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Genetic predictors of severe intraventricular hemorrhage in extremely low-birthweight infants

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Abstract

Objective—To test associations between grades 3 or 4 (severe) intraventricular hemorrhage (IVH) and single nucleotide polymorphisms (SNPs) associated with coagulation, inflammation, angiogenesis, and organ development in an exploratory study.

Study design—Extremely low-birthweight (ELBW) infants enrolled in the Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network's (NRN) Cytokines Study were included if they had cranial ultrasound (CUS) and genotyping data

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Author contributions CDT and CMC conceptualized and designed the analysis and drafted, reviewed, and revised the manuscript. SWE and GPP conducted the data analysis and reviewed and revised the manuscript. EASC, MMD, MEH, RFG, JMD, JCM, BBP, and AD collected data and critically reviewed the manuscript.

Conflict of interest The authors have no conflict of interest to report. The contents of this report represent the views of the authors and do not represent the views of the Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network or the National Institutes of Health.

Members of the Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network and their affiliations are presented in Supplementary information.

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available in the NRN Anonymized DNA Repository and Database. Associations between SNPs and IVH severity were tested with multivariable logistic regression analysis.

Result—One hundred thirty-nine infants with severe IVH and 687 infants with grade 1 or 0 IVH were included. One thousand two hundred seventy-nine SNPs were genotyped. Thirteen were preliminarily associated with severe IVH including five related to central nervous system (CNS) neuronal and neurovascular development.

Conclusion—Genetic variants for CNS neuronal and neurovascular development may be associated with severe IVH in premature infants.

Introduction

Intraventricular hemorrhage (IVH) is a major contributor to mortality and neurodevelopmental morbidity in extremely low-birthweight (ELBW) infants [1]. IVH increases with decreasing gestational age, with overall rates of severe IVH approaching 20% among those born before 28 weeks gestation [2]. In 2006, Bhandari et al. provided evidence that inherited risk factors contribute to severe IVH risk in preterm twins [3]. Since IVH may occur when cerebral venous thrombosis causes venous congestion, hypertension, and tissue edema, investigators have hypothesized that inherited hypercoagulable states contribute to IVH [4]. However, associations between IVH and thrombophilia gene variants have not been consistently demonstrated [4–7]. In addition, it is unclear if coagulopathy or platelet dysfunction contribute to the etiology of IVH [8–11], or whether hemostatic agents can prevent IVH [12–15]. In addition, chorioamnionitis and postpartum markers of inflammation are also associated with increased risk of IVH in preterm infants [16, 17]. Evolving data have provided evidence of gene–environment interactions on the impact of coagulation, vascular, and inflammatory pathways on IVH [7, 18, 19].

This was an exploratory, hypothesis generating, study utilizing a *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) Neonatal Research Network (NRN) database to identify genetic contributions to severe IVH in a cohort of ELBW infants from U. S. academic tertiary centers. The objective was to evaluate the association between severe IVH and single nucleotide polymorphisms (SNPs) in genes associated with coagulation, angiogenesis, inflammation, and organ (i.e., brain, kidney, eye) development in this study population. We hope that this work will also serve as a resource for other investigators who wish to explore genetic associations in this legacy cohort.

Subjects and methods

Source of study population and materials

The source of the study population and materials was the NICHD NRN Anonymized DNA Repository and Database, which was constructed from samples and data used for the Cytokines Study. The Cytokines Study (1999–2002) was a prospective study of predictors of neurodevelopmental outcomes in preterm infants in 1067 infants at 17 centers of the NRN, which included clinical data and whole blood spots on filter paper allowing for DNA extraction and analysis [20]. Consent was obtained for all subjects. Whole blood spots were collected on days 0 (cord blood or on day 0–1), 3 ± 1, 7 ± 1, 14 ± 3, and 21 ± 3 and frozen to

−70 °C. To construct the NRN Anonymized DNA Repository and Database from samples and data used for the Cytokines study, original NRN code numbers for each infant's data and their filter card samples were deleted and replaced with a new study number with no linkage between the new and the original study numbers. Dates were replaced with calculated postnatal age for specific time points, and center identification was deleted. All 17 NRN center IRBs and the IRB from the NRN' s Data Coordinating Center at RTI International (Research Triangle Park, NC) approved development of the NRN Anonymized DNA Repository and Database from the Cytokines study data and samples. Development of the NRN Anonymized Repository and Database was also reviewed and approved by a panel of outside reviewers for NICHD.

