Genetic Variants Associated With Severe Retinopathy of Prematurity in Extremely Low Birth Weight Infants

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See the appendix for the members of the Genomics Subcommittee.

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Purpose. To determine genetic variants associated with severe retinopathy of prematurity (ROP) in a candidate gene cohort study of US preterm infants.

Methods. Preterm infants in the discovery cohort were enrolled through the Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network, and those in the replication cohort were from the University of Iowa. All infants were phenotyped for ROP severity. Because of differences in the durations of enrollment between cohorts, severe ROP was defined as threshold disease in the discovery cohort and as threshold disease or type 1 ROP in the replication cohort. Whole genome amplified DNA from stored blood spot samples from the Neonatal Research Network biorepository was genotyped using an Illumina GoldenGate platform for candidate gene single nucleotide polymorphisms (SNPs) involving angiogenic, developmental, inflammatory, and oxidative pathways. Three analyses were performed to determine significant epidemiologic variables and SNPs associated with levels of ROP severity. Analyses controlled for multiple comparisons, ancestral eigenvalues, family relatedness, and significant epidemiologic variables. Single nucleotide polymorphisms significantly associated with ROP severity from the discovery cohort were analyzed in the replication cohort and in meta-analysis.

Results. Eight hundred seventeen infants in the discovery cohort and 543 in the replication cohort were analyzed. Severe ROP occurred in 126 infants in the discovery and in 14 in the replication cohort. In both cohorts, ventilation days and seizure occurrence were associated with severe ROP. After controlling for significant factors and multiple comparisons, two intronic SNPs in the gene *BDNF* (rs7934165 and rs2049046, $P < 3.1 \times 10^{-5}$) were associated with severe ROP in the discovery cohort and were not associated with severe ROP in the replication cohort. However, when the cohorts were analyzed together in an exploratory meta-analysis, rs7934165 increased in associated significance with severe ROP ($P = 2.9 \times 10^{-7}$).

Conclusions. Variants in *BDNF* encoding brain-derived neurotrophic factor were associated with severe ROP in a large candidate gene study of infants with threshold ROP.

Keywords: retinopathy of prematurity, extremely low birth weight, brain-derived neurotrophic factor, genetic associations, neurovascular

Retinopathy of prematurity (ROP) is a leading cause of childhood blindness worldwide.[1,2] Retinopathy of prematurity has been associated with environmental factors, including high supplemental oxygen at birth,[3] fluctuations in oxygenation,[4] oxidative stress,[5] and more recently with genetic predisposition based on racial and regional risk profiles[6–9] and a heritability estimate of 70%.[10] Candidate gene analyses reported ROP associated with gene variants in the WNT signaling pathway (e.g., *NDP*, *FZD4*, *LRP5*),[11–17] which is important in development; in *EPAS1*[18] or *VEGF*,[19] which are regulated by hypoxia and involved in angiogenesis; and in *SOD*,[20,21] which transcribes the antioxidant enzyme, superoxide dismutase. However, these studies varied as to whether significant epidemiologic factors or multiple comparisons were taken into account and/or whether severe, treatment-warranted ROP was distinguished from nonsevere ROP. Severe ROP can cause

blindness from scarring or retinal detachment if not treated, whereas most ROP is nonsevere and resolves without treatment. In addition, previous candidate gene studies enrolled infants from a broad range of birth weights and gestational ages. As ROP can develop in large preterm infants of young gestational ages when high, unregulated oxygen is delivered at the time of birth,[22] studies of genetic variants associated with ROP that include preterm infants in unregulated oxygen may introduce variables that mask genetic variants associated with severe ROP under regulated oxygen conditions. Also, extreme prematurity is a strong independent risk factor for ROP,[23] and studies including older and larger preterm infants may have failed to distinguish genetic variants associated with severe ROP from those of preterm birth. Therefore, a study is needed that analyzes genetic variants associated with different levels of ROP severity in a group of extremely premature infants and that is controlled for significant epidemiologic variables and multiple comparisons in order to tease out the potential role of heritability in the risk of developing severe ROP.

To address this need, we analyzed associations between ROP of different levels of severity, including no ROP, and genetic variants in a unique discovery cohort restricted to US preterm infants born at less than 1000 grams birth weight, in whom the most severe level of ROP analyzed was "threshold" ROP, at which the risk of a bad outcome approached 50%.[24] It would no longer be ethical to wait for threshold ROP to develop before treating infants with ROP, because benefit has been found for earlier treatment.[25] In addition, the cohort comprised candidate genes that included some previously reported in genetic studies as well as others reported to be important in the development of biologic features of severe ROP or in conditions associated with ROP in human preterm infants. Candidates generally involved pathways in neurodevelopment, angiogenesis, or inflammatory and oxidative pathways.[24] We also controlled for significant epidemiologic factors and multiple comparisons. We report two intronic variants in the gene encoding brain-derived neurotrophic factor (*BDNF*) in association with severe ROP.

Methods

All work was approved by Institutional Human Subjects Committees, adhered to the Declaration of Helsinki, and was compliant with the Health Insurance Portability and Accountability Act (HIPAA). The discovery cohort was a multiracial population of 1013 related and unrelated preterm infants born at less than 1000 grams and who had been enrolled between 1998 and 2001 as part of a study of associations between serial cytokine levels and neurodevelopmental delay.[26] Genomic DNA was extracted from infant blood spot samples obtained for the cytokines study and stored in an anonymized DNA biorepository by the Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network. Replication of significant findings was performed in a separate cohort of 544 preterm infants from the University of Iowa enrolled between 1999 and 2013 in a genetic discovery protocol approved by the University of Iowa institutional human subjects committee; this cohort included premature infants of extremely low and very low birth weights (Table 1).

