289 (92.0)	<.0001
Preeclampsia (%) ^a	248/1321 (18.8)	66/290 (22.8)	0.12
Clinical chorioamnionitis (%)	330/1323 (24.9)	N/A	
Any ANS w/in 7 d prior to delivery (%)	1147/1321 (86.8)	286/289 (99.0)	<.0001
Complete ANS within 7 d (%)	788/1324 (59.5)	148/286 (51.7)	0.017
Cesarean section (%)	876/1323 (66.2)	154/290 (53.1)	<.0001
Delivery room variables			
Apgar 1 minute < 3 (%)	384/1321 (29.1)	80/289 (27.7)	0.68
Apgar 5 minutes < 3 (%)	84/1322 (6.4)	11/290 (3.8)	0.01
Intubation for resuscitation (%)	960/1261 (76.1)	222/290 (76.6)	0.94
Neonatal variables			
High frequency ventilation (%)	401/1183 (33.9)	N/A	
Pneumothorax (%)	81/1183 (6.9)	N/A	
PVL (%) ^b	99/893 (11.1)	17/290 (5.9)	0.009
Ventriculomegaly (%)	191/542 (35.2)	N/A	
Death before discharge (%)	72/1289 (5.6)	42/289 (14.5)	<.0001

^a GTI uses preeclampsia, while NRN combines hypertension/preeclampsia/eclampsia into one variable;

^b PVL defined at 36–44 weeks postmenstrual age for GTI and at either 38 days or 36 weeks for NRN neonates