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Genomics in the Neonatal Nursery: Focus on ROP

Mary Elizabeth Hartnett, MD and

John A. Moran Eye Center, University of Utah, 65 Mario Capecchi Dr., SLC, UT 84108, 801-213-4152

C. Michael Cotten, MD

Duke University Medical Center, Box 2739 DUMC, Durham NC 27710, 2424 Erwin Road Suite 504 Durham, NC 27705, 919-681-4844

Mary Elizabeth Hartnett: me.hartnett@hsc.utah.edu; C. Michael Cotten: michael.cotten@duke.edu

Abstract

Retinopathy of prematurity (ROP) is a complex disease that is influenced by both genetic and environmental factors. Several small studies have found genetic variants in *EPAS1*, *VEGF*, *SOD* and members of the WNT family in association with ROP. Design in genetic studies is challenging because of changing recommendations for the management of prematurity and ROP, the fact ROP is rare, and that availability of resources for managing premature infants can vary throughout the world. In addition, there is a shortage of ophthalmologists with the ability to diagnose and characterize severe ROP. Careful determination of the degree of prematurity is important when evaluating genetic studies. Controlling for significant epidemiologic factors and multiple comparisons is also important to consider when evaluating genetic studies. One large candidate gene study controlled for degree of prematurity, significant epidemiologic factors, and multiple comparisons and found variants within the intron of *BDNF* associated with severe ROP. Future studies using unbiased techniques to assess genetic risk are important as are in-depth study of *BDNF* through deep sequencing and associated mechanistic studies using appropriate experimental models.

Introduction

Retinopathy of prematurity (ROP) is a leading cause of childhood blindness. Premature birth accounts for approximately 11% of all live births in the US and is on the rise in developing countries throughout the world.[1] For this and other reasons, ROP is a world-wide concern.

ROP only occurs in premature infants and has been strongly linked to extreme prematurity.[2] In the setting of premature birth, ROP has also been associated with other external factors, including those aligned with causes of preterm birth, such as poor prenatal nutrition or prenatal illicit drug use, and with perinatal associations and risks, including high supplemental oxygen at birth[3] and increased “oxidative stress”.[4] Despite advances in

neonatology and in the ability to regulate and monitor oxygen levels, ROP remains a cause of blindness and vision loss. In some developing countries, this may be due to insufficient resources to provide equipment to monitor and regulate oxygen, to provide staff to optimize settings for oxygen, or to provide adequate prenatal care, creating an appearance of ROP perhaps similar to that experienced in the US and UK in the 1950's.[5,6] In developed countries, survival of infants at the extremes of birth weight and gestational age present new problems associated with inadequate postnatal growth and conditions that increase the generation of reactive oxygen and nitrogen species in premature infants, who have reduced oxidative reserve.[7] However, there is also evidence that suggests a strong heritable component to ROP or that genetic variants may predispose premature infants of certain risk strata to ROP.[8]

Racial influence

Studies have reported that ROP occurs in Caucasians more than in African Americans[9] or Asians.[10] There are reports of other susceptibilities as well. Recently, a study reported differences in genetic susceptibility based on race. In the study, single nucleotide polymorphisms (SNPs) in endothelial nitric oxide synthetase were determined and found to have different gene frequencies in Caucasians than in African Americans.[11] The study supports the notion that underlying frequency of genetic variants may influence the susceptibility of preterm infants to ROP.