All infants were prospectively assessed by qualified ophthalmologists for ROP zone and stage[27] in at least one examination prior to death or discharge. In infants who were examined multiple times, the zone and stage were recorded for analysis from the examination having the most severe level of ROP. An infant was defined as having ROP when it was present in one eye. The diagnosis of severe, treatment-warranted ROP was made when an infant required treatment in at least one eye. For the discovery cohort, treatment was performed for threshold ROP, which carries a 50% risk of a poor outcome without treatment,[24] because infants were enrolled before results of the Early Treatment for Retinopathy of Prematurity Study (ETROP)[25] had been obtained. In the replication cohort, treatment was performed for threshold ROP or in infants enrolled after 2003, for type 1 ROP, which carries a 15% risk of a poor outcome, because of changes in treatment guidelines after ETROP.[25] Hereafter, severe ROP refers to ROP that warranted treatment.

Discovery Cohort

A pathway-based approach was used to identify candidate genes implicated in the development of the phases of severe ROP[28] or in conditions of prematurity associated with ROP. Previously reported candidate genes[11–21] and additional ones involving pathways in neurodevelopment, inflammation, angiogenesis, or oxidation were included. Tagging single nucleotide polymorphisms (TagSNPs) were chosen for genotyping using HapMap (provided in the public domain at http://www.hapmap.org/) and the following criteria: minor allele frequency greater than 10%, r^2 value of at least 0.8, and TagSNP tagged for at least six other SNPs. To represent the entire variation within a gene, additional SNPs approximately every 3000 to 5000 base pairs were included. Whole genome amplified DNA from stored blood spot samples was genotyped with the Illumina GoldenGate platform (Illumina, Inc., San Diego, CA, USA) for 1614 TagSNPs of the candidate genes. Data cleaning and analysis were performed using PLINK v1.07 (provided in the public domain at http://pngu.mgh.harvard.edu/~purcell/plink/).[32] Single nucleotide polymorphisms were removed that had a low genotyping pass rate (greater than 10% of genotypes missing) and/or that were not in Hardy-Weinberg equilibrium (HWE) in infants without ROP (HWE $P < 3 \times 10^{-5}$ based on $P = 0.05/1614$ SNPs). Individuals with more than 10% of genotypes missing were also removed. Predefined separate association tests were performed for different ROP outcomes: analysis 1 (ROP versus no ROP), analysis 2 (severe ROP versus nonsevere ROP), and analysis 3 (severe ROP versus nonsevere ROP or no ROP). Although analysis 3 was considered the strongest to determine variants associated with the most severe form, threshold ROP, the three analyses were performed to detect variants associated with visual morbidity, which also can occur with lesser levels of severity of ROP. Single nucleotide polymorphisms with $P < 3.3 \times 10^{-5}$ were considered significant based on the 1494 TagSNPs that remained after data cleaning ($P = 0.05/1494 = 3.3 \times 10^{-5}$), recognizing that this method may be conservative in the presence of linkage disequilibrium.[29]

Continental ancestry can strongly influence variant frequency at any one locus. For this reason, we used eigenvector values[30] as covariables in subsequent association analyses of the discovery cohort, as calculated using a previous genome-wide scan (GWAS) for 800 infants from the discovery cohort.[31]

Epidemiologic variables were tested for association with ROP outcomes in each analysis using logistic regression in SAS v9.3 (SAS Institute, Cary, NC, USA). Subsequently, stepwise logistic regression was performed in order to determine the most significantly associated epidemiologic risk factors. For SNP association analyses, the minor, less frequent, allele for each SNP was tested for association with ROP or severe ROP in each analysis using QFAM-total in PLINK,[32] which is a total association test that uses between- and within-family components and performs a linear regression of phenotype on genotype. QFAM-total uses a permutation test to correct for family relatedness; 100,000 permutations were used to correct.

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Table 1. Subject Characteristics

Characteristic	Discovery Cohort			Replication Cohort†		
	No ROP, *n* = 264	Nonsevere ROP, *n* = 467	Severe ROP, *n* = 126	No ROP, *n* = 331	Nonsevere ROP, *n* = 195	Severe ROP, *n* = 14
Gestational age, wk (SD)	27.1 (1.9)	25.7 (1.7)	24.5 (1.2)	28.7 (1.9)	26.2 (2.1)	24.4 (1.0)
Birth weight, g (SD)	823.6 (126.0)	758 (133.0)	697 (125.0)	1242 (356.0)	904 (291.0)	678 (167.0)
Small for gestational age, *n* (%)	64 (24.3)	55 (12.1)	6 (4.8)	36 (10.9)	23 (11.8)	3 (21.4)
Male, *n* (%)	113 (43.0)	222 (48.8)	62 (50.0)	195 (58.9)	109 (50.0)	8 (57.1)
Mean days in ventilation (SD)	8.2 (9.2)	17.5 (10.2)	25.4 (5.7)	13.7 (18.5)	41.8 (32.2)	76.3 (24.7)
Occurrence of seizures, *n* (%)	13 (4.9)	43 (9.4)	25 (20.2)	2 (0.6)	3 (1.5)	3 (23.1)
Antenatal steroids, *n* using (%)	218 (82.9)	351 (77.1)	90 (73.2)	312 (94.3)	181 (93.3)	13 (92.9)
Race, self-reported						
Black, *n* (%)	136 (51.5)	219 (46.9)	60 (47.6)	32 (9.7)	155 (71.1)	10 (71.4)
White, *n* (%)	122 (46.2)	240 (51.4)	65 (51.6)	256 (77.3)	19 (8.7)	1 (7.1)
Other,* *n* (%)	6 (2.2)	8 (1.7)	1 (0.7)	43 (12.9)	44 (20.1)	3 (21.4)
Ethnicity						
Hispanic, *n* (%)	29 (11.0)	101 (21.6)	24 (19.0)	20 (6.0)	8 (3.7)	1 (7.1)
Non-Hispanic, *n* (%)	235 (89.0)	366 (78.4)	102 (80.9)	311 (94)	210 (96.3)	13 (92.9)