Study population

The study population included preterm infants, 400–1000 g at birth, who were enrolled in the Cytokines Study and had available DNA and at least one cranial ultrasound (CUS) performed. The CUS closest to 28 days of life and 36 weeks postmenstrual age was included. Infants were excluded if major congenital anomalies were documented.

Clinical data

Clinical data were collected by trained research coordinators using standardized data collection forms. Data included gestational age, SGA (small for gestational age) status, sex, maternal self-declared race and ethnicity, maternal hypertension, exposure to indomethacin in the first 24 postnatal hours, antenatal steroids, 5-min Apgar score, and CUS results. CUS were read by the clinical center radiologists. IVH was graded based on Papile's classification; grade I was evidence of blood confined to the subependymal germinal matrix, grade II was defined as the presence of blood within the ventricular lumen, grade III consisted of IVH with ventricular dilatation, and grade IV had parenchymal hemorrhagic infarction [21]. The grading was based on the most severe abnormality seen in the CUS, including the location of the bleed and ventricular size. For this study, cases of severe IVH included infants with grades III and IV, while controls included those with grades 0 and I. Infants with grade II IVH were excluded from this study in an attempt to only evaluate extreme phenotypes without overlap. Based on sample size, we would not have enough power to detect associations with smaller subgroups such as comparing grades III versus IV IVH. At the time of this study brain magnetic resonance imaging (MRI) was not routinely performed for evaluation of IVH; therefore, no data on MRI are included.

Selection of candidate genes and SNPs

A list of SNPs in candidate genes involved with coagulation, angiogenesis, inflammation, and organ development was generated, as we hypothesized that these processes are important in multiple organ systems at risk for morbidities of prematurity, including severe IVH.

The full list of genes is listed in Supplementary information Table 1. SNPs were prioritized based on function (coding vs. non-coding), potential regulatory role, and repetitive score (SNPs in non-repetitive regions are ranked higher than those in repetitive regions).

Our study was not powered to detect genetic effects which varied substantially by ancestry. The purpose of this study, therefore, was to identify genetic variants which influenced IVH risk on a background of multiple genetic ancestries. To maximize power, therefore, our priority was to identify those SNPs with the highest overall minor allele frequency (MAF) across multiple ancestries. In addition to high MAF, SNPs providing the best coverage of gene haplotypes were used as secondary criteria.

We had a goal of genotyping 20 SNPs per candidate gene. For two of the coagulation pathway genes (factor V and factor VIII), more than 200 SNPs are required for complete coverage (one has 25 exons, the other covers more than 160 kb). For these two large genes, we attempted to genotype SNPs with previous associations, recognizing that these two genes would not be fully interrogated. For three “smaller” coagulation-related genes (prothrombin, methylene tetra-hydrofolate reductase (MTHFR including C667T and A1298C), and factor VII), we attempted to test 20 SNPs each.

DNA amplification and genotyping

Whole-genome DNA was amplified from the filter card samples using Qiagen’s Repli-G system (Qiagen N.V. Netherlands). Whole-genome amplified DNA (WGA DNA) was genotyped with an Illumina GoldenGate assay (Illumina Inc., San Diego, CA) at the Duke Center for Human Genetics. In this study X, Y markers were not included for sex check. A manual of operations provided detailed protocol for handling samples in order to avoid sample swaps.