Twin studies

A retrospective 3 center study determined the risk of any ROP between mono- and dizygotic twins over a 10-year period. The study used a mixed effects logistic regression analysis and covariates of gestational age, birth weight, duration of respiratory distress syndrome or of oxygen use, presence of bronchopulmonary dysplasia and what the treating institution was. The study found a 70% variance in developing any level of ROP due to heritable factors.[8] The majority of infants who developed ROP were <1000g birth weight. The study did not analyze heritable effect risk based on different levels of ROP severity. However, other studies reported significant inter-sibling variability in twins[12] or no association between very low birth weight and retinopathy of prematurity in twin infants.[13] Other conditions associated with twin or multiple gestations may play a role. Assistive reproductive technology (ART) has been associated with increased risk of ROP for reasons that are peculiar to ART[14] or that ROP was not associated with genetics involving multiple gestation births.[15]

External environmental influences in ROP

ROP was first described as retrolental fibroplasia, probably representing current stage 5 ROP, in the 1940's by Terry.[16] From studies that exposed animals to conditions in incubators that were similar to what premature infants experienced, the hypothesis of high oxygen at birth was proposed[17–19] and then later tested in a clinical trial.[20] Avoidance of high oxygen at birth nearly obliterated ROP, but as smaller and younger preterm infants were able to survive due to advances in neonatal care, ROP reappeared.[21] The influence of oxygen on ROP is complex, and except for avoiding high supplemental oxygen at birth, no

clear recommendations on oxygen saturation profiles are universally accepted that reduce the incidence of ROP without adversely affecting neural development and survival.[22,23] It has also been proposed that oxygen fluctuations[24] may increase the generation of reactive oxygen and nitrogen species and along with reduced oxidative reserve of preterm infants, lead to oxidative stress and increased signaling triggered by oxidative compounds that can lead to cell damage and neuronal death or can trigger angiogenesis, as examples.[7,25] Inflammatory cytokines are also increased in ROP.[26] Crosstalk among angiogenic, inflammatory and oxidative signaling pathways can potentially accentuate pathology by overactivating angiogenic signaling pathways.[27]

Recent evidence has shown the importance of neurovascular interactions in ROP. Crosstalk between molecular pathways of endothelial cells and glial cells can affect glial, neural and vascular development. Examples include the effects of semaphorins that provide neural or endothelial cells cues regarding migration.[28–30] In addition, VEGF, which is an important angiogenic factor, is produced by M ller cell glia and can influence the health of neurons and glia within the retina.[31–33] In experimental models, light levels can influence the maturation of certain types of ganglion cells and influence developmental angiogenesis in the retina in experimental models.[30]

Additional studies report the association of poor postnatal growth and ROP.[34] Poor growth and low IGF-1 levels are associated with large avascular areas of retina (resulting in low ROP zone) and a greater risk of severe ROP.[35] Other studies report the role of specific nutritional components, including derivatives of omega 3 fatty acids,[36] or signaling effectors of the PPARα transcription factor[37] as having protective effects on ROP. In addition, studies report changes in metabolites. For example, succinate signaling through its receptor, GPR91, in ganglion cells in experimental models of ROP.[28,38] These are a few examples of associations reported with ROP.

ROP is strongly linked to extreme prematurity,[2] so delineating ROP from prematurity can be problematic. Studies that suggest genetic associations will be discussed below. Although a single genetic variant causing ROP in multiple different samples of patients has not reported, it is possible that genetic susceptibility in the setting of various external environments might lead to differential gene expression and suggests that different extremes of prematurity in the setting of a genetic susceptibility can influence ROP development through modifications in gene expression.

Considerations of genetic studies in ROP

There are several considerations when reviewing studies performed to assess genetic variants associated with ROP and severity of ROP. First, ROP is inherently associated with level of prematurity. Both young gestational age and low birth weight are associated with increased severity of ROP.[2] Therefore, it is nearly impossible to distinguish between variants affecting prematurity and those affecting severity in ROP. However, one can limit study to infants of more defined levels of prematurity and/or control for birth weight or gestational age in analyses. ROP is a rare condition even though it is one of the most common causes of vision loss in premature infants. Neonatal care is constantly advancing,