* Other, self-report of race other than non-Hispanic white or non-Hispanic black or Hispanic.
† Four infants with ROP lacked information as to whether treatment was performed and are not included.

To control for significant epidemiologic factors determined from stepwise regression and the four previously identified eigenvector values, residuals were calculated in R statistical software (provided in the public domain at http://www.r-project.org/) and used in QFAM. Linkage disequilibrium between genotyped SNPs was determined using Haploview (provided in the public domain at http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview).

Replication Cohort

In the replication cohort, SNP association analyses were performed using the minor allele defined in the discovery cohort and controlling for epidemiologic factors found significant in the discovery cohort. For infants from related families, conditional logistic regression was used, and for unrelated infants, logistic regression was used. All analyses were performed using SAS. Meta-analysis of the discovery and replication cohorts, based on individually calculated odds ratios and confidence intervals, was performed using Comprehensive Meta-Analysis v2 (Biostat, Inc., Englewood, NJ, USA). Fixed and random effects models were used where appropriate based on the results of Cochran's Q test for population heterogeneity.

Results

Discovery Cohort

Infants requiring treatment for severe ROP were born younger than 28 weeks (24.5 weeks mean gestation) (Table 1). A total of 1614 SNPs were successfully genotyped in 1013 infants. Of these 1013 infants, 14 were removed for major birth defects (structural congenital heart defects, diaphragmatic hernia, duodenal atresia or gastroschisis, obstructive uropathy and genitourinary defects, skeletal dysplasia, defects of the central nervous system, or other life-threatening birth defects) and 141 for incomplete eye examination data because of death before first eye examination, transfer to another out-of-network nursery, or having an unstable course. One infant had both circumstances and was subsequently removed. An additional 40 infants were removed for low genotyping rate (greater than 10% of genotypes missing). After data cleaning in PLINK, 1494 SNPs were tested for association with ROP in 817 subjects; 122 were related and 695 were not related.

Forty-three epidemiologic variables were tested for association in each of the three ROP analyses (Table 2). The related and unrelated subjects were analyzed separately. In many related cases there were no informative families and no epidemiologic variables with nominal significance. In univariate analysis of the unrelated subjects, 23 variables were significantly associated with ROP (versus no ROP) in analysis 1, 20 with severe ROP (versus nonsevere ROP) in analysis 2, and 22 with severe ROP (versus nonsevere ROP or no ROP) in analysis 3.

For each analysis, there were no informative families for stepwise regression. Stepwise regression of the unrelated subjects for analysis 1 showed that the most parsimonious model included only days of ventilation within the first 28 postnatal days in association with ROP. After single SNP analysis adjusted for ventilation days and the four eigenvector values, 129 SNPs were nominally significant ($P < 0.05$), and after correction for multiple testing, no SNPs were significantly associated with ROP ($P > 10^{-5}$). In analysis 2, the stepwise regression model showed that the most parsimonious model included only occurrence of seizures in association with severe ROP. Single SNP analysis adjusted for seizure occurrence and the four eigenvector values showed 97 SNPs to be nominally significant ($P < 0.05$). After correction for multiple testing, two SNPs in *BDNF* were significantly associated with severe ROP: rs7934165 and rs2049046 ($P = 3 \times 10^{-5}$ and 6×10^{-5}, respectively, Table 3). Stepwise regression for analysis 3 revealed that both ventilation days and seizure occurrence were significantly associated with severe ROP after adjusting for all other variables ($P < 0.05$). Single SNP analysis adjusted for these variables and the four eigenvector values found 99 nominally significant SNPs ($P < 0.05$). After correction for multiple testing, the same two SNPs in *BDNF* as found in analysis 2 were significantly associated with severe ROP: rs7934165 and rs2049046 ($P = 2 \times 10^{-5}$ and 3×10^{-5}, respectively, Table 3). Because small for gestational age, low birth weight, sex, and use of antenatal corticosteroids have been associated with ROP,[23] analyses were also performed after controlling for these variables and had similar outcomes (data not shown). The two *BDNF* SNPs were found to be in high

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Table 2. Association of Epidemiologic Variables in the Discovery Cohort