Statistical analysis

Categorical baseline clinical characteristics were compared between the control group and IVH group with logistic regression, adjusted for ancestry using the first ten eigenvalues. Ancestry eigenvalues were calculated by principal components (PC) calculated from the linkage disequilibrium-pruned full SNP list.

P values and false discovery rate (FDR) for SNPs were calculated from multivariable analyses using gestational age, small for gestational age, antenatal steroids, ancestry, sex, use of early indomethacin, and 5 min Apgar score <5 as covariables for tests of association for all SNPs, comparing those with grades 0 and 1 IVH with infants with grades III and IV IVH. SNPs with p values <0.01 and FDR <0.75 were considered informative, consistent with the exploratory nature of this study. The criteria were selected to allow for some control of false positive results, but also allowing for the ability to discover findings for follow up in a study with moderate power.

For these analyses, the genes are named by using the official Guidelines for Human Gene Nomenclature symbol. Genetic association testing was performed using PLINK v1.07, PC analysis was performed using EIGENSTRAT [22], and regional association plots (Fig. 1 and Supplementary information Fig. 1) were produced using LocusZoom [23].

Results

Of the 1067 ELBW infants enrolled in the Cytokines study, 899 with available CUS results and adequate DNA amplification and genotyping were included in the final study cohort. One hundred and thirty-nine infants had grades III–IV IVH, 73 had grade II IVH, and 687 had grades 0–I. Clinical characteristics and frequency of IVH are shown in Table 1. One thousand two hundred seventy-nine of the 1634 proposed candidate SNPs were successfully genotyped with the GoldenGate assay in the 899 samples. SNPs that were called in >90% of samples were included in our analyses (21.8% removed). The SNPs for Factor V Leiden (rs6025) and the MTHFR 1298A>C SNP (rs1801131) were not successfully genotyped by the GoldenGate assay but were successfully genotyped using TaqMan® SNP Genotyping Assays (Life Technologies, Grand Island, NY).

In multivariable analysis with covariables for gestational age, SGA status, sex, ancestry, maternal hypertension, exposure to indomethacin in the first 24 postnatal hours, antenatal steroids and a 5-min Apgar score ≤5, 35 of the 36 successfully genotyped SNPs from coagulation factor genes: prothrombin (*F2*), *MTHFR*, Factor V (*F5*), Factor 13A1 (*F13A1*), and Factor 7 (*F7*) had a p value of > 0.01. Factor V Leiden (rs6025) was quite similar in frequency between cases and controls, with a relatively high p value (0.94) in the multivariable analysis. The association between the missense variant SNP in the MTHFR gene (rs1801131) was also weak in the multivariable analysis ($p = 0.92$). One intronic variant SNP in *F7*, rs488703, had a MAF of 22% among infants with severe IVH, while ~15% of control infants had the minor allele ($p < 0.003$).

The other 12 SNPs with p values <0.01 in the multivariable analyses were from genes involved with organ development and cell function, except one SNP (rs4240872) in the gene for IL6 receptor (IL6R). The 13 SNPs with p values < 0.01 and FDR estimates < 0.75 are presented in Table 2, with MAFs among cases and controls included. In addition, we have included Supplementary information Table 1, which lists all the p values and FDRs for each successfully genotyped SNP.

IGF1R has a number of informative SNPs including SNPs rs12442623, rs1513643, and rs1810225. Figure 1 is a LocusZoom plot of *IGF1R*, which include local association results and information about the location and orientation of the gene, linkage disequilibrium coefficients, and local estimates of recombination rates. Plots of the rest of the genes in Table 2 are included as Supplementary information Fig. 1.

We have added a scatterplot matrix of all ten PC, color-coded by self-declared race/ethnicity, as Supplemental Fig. 2a, to demonstrate that the ten PC sufficiently describe ancestry. As seen in the PC1 vs PC3 panel, shown in greater detail in Supplemental Fig. 2b, self-identified race/ethnicity is largely concordant with clusters of subjects. For this analysis, race/ethnicity is grouped into non-Hispanic Black, non-Hispanic White, Hispanic, and other/missing.