making it difficult to enroll premature infants from homogeneous groups. To obtain sufficient numbers of infants at similar levels of prematurity, studies may wish to enroll infants over long durations of time during which neonatal care and treatment of ROP can change. Another way to increase sample size over shorter enrollment periods is to enroll from multiple centers, but there can be differences in the strategies of neonatal care. An example can be related to oxygen saturation targets. If high oxygen is delivered at birth, severe ROP might be seen in infants who would not usually develop it. There can also be differences in the survival rate of extremely premature infants, those often at high risk of severe ROP. If infants die before ROP can be diagnosed, these infants are often excluded from the assessment of ROP severity. If infants who do not survive to have an ROP examination are included in the infants analyzed, the percentage of infants with ROP may also be erroneously determined. These considerations can further complicate the interpretation of outcomes in a study. An additional factor is erroneous diagnosis or classification of ROP. Studies report insufficient numbers of adequately trained ophthalmologists to diagnose ROP within recommended time frames.[39] If the recommendation for treatment of ROP changes over the course of enrollment for a study, additional variability in classifying severe ROP can be introduced. This has occurred, for example, when cryotherapy was recommended for threshold ROP in the Cryo-ROP study,[40] and again when treatment for less severe forms of ROP was recommended with ETROP.[41] For all these reasons, it becomes difficult to design and interpret studies about genetic influence on ROP or its severity.

Many external factors can also affect expression of gene variants through epigenetic mechanisms or post-translational modifications that only affect gene expression under certain stresses or conditions.

Definitions

To provide greater understanding when reading genetic studies, definitions of terms often used are provided. Many first steps in genetic studies look at the association of variants in SNPs (single-nucleotide polymorphisms) with a certain condition or disease. SNPs are common in that the minor allele (the less common allele) occurs in no less than 1% of the population. Candidate gene studies seek to find significantly higher prevalence of in SNPs in predetermined genes, which are biologically plausible in causing a condition or disease, with a condition compared to an otherwise similar group of subjects without the condition or disease. A genome wide association scan (GWAS) looks at SNPs throughout the genome and may identify SNPs in genes that have not been predetermined or predicted. Both GWAS and candidate gene studies are done on SNPs that occur within coding and non-coding sequences of the genes or genome.

Whole exome sequencing is performed on the translated part of the genome, whereas whole genome sequencing is performed on both the transcribed and non-coding regions of the genome. Whole exome sequencing is useful when there is a family history of the disease or a manifestation of it within family members, and then analyses of gene variants of affected and non-affected family members are performed. Whole genome sequencing may be done when family involvement is not present. Deep sequencing of the genome around an

identified gene variant is performed to tease out potential causal variants that may not have been included in the original genome wide analysis of SNPs. For example, in genome wide or exome wide studies, SNPs can be taken every 3000 to 5000 base pairs to reduce overlap between SNPs and represent the entire variation within a gene. However missing base pairs may contain the essential disease causing gene variants, and deep sequencing can be beneficial for identifying rarer disease-causing variants that are linked with the genotyped variants that identify the region for deep sequencing.

Studies of Genetics in ROP

Most genetic studies in ROP have been performed on candidate genes. The strength of candidate gene studies is that there is often biologic plausibility in choosing certain pathways in association with a disease. However, a limitation is that only those genes that are hypothesized to be involved are analyzed, and as more genome wide analyses are performed it is recognized that new pathways arise that turn out to contain important or causative variants based on follow up mechanistic studies. The Neonatal Research Network is in the process of analyzing a genome wide association study on extremely low birth weight infants. Candidate gene studies have been performed throughout the world, but studies generally had small sample sizes, did not control for multiple comparisons or have not been replicated. Many studies include infants with a wide range of gestational ages and birth weights, i.e. different degrees of prematurity. Studies that assessed the association of genetic variants with any level of ROP severity reported variants in the WNT signaling pathway,[42–48] which is important in development, in *EPAS1*[49] or *VEGF*,[50] which are regulated by hypoxia and involved in angiogenesis, and in *SOD*,[51,52] which transcribes an antioxidant enzyme. In studies specifically considering the risk associated with severe ROP, variants within the