Description	Measurement	Analysis 1: ROP vs. No ROP		Analysis 2: Severe ROP vs. Nonsevere ROP		Analysis 3: Severe ROP vs. Nonsevere ROP or No ROP	
		Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value
Antenatal steroids administered	Yes/No	0.56 (0.37–0.85)	0.0067	0.78 (0.47–1.25)	0.2907	0.65 (0.41–1.04)	0.071
Any mechanical ventilation prior to status	Yes/No	8.28 (4.42–15.52)	4.02E-11	3.58 (0.46–27.64)	0.2216	10.48 (1.44–76.57)	0.0206
Infant's birth weight, g	Continuous	0.996 (0.994–0.997)	1.49E-11	0.996 (0.994–0.998)	1.22E-05	0.995 (0.99–0.997)	4.12E-09
Consensus bronchopulmonary dysplasia	Yes/No	2.56 (2.11–3.10)	5.21E-22	1.67 (1.31–2.14)	4.68E-05	2.04 (1.63–2.56)	6.79E-10
Traditional bronchopulmonary dysplasia	Yes/No	4.77 (3.30–6.90)	8.05E-17	2.26 (1.39–3.66)	0.001	3.56 (2.23–5.68)	1.07E-07
Positive culture for Candida	Yes/No	1.97 (0.998–3.88)	0.0505	2.69 (1.42–5.08)	0.0024	3.05 (1.67–5.58)	0.0003
Cerebral palsy	Yes/No	3.57 (1.80–7.10)	0.0003	3.82 (2.20–6.65)	2.08E-06	4.91 (2.89–8.36)	4.50E-09
Early-onset septicemia/bacteremia	Yes/No	>999.999(−<0.001–>999.999)	0.984	1.07 (0.22–5.20)	0.9383	1.68 (0.34–8.20)	0.5212
Gestational age, wk	Continuous	0.60 (0.54–0.66)	5.14E-23	0.58 (0.49–0.69)	1.27E-09	0.52 (0.44–0.61)	5.24E-15
Intraventricular hemorrhage	Yes/No	1.35 (0.27–6.76)	0.7128	1.87 (0.34–10.36)	0.473	1.96 (0.39–9.85)	0.4137
Severe IVH, grade 3 or 4	Yes/No	1.998 (1.13–3.54)	0.0176	2.07 (1.18–3.65)	0.0116	2.44 (1.42–4.19)	0.0013
Most severe grade of IVH	Continuous	1.31 (1.13–1.53)	0.0006	1.21 (1.04–1.42)	0.0174	1.295 (1.11–1.51)	0.0008
Late-onset culture-positive septicemia/bacteremia	Yes/No	1.87 (1.33–2.62)	0.0003	1.98 (1.27–3.08)	0.0024	2.34 (1.53–3.59)	8.98E-05
Mechanical ventilation	Yes/No	8.28 (4.42–15.52)	4.02E-11	3.58 (0.46–27.64)	0.2216	10.48 (1.44–76.57)	0.0206
Meningitis	Yes/No	0.70 (0.33–1.47)	0.3453	1.34 (0.47–3.81)	0.5819	1.13 (0.42–3.004)	0.812
Moderate or severe cerebral palsy	Yes/No	5.69 (1.73–18.74)	0.0042	3.565 (1.76–7.24)	0.0004	4.90 (2.47–9.74)	5.74E-06
Proven NEC (necrotizing enterocolitis)	Yes/No	1.03 (0.57–1.87)	0.9188	0.80 (0.34–1.87)	0.6059	0.82 (0.36–1.87)	0.6387
Age when NEC diagnosed, d	Continuous	0.98 (0.95–1.01)	0.1021	1.01 (0.98–1.05)	0.4874	1.004 (0.97–1.04)	0.8324
NEC: no (0), medical (1), or surgical (2)	Continuous	1.11 (0.74–1.66)	0.6215	0.925 (0.54–1.58)	0.7762	0.96 (0.57–1.63)	0.8834
NEC surgery	Yes/No	1.64 (0.60–4.47)	0.3356	1.065 (0.34–3.31)	0.9135	1.24 (0.41–3.72)	0.7037
Antibiotics used during admission	Yes/No	1.39 (0.995–1.95)	0.0537	1.43 (0.87–2.36)	0.1583	1.58 (0.98–2.56)	0.0632
Had at least 1 prenatal care visit	Yes/No	0.48 (0.25–0.91)	0.0243	0.71 (0.37–1.37)	0.3097	0.58 (0.31–1.097)	0.0945
Gravida	Yes/No	1.06 (0.98–1.15)	0.1717	1.001 (0.90–1.11)	0.9887	1.02 (0.92–1.13)	0.6925
Head circumference, cm	Continuous	0.63 (0.57–0.71)	1.04E-15	0.73 (0.64–0.84)	9.14E-06	0.655 (0.57–0.75)	4.20E-10
Antepartum hemorrhage	Yes/No	2.29 (1.36–3.87)	0.002	1.245 (0.72–2.16)	0.433	1.57 (0.93–2.68)	0.0947
Hypertension/pre-eclampsia/eclampsia	Yes/No	0.36 (0.25–0.50)	3.53E-09	0.36 (0.19–0.70)	0.0028	0.26 (0.13–0.49)	3.82E-05
Diabetes, insulin dependent	Yes/No	0.73 (0.34–1.56)	0.4123	0.73 (0.21–2.59)	0.6305	0.66 (0.197–2.23)	0.5059
Mother's age, y	Continuous	0.98 (0.95–0.999)	0.0418	0.99 (0.96–1.02)	0.5763	0.98 (0.95–1.01)	0.272
Parity	Yes/No	1.04 (0.93–1.16)	0.5359	0.98 (0.85–1.14)	0.8172	0.995 (0.86–1.15)	0.9515
Tocolytics use	Yes/No	1.13 (0.81–1.58)	0.488	1.595 (1.02–2.49)	0.0401	1.605 (1.05–2.46)	0.03
Head circumference at 36 wk, cm	Continuous	0.94 (0.85–1.04)	0.2404	0.84 (0.74–0.95)	0.0064	0.82 (0.73–0.93)	0.0022
Weight at 36 wk, g	Continuous	1.00 (1.00–1.00)	0.2339	1.00 (0.999–1.000)	0.5431	1.00 (0.999–1.00)	0.7982
Patent ductus arteriosus	Yes/No	2.84 (2.03–3.98)	1.33E-09	3.11 (1.89–5.12)	8.38E-06	4.145 (2.55–6.73)	8.72E-09
Periventricular leukomalacia	Yes/No	1.25 (0.55–2.84)	0.6034	1.42 (0.54–3.72)	0.4769	1.49 (0.59–3.73)	0.3979
Race, 3 levels: 1 = black, 2 = white, 3 = other	Continuous	1.06 (0.78–1.43)	0.7141	0.93 (0.62–1.41)	0.7334	0.96 (0.65–1.42)	0.8252
Race, 6 levels: 1 = black or African American; 2 = white; 3 = American Indian or Alaska Native; 4 = Asian, Native Hawaiian, or Other Pacific Islander; 5 = more than 1 race; 6 = Other	Continuous	1.00 (0.77–1.31)	0.9862	0.93 (0.63–1.37)	0.7137	0.94 (0.66–1.34)	0.7382
Race/Hispanic origin, levels: 1 = non-Hispanic black; 2 = non-Hispanic white; 3 = Hispanic or Latino; 4 = other	Continuous	1.16 (0.95–1.42)	0.1348	0.92 (0.70–1.19)	0.51	0.97 (0.75–1.25)	0.8206
Indicator of seizure activity	Yes/No	2.60 (1.30–5.21)	0.007	2.72 (1.49–4.94)	0.001	3.32 (1.88–5.88)	3.74E-05