Discussion

We describe a candidate gene analysis of severe IVH using samples and data from the NRN Cytokines Study cohort of ELBW infants. Our results identified 13 SNPs associated with severe IVH. Eleven SNPs were associated with organ development and cell function, one was associated with coagulation (*F7*), and one was associated with inflammation (*IL6R*). However, overall, no association was found among most inflammatory cytokines, and coagulation pathway or vascular associated SNPs were not associated with severe IVH.

Coagulation and vascular structures

Consistent with prior investigations of hemostatic genes reported by Hartel et al. and Aden et al. [24, 25], we did not find strong associations between severe IVH and common variants in clotting factor genes. We did identify an association between one SNP in *F7*, rs488703, and severe IVH. The risk allele SNP has been associated with lower levels of circulating factor VII in healthy children [26], and in adults with heart disease [27]. Future genomic and proteomic studies may focus on validating this *F7* association, as well as assessing other components of the hemostatic system including the platelet function and structure, microparticles, endothelium and the underlying collagen structure of blood vessels, such as the rare variant in *COL4A1* identified in dizygotic twins with severe IVH [28]. Consistent with prior findings by Aden et al. [25], we found no association between severe IVH and our tested SNPs in *COL4A1*. However, more extensive sequencing may reveal rare variants (variants found in less than 1% of the population) associated with IVH.

Genes related to inflammation

Inflammation plays a key role in the pathophysiology of neonatal disease and is a likely contributor to IVH risk [16, 17, 29, 30]. Baier reported preliminary evidence that cytokine gene variants are risk factors for IVH and periventricular leukomalacia [30]. In our cohort, we did not identify associations between the evaluated inflammatory gene SNPs and IVH. As with the coagulation factor genes, common variants in inflammation genes were not associated with severe IVH in this cohort. The intronic SNP in *IL6R* with higher prevalence among infants with severe IVH is not projected to be pathogenic with relation to protein structure and function [31]. Of interest, however, *IL6* has been associated with preterm birth and neurocognitive outcomes, and amniotic fluid levels of IL6 seem to be influenced by both *IL6* and *IL6R* polymorphisms [32]. In addition, the *IL6R* polymorphism has been previously associated with psychomotor delay in preterm infants [33]. Examination of allele frequency in larger cohorts and genotyping inclusive of rare variants may be necessary to identify if genetic variants in inflammatory pathway genes are associated with IVH and related outcomes.

Genes related to cellular growth

In this cohort, SNPs associated with several proteins that mediate cell growth and survival were associated with IVH. Our analyses identified a SNP, rs10847980, in the intragenic region on chromosome 12 between *ABCB9* and *VPS37B* on chromosome 12, which was more commonly noted among infants with severe IVH (29%) compared with those without severe IVH (20%). ABCB9 is an ATP-dependent low-affinity peptide transporter, which

translocates peptides from the cytosol to the lysosomal lumen. VPS37B, vacuolar protein sorting 37 homolog B, is a component of a complex that regulates vesicular trafficking and may be involved in cell growth and differentiation. This SNP has not been reported to be clinically significant [34].

Other SNPs with p values < 0.01 are also in or close to genes related to cell functions and organ development (Table 3). All are intronic variants. Of particular interest are *NAA15*, *IGF1R* (Fig. 1), and *NOS2*, which are linked to coordinated neuronal, and possibly neurovascular development. *IGF1R* has also been previously linked to preterm birth in a Finnish cohort [35]. Since we have a significant difference in gestational ages between our controls and cases, we cannot exclude the possibility that this finding could represent as association with gestational length, small for gestational age, or gestational age rather than IVH.

Strengths of our study include a well characterized and ethnically diverse study cohort. Clinical data were prospectively collected, and the data collected allowed for multivariate adjustment controlling for clinical factors and quantitative ancestry variables.