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Table 2. Continued

Description	Measurement	Analysis 1: ROP vs. No ROP		Analysis 2: Severe ROP vs. Nonsevere ROP		Analysis 3: Severe ROP vs. Nonsevere ROP or No ROP	
		Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value
Any sepsis, early or late onset	Yes/No	1.98 (1.41–2.78)	7.07E-05	2.04 (1.31–3.18)	0.0017	2.45 (1.60–3.77)	4.10E-05
1 = male, 2 = female, 3 = ambiguous	Continuous	0.74 (0.53–1.02)	0.0643	0.85 (0.55–1.32)	0.4781	0.775 (0.51–1.18)	0.2319
Small for gestational age (<10th percentile for gestational age)	Yes/No	0.38 (0.25–0.57)	2.71E-06	0.40 (0.17–0.97)	0.0418	0.28 (0.12–0.66)	0.0036
Days of ventilation within 28 d	Continuous	1.11 (1.09–1.13)	1.06E-30	1.12 (1.08–1.16)	1.12E-09	1.14 (1.10–1.18)	1.07E-14
Total days of ventilation	Continuous	1.05 (1.04–1.06)	1.48E-21	1.03 (1.02–1.04)	1.52E-11	1.03 (1.03–1.04)	1.55E-18

CI, confidence interval; IVH, intraventricular hemorrhage.

linkage disequilibrium ($r^2 = 0.87$, data not shown), but the combined effect of both SNPs produced no greater association with ROP or severe ROP than either SNP alone (data not shown).

Replication and Meta-Analysis

The two *BDNF* SNPs were genotyped in the Iowa replication cohort of 118 related and 426 unrelated infants and were in HWE in unaffected individuals ($P > 0.05$). Subject characteristics between the discovery and replication cohorts are shown in Table 1. Both seizure occurrence and ventilation days were significant risk factors for all analyses in the replication cohort (data not shown). Neither rs7934165 nor rs2049046 was significantly associated with ROP in unrelated or related infants in any of the analyses (Table 4) in the replication cohort. In many instances, there were no informative families. The enrollment durations for the discovery and replication cohorts differed, but there was overlap in the years of enrollment for infants in the cohorts. The treatment indications for severe ROP in the replication cohort also overlapped with the discovery cohort in that infants were treated for threshold disease but also for type 1 ROP. An exploratory meta-analysis[33] found that *BDNF* SNPs remained significantly associated with severe ROP; and in analysis 3, under a recessive model, rs7934165 increased in associated significance with severe ROP ($P = 2.9 \times 10^{-7}$; Table 4). For this analysis, only the unrelated discovery and replication cohorts contributed. In every analysis, Cochran's Q was not significant, confirming no population heterogeneity; therefore, a fixed effects model was used for meta-analysis (Table 4).

Discussion

This is the largest candidate gene study to date that analyzed SNP variants in preterm infants for associations with ROP or severe ROP after controlling for epidemiologic factors, continental ancestral eigenvector values, and multiple comparisons. Another strength was the inclusion of candidate genes chosen for involvement in the pathomechanisms of biologic features of ROP or with conditions in prematurity associated with ROP, or ones that had been reported in previous candidate gene studies.[11–21] Generally the pathways involved inflammation, oxidation, angiogenesis, and development. Additionally, TagSNPs were chosen to represent the entire variation within a gene and to limit testing of repetitive signals by SNPs in high linkage disequilibrium. No SNPs were significantly associated with the presence versus absence of ROP. However, in the discovery cohort and after meta-analysis with a separate cohort, two SNPs in *BDNF* were highly associated with severe ROP. In addition, in the same discovery cohort, low serum BDNF had previously been reported in association with severe ROP, providing evidence for a plausible functional association.[34]

Retinopathy of prematurity and preterm birth are highly associated.[23] Most nonsevere ROP resolves, whereas severe ROP can lead to blindness but affects only approximately 10% of extremely low birth weight infants. We sought to discriminate risk of severe ROP from that of preterm birth. In the discovery cohort, we restricted the range of birth weights and included only preterm infants born weighing less than 1000 grams. This may have resulted in lost significance between severe ROP and low birth weight and young gestational age, associations previously reported with ROP.[23] The analysis of severe versus nonsevere or no ROP provides the strongest comparison between infants of extremely low birth weight with the most severe form of ROP and those

Table 3. Top10 SNPs From Each Analysis, Adjusted for Significant Covariates

					Analysis 1: Any ROP vs. No ROP				Analysis 2: Severe ROP vs. Nonsevere ROP				Analysis 3: Severe ROP vs. Nonsevere ROP or No ROP			
C H R	**SNP**	**BP**	**Gene**	**A1**	**NIND**	**BETA**	***P***	**EMP1**	**NIND**	**BETA**	***P***	**EMP1**	**NIND**	**BETA**	***P***	**EMP1**
1	rs9332681	169555791	*F5*	A	747	−0.202	0.0004	0.0008	510	0.082	0.2407	0.2386	747	0.001	0.9793	0.9789
1	rs379489	196693451	*CFH*	T	816	−0.021	0.3926	0.4127	561	0.087	0.001	0.0009	816	0.055	0.0036	0.0038
1	rs395544	196698272	*CFH*	A	817	−0.021	0.403	0.4193	561	0.085	0.0014	0.0015	817	0.053	0.0048	0.0054
1	rs11587174	197012491	*F13B*	A	780	−0.181	0.0003	0.0006	538	0.085	0.1637	0.1639	780	0.007	0.8549	0.8561
2	rs1467199	191880502	*STAT1*	C	817	−0.086	0.0009	0.0017	561	0.022	0.454	0.4545	817	−0.004	0.8218	0.8208
5	rs34417936	55248135	*IL*	T	798	−0.155	0.0006	0.0011	549	0.03	0.5634	0.5675	798	−0.015	0.6599	0.6632
7	rs2299386	103419656	*RELN*	A	815	0.05	0.0379	0.0451	561	0.067	0.0078	0.0076	815	0.058	0.0015	0.0016
7	rs10251365	103420619	*RELN*	A	817	−0.013	0.5943	0.6112	561	−0.086	0.0013	0.0014	817	−0.062	0.0012	0.001
8	rs16879811	32573039	*NRG1*	A	817	0.014	0.5809	0.599	561	0.095	0.0005	0.0008	817	0.07	0.0004	0.0004
8	rs16879814	32573583	*NRG1*	G	817	0.02	0.4471	0.4658	561	0.096	0.0005	0.0006	817	0.071	0.0003	0.0003
11	rs2353512	27679662	*BDNF-AS*	A	795	−0.19	0.0007	0.0012	550	0.1	0.142	0.1451	795	0.013	0.7674	0.7686
11	rs7127507	27714884	*BDNF*	G	817	0.017	0.4644	0.4829	561	0.086	0.0004	0.0004	817	0.063	0.0003	0.0002
11	rs2049046	27723775	*BDNF*	A	817	0.028	0.2346	0.2482	561	0.097	6.26E-05	6.00E-05	817	0.074	2.43E-05	3.00E-05
11	rs7934165	27731983	*BDNF*	C	816	0.023	0.3002	0.3139	560	0.1	2.47E-05	3.00E-05	816	0.075	1.25E-05	2.00E-05
11	rs12281784	46908250	*LRP4*	A	817	−0.095	0.0003	0.0005	561	0.067	0.0078	0.0076	817	−0.012	0.5393	0.5422
12	rs9989002	102850223	*IGF1*	T	808	−0.02	0.7562	0.7638	554	0.244	0.0006	0.0009	808	0.158	0.0015	0.0022
13	rs11620315	28997886	*FLT1*	C	817	0.13	0.0013	0.0018	561	−0.004	0.914	0.9162	817	0.024	0.432	0.4467
15	rs1319859	99230263	*IGF1R*	A	814	0.072	0.0008	0.0013	559	0.015	0.502	0.5032	814	0.026	0.1134	0.1155
15	rs884636	99513784	*PGPEP1L*	A	814	−0.383	0.001	0.0015	560	−0.216	0.2416	0.2562	814	−0.15	0.0932	0.1003
16	rs7204874	27303287	*NSMCE1|IL4R*	A	817	0.041	0.2656	0.2814	561	0.114	0.0033	0.0034	817	0.088	0.0016	0.0014
16	rs2057768	27322095	*IL4R*	T	816	−0.016	0.5255	0.5422	561	−0.092	0.0007	0.0007	816	−0.068	0.0005	0.0007
18	rs1551005	29171690	*TTR*	C	808	−0.204	0.0009	0.0014	556	0.081	0.2873	0.2906	808	−0.002	0.961	0.9603

CHR, chromosome; BP, base pair location; A1, minor allele; NIND, number of nonmissing individuals in analysis; BETA, regression coefficient; EMP1, point-wise empirical *P* value.