Limitations to the study included the genetic epidemiology approach, including the challenge of multiple comparisons with modest sample sizes. The statistical analysis results were not corrected for multiple comparisons since the study was designed as exploratory and hypothesis generating, although we did apply a more stringent cutoff of 0.01 for additional discussion. Due to the modest sample size, grades 0/I and grades III/IV IVH were compared which limits genetic associations along a continuum and limits evaluation of genetic contributions to potentially different underlying pathophysiology between grades of IVH. Also, in this targeted genetic evaluation, we did not evaluate for all previously described SNPs associated with IVH and were not able to evaluate for gene–environment interactions.

In addition, CUS was used instead of MRI for evaluation of IVH based on clinical practice at the time and feasibility in VLBW. It is possible that low-grade IVH were missed in this VLBW population and future study could include MRI in VLBW at older age [36]. Also, CUS were read locally by clinical site radiologists rather by central review.

While the candidate gene and SNP approach may identify valid associations, due to the complexity of the interactions of brain development, angiogenesis, coagulation, and inflammation, as well as the interactions with the Neonatal Intensive Care Unit (NICU) environment, the odds of identifying a causative allele among more than 20,000 genes and over 300 million common (and uncommon) variants [37] with our modest sample size are low. Ment et al. conducted a multicenter genome-wide association study (GWAS) in inborn infants with birthweights 500–1250 g and either severe IVH or normal CUS and a replication study using a NRN validation cohort to evaluate determinants of IVH [7]. No significant SNPs were identified. In order to identify and validate genetic variants associated with disorders of prematurity, multicenter data and sample collection, with sample sizes in the many thousands, will be necessary. Alternatively, whole-exome or whole-genome sequencing plus transcriptomics may disclose rare variants of large impact with more modest sample numbers.