Table 4. Results of Meta-Analysis of Discovery and Replication Cohorts

				Analysis 2, Adjusted			Analysis 3, Adjusted		
Model	**SNP**	**Gene**	**Study Name**	**Odds Ratio (95% CI)**	***Z* Value**	***P* Value**	**Odds Ratio (95% CI)**	***Z* Value**	***P* Value**
	rs7934165, add	*BDNF*	Discovery - Unrelated	2.0 (1.4–2.7)	4.1	4.04E-05	2.3 (1.6–3.2)	4.6	3.80E-06
	rs7934165, add	*BDNF*	Discovery - Related	1.0 (0.01–15.9)	0.0	1.0000	142.4 (0.0–5070080.8)	0.9	0.3537
	rs7934165, add	*BDNF*	Replication - Unrelated	0.9 (0.6–2.3)	−0.2	0.8450	1.1 (0.4–3.4)	0.3	0.8021
Fixed	rs7934165, add	*BDNF*		1.8 (1.3–2.4)	3.8	0.0002	2.1 (1.5–3.0)	4.5	6.80E-06
	rs7934165, dom	*BDNF*	Discovery - Unrelated	2.1 (1.2–3.8)	2.5	0.0121	2.1 (1.1–3.9)	2.5	0.0129
	rs7934165, dom	*BDNF*	Replication - Unrelated	0.6 (0.1–2.3)	−0.8	0.4373	0.6 (0.1–2.9)	−0.7	0.5068
Fixed	rs7934165, dom	*BDNF*		1.7 (1.0–3.0)	2.0	0.0431	1.8 (1.0–3.2)	2.1	0.0359
	rs7934165, rec	*BDNF*	Discovery - Unrelated	2.7 (1.7–4.3)	4.2	2.76E-05	3.6 (2.2–5.9)	5.0	5.11E-07
	rs7934165, rec	*BDNF*	Replication - Unrelated	1.5 (0.3–6.5)	0.5	0.6255	2.6(0.5–13.6)	1.1	0.2670
Fixed	rs7934165, rec	*BDNF*		2.6 (1.6–4.0)	4.1	3.34E-05	3.5 (2.2–5.7)	5.1	2.91E-07
	rs2049046, add	*BDNF*	Discovery - Unrelated	1.9 (1.4–2.7)	3.9	1.06E-04	2.3 (1.6–3.3)	4.5	6.33E-06
	rs2049046, add	*BDNF*	Discovery - Related	1.0 (0.1–15.9)	0.0	1	142.4 (0.0–5070080.8)	0.9	0.3537
	rs2049046, add	*BDNF*	Replication - Unrelated	0.9 (0.4–2.2)	−0.2	0.8404	0.9 (0.3–2.6)	−0.2	0.8534
Fixed	rs2049046, add	*BDNF*		1.7 (1.3–2.4)	3.5	0.0004	2.1 (1.5–2.9)	4.3	2.13E-05
	rs2049046, dom	*BDNF*	Discovery - Unrelated	1.9 (1.1–3.4)	2.2	0.0270	1.9 (1.1–3.5)	2.2	0.0259
	rs2049046, dom	*BDNF*	Replication - Unrelated	0.5 (0.1–2.0)	−0.9	0.3469	0.4 (0.1–2.2)	−1.0	0.3092
Fixed	rs2049046, dom	*BDNF*		1.6 (0.9–2.7)	1.7	0.0926	1.6 (0.9–2.9)	1.8	0.0779
	rs2049046, rec	*BDNF*	Discovery - Unrelated	2.7 (1.7–4.3)	4.1	4.65E-05	3.8 (2.3–6.3)	5.1	4.00E-07
	rs2049046, rec	*BDNF*	Replication - Unrelated	1.5 (0.4–5.9)	0.6	0.5529	1.8 (0.4–8.8)	0.7	0.4887
Fixed	rs2049046, rec	*BDNF*		2.5 (1.6–3.9)	4.0	5.30E-05	3.5 (2.2–5.7)	5.0	4.65E-07

Data not shown for analyses without informative observations. Analysis 2 (severe ROP versus nonsevere ROP) was adjusted for occurrence of seizures. Analysis 3 (severe ROP versus nonsevere or no ROP) was adjusted for occurrence of seizures and number of days of ventilation within 28 days. In analysis 3, under a recessive model, rs7934165 increased in associated significance with severe ROP in meta-analysis compared to the discovery cohort. Add, additive genetic model; dom, dominant genetic model; rec, recessive genetic model.

without. In the discovery cohort and in meta-analysis with a different cohort of preterm infants of lesser severity, intronic variants in *BDNF* were significant. Study enrollment for the discovery cohort occurred when treatment for severe ROP was performed for threshold ROP, which if left untreated would cause a poor outcome 50% of the time. The replication cohort included larger infants of older gestational age and infants enrolled after treatment guidelines had been expanded to include ROP with an approximately 15% risk of a poor outcome.[25] The durations of enrollment overlapped but differed between the discovery and replication cohorts. Therefore, neonatal care had changed for some infants in the replication cohort in ways that did not occur for infants in the discovery cohort. Only 14 infants in the replication cohort developed severe ROP, whereas 126 infants in the discovery cohort developed severe ROP, and all these had threshold ROP. These facts may account for why analysis of the replication cohort did not find an association between severe ROP and either SNP identified in the discovery cohort. Finally, since ROP phenotype and incidence can vary throughout the world based not only on gene pool but also on resources available to regulate and monitor oxygen and the ability to diagnose ROP accurately in preterm infants,[3,22] we included only US infants in both cohorts enrolled from neonatal units with resources to regulate oxygen and avoid high oxygen at birth. Therefore, the attributes of our population and analyses may have allowed us to better discriminate between risk of severe ROP and that of prematurity compared to previous candidate gene studies.[13–17]

Retinopathy of prematurity is included among rare diseases even though it is one of the most common pediatric retinal conditions. Retinopathy of prematurity also has different phenotypes based on resources for prenatal and perinatal care. Therefore, the contribution of different causal factors to the severity of ROP has varied historically as well as by region.[3,22] For these reasons, studies can have small sample sizes and may not be comparable. The discovery cohort in this study is the largest one to date consisting only of extremely low birth weight infants (<1000 grams) who were managed in the United States prior to the adoption of early laser treatment to prevent blindness from stage 3 ROP with intravitreal neovascularization. This unique cohort represents premature infants with an extremely severe form of ROP, threshold disease, in which the risk of blindness approaches 50%. This cohort is unlikely to be replicated in the future. The finding of a variant in a neural growth factor was novel and also aligned with several current experimental and preclinical studies showing the importance of neurovascular interactions in retinal vascular development and ROP.[35–40]

It was not ethically possible to enroll preterm infants in the replication cohort who were similar to those in the discovery cohort, because since the ETROP study ophthalmologists treat infants for ROP less severe than threshold disease. Infants enrolled in the replication cohort represented the mix seen in many studies. We did not find significance for the same SNPs in *BDNF* in the replication cohort as in discovery. The reason may be that other factors are involved in ROP development in infants with larger than 1000 grams birth weight or that type 1 ROP, which carries a 15% risk of blindness, would require a much larger sample size than would threshold disease to find significance in the same variants. Also, there were only 14 infants with severe ROP in the replication cohort. This may reflect regional differences and a different time of enrollment of infants experiencing improvements in neonatal care. In countries that lack resources for optimal nutrition, prenatal

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care, or oxygen regulation, severe ROP occurs in infants of greater birth weights and older gestational ages than are even screened in the United States.[3,22] It is possible that many infants with variants in *BDNF* may not survive in regions lacking resources for certain elements of prenatal and perinatal care.

Although significance was not found in the replication cohort alone, in an exploratory meta-analysis, one intronic SNP in *BDNF* gained significance compared to findings in the discovery cohort. This supports the hypothesis that BDNF may be involved in protection against severe ROP.

The *BDNF* SNPs significant in analysis 3, rs2049046 and rs7934165, are located within introns of the *BDNF* gene on chromosome 11. Brain-derived neurotrophic factor is a neural growth factor involved in promoting neuronal survival in brain and retina.[41] Retinopathy of prematurity develops when retinal neurons and vasculature are developing in the preterm infant. It is recognized that neural factors provide guidance cues for both neurons and vascular cells, suggesting the importance of neurovascular interactions in retinal and vascular development.[35,40] As an example related to ROP, BDNF is important in ganglion cell maturation in the retina and is reduced during dark rearing of mice.[36] Recently, it was found that mice in utero reared in the dark or lacking the gene encoding melanopsin, which is involved in certain ganglion cell responses to light,[37] developed vascular anomalies that could predispose to conditions like ROP.[38] Additional clinical evidence that higher average day length during early gestation was associated with lower risk of ROP[42] provides an additional link among light, ganglion cell maturation, retinal vascular development, and reduced risk of later ROP. There is strong clinical evidence of reduced circulating BDNF levels in severe ROP.[34,43,44] Blood spot samples of infants from the same population as the discovery cohort in this report were analyzed for cytokines,[34] and reduced serum protein BDNF was found associated with severe ROP.[34] Although low serum BDNF in the cytokine analysis cannot be causally related to the intronic *BDNF* SNP variants reported here, the finding of reduced BDNF protein in the same cohort as the discovery cohort supports the thinking that variants in *BDNF* may be important in the pathophysiology of severe ROP. In the first report of BDNF concentrations and ROP, Rao et al.[43] found that BDNF levels on postnatal day 60 in infants who developed ROP were lower than in those who did not develop ROP. In a separate study from Sweden, serum BDNF and RANTES levels measured within 14 days of birth were lower in preterm infants who developed severe ROP than in those who did not.[44] However, to determine if the intronic variants we report in *BDNF* affect protein function and biologic outcomes, deep sequencing of the gene in future nonbiased human studies and studies in cultured cells and/or animal models must be performed.

Although other studies reported significance with genes within the WNT signaling pathway, we did not find these associated with severe ROP. Another condition, familial exudative vitreoretinopathy (FEVR), is caused by variants in genes of the WNT pathway and has characteristics similar to ROP except that it occurs in full-term infants. It is possible that the studies reporting WNT pathway variants associated with severe ROP included infants with FEVR who were also premature. However, more study is needed.

Candidate gene approaches by design limit the choices of genes analyzed, and future studies using unbiased whole genome approaches will be important. However, our study strengths are the TagSNP approach, which covered genes of the candidates chosen; the focus on pathways implicated in the pathogenesis of severe ROP or in conditions of prematurity associated with ROP; and the discovery of the highly significant variant in *BDNF*, rs7934165, in meta-analysis with a replication cohort. The previously reported associations of reduced serum BDNF and severe ROP in the discovery cohort population also support a role for reduced BDNF function in severe ROP. This work may improve our understanding of neurovascular interactions in the pathogenesis of ROP and lead to future therapies for prevention and treatment of ROP.

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Appendix

Genomics Subcommittee

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