Conclusion

Thirteen SNPs were tentatively associated with severe IVH including five related to neuronal and neurovascular development (*NAA15*, *IGF1R*, and *NOS2*) that may contribute to the risk of severe IVH in premature infants. More generally, the findings suggest links between genetic variants that contribute to brain development and development of the neurovascular system that, when superimposed on the NICU environment, influence an individual infant's risk of severe IVH. Because of the limitations in our cohort and analysis, and the high FDRs, these findings need both epidemiologic and physiologic validation. If the findings are validated, then they can inform risk-based interventions to prevent IVH in this vulnerable population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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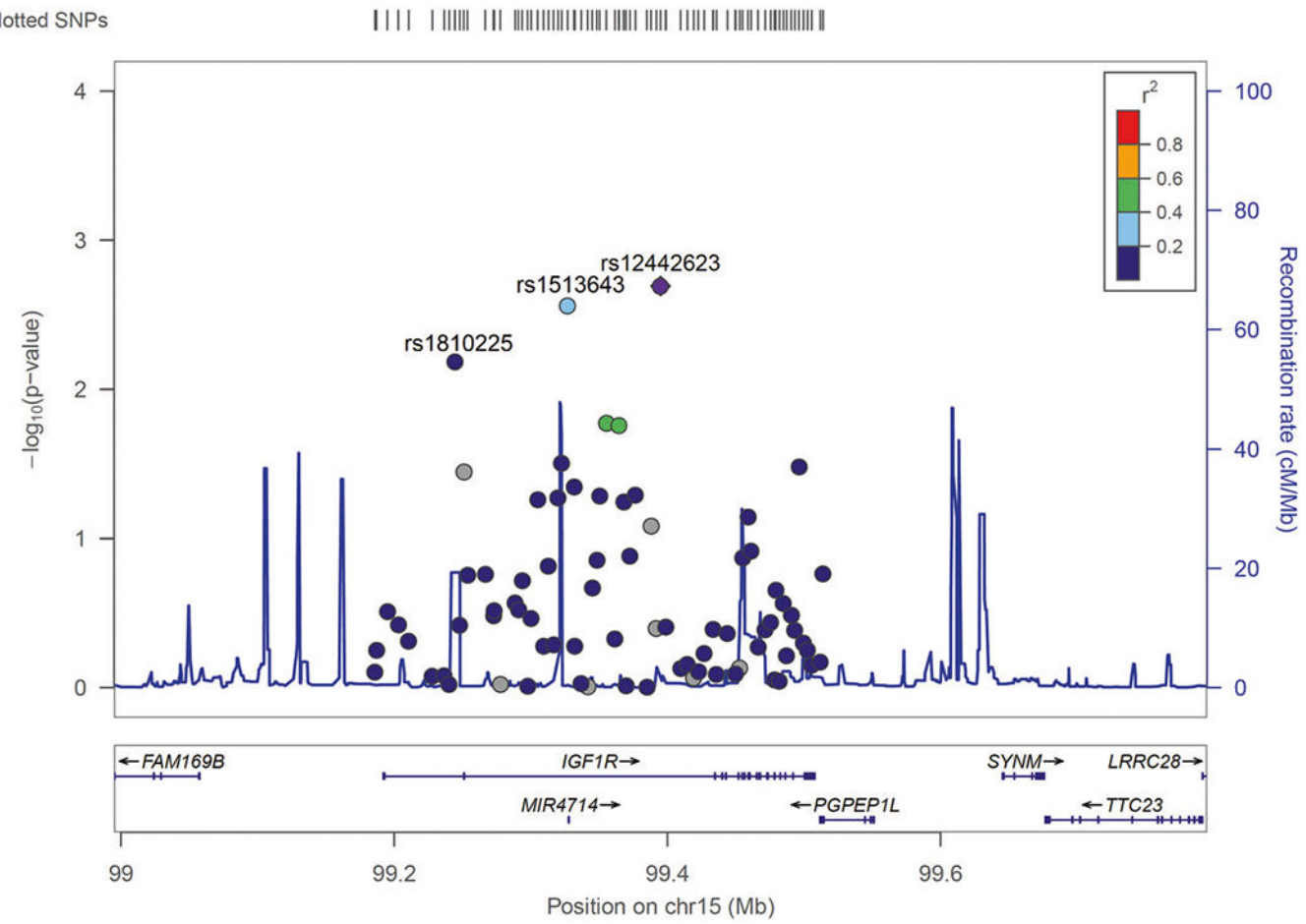

Fig. 1. LocusZoom plot of IGFR1.

The plot includes the local association results including rs1810225, rs1513643, and rs12442623; information about the location and orientation of the gene on chr15; linkage disequilibrium with rs12442623 (r2 color-coded according to inset legend); and local estimates of recombination rates (cM/Mb, blue line).

Table 1

Patient characteristics comparing controls (grades 0/I IVH) to cases (grades III/IV IVH).

	Controls Grades 0/I *N* = 687	**Cases Grades III/IV *N* = 139**	***p* value**
Gestational age (weeks), mean (SD)	26.1 (1.9)	24.9 (1.8)	<0.001
Small gestational age, *N*(%)	119 (17.3)	11 (7.9)	0.008
Male gender, *N*(%)	318 (46.3)	78 (56.1)	0.043
Non-Hispanic Black *N*(%)	135 (19.7)	31 (22.3)	0.552
Non-Hispanic White *N*(%)	293 (42.6)	66 (47.5)	0.340
Hispanic ethnicity *N*(%)	92 (13.4)	17 (12.2)	0.817
Other/Unknown Race/Ethnicity *N*(%)	167 (24.3)	25 (18.0)	0.134
Antenatal steroids, *N*(%)	544 (79.2)	98 (70.5)	0.033
Apgar <5, *N*(%)	70 (10.2)	30 (21.6)	<0.001

Table 2

Comparison of minor allele frequencies and false discovery rate for each gene/ SNP between cases (grades 0/I IVH) and controls (grades III/ IV IVH).

Gene	SNP	Cases MAF	Controls MAF	*p* value	FDR
LOC105371989	rs367024	0.173	0.275	0.00015	0.194
VPS37B/ABCB9	rs10847980	0.291	0.205	0.00047	0.303
IGF1R	rs12442623	0.212	0.286	0.00203	0.723
IGF1R	rs1513643	0.313	0.411	0.00276	0.723
F7	rs488703	0.223	0.147	0.00293	0.723
NOS2	rs8072199	0.342	0.273	0.00371	0.723
NAA15	rs747004	0.417	0.348	0.00413	0.723
RELN	rs736707	0.396	0.309	0.00472	0.723
IGF1R	rs1810225	0.148	0.087	0.00651	0.723
IL6R	rs4240872	0.450	0.370	0.00814	0.723
PAN3	rs1886233	0.297	0.360	0.00889	0.723
GRIN3A	rs13298667	0.250	0.189	0.00939	0.723
TFAP2B	rs2817399	0.327	0.400	0.00999	0.723

MAF minor allele frequency, *FDR* false discovery rate.

Table 3

Other SNPs associated with severe IVH (grades III/IV).

SNP	Gene	Potential significance
rs367024	RNA gene	Minor allele may be protective (17% among case infants and 27% among control infants. LOC105371989, the gene on chromosome 11 harboring this SNP, is designated only as an RNA gene, and the SNP has not been reported to have clinical significance [38].
rs747004	*NAA15*	NAA15 (N (alpha)-acetyltransferase 15) may be important for vascular, hematopoietic and neuronal growth and development. It is required to control retinal neovascularization in adult ocular endothelial cells; however, the intronic variant SNP, rs747004, which was noted more frequently among cases in our cohort than babies with grade 0 or 1 IVH, has not been previously associated with clinical disease [39].
rs12442623, rs1513643, rs1810225	*IGF1R*	IGF1R (insulin growth factor-1 receptor) binds insulin-like growth factor with a high affinity. It has tyrosine kinase activity. It plays a critical role in transformation events. Cleavage of the precursor generates alpha and beta subunits. It is highly overexpressed in malignant tissues where it functions as an anti-apoptotic agent by enhancing cell survival [40]. It has been linked to preterm birth [35, 41–43].
rs8072199	*NOS2*	NOS2 is the gene for nitric oxide synthase. Levels of nitric oxide contribute to microvascular resistance, and can contribute to synthesis of proinflammatory mediators such as IL6 and IL8 [44]. The variant has been associated with lower FeNO in children [45].
rs736707	*RELN*	RELN (reelin) encodes a large secreted extracellular matrix protein thought to control cell-cell interactions critical for cell positioning and neuronal migration during brain development [46, 47]. The variant has previously been associated psychiatric disorders including schizophrenia and autism spectrum disorder [48].
rs1886233	*PAN3*	PAN3 (poly(A) specific ribonuclease subunit gene), is one of two cytoplasmic mRNA deadenylases involved in general and miRNA-mediated mRNA turnover. The variant has not been associated with disease [49].
rs13298667	*GRIN3A*	The Glutamate Ionotropic Receptor NMDA Type Subunit 3A GRIN3A encodes a subunit of the N-methyl-D-aspartate (NMDA) receptors, which belong to the superfamily of glutamate-regulated ion channels, and function in physiological and pathological processes in the central nervous system [50]. The variant is associated with severe IVH in our study cohort, has not previously been associated with disease [51].
rs2817399	*TFAP2B*	The Transcription Factor AP-2 Beta gene, TFAP2B encodes a member of the AP-2 family of transcription factors. TFAP-2-beta appears to be required for normal face and limb development and for proper terminal differentiation and function of renal tubular epithelia [52]. The intronic variant rs2817399 associated with severe IVH in this cohort has been associated with patent ductus arteriosus which does not respond to indomethacin, although this association was not replicated in this NRN cohort [53, 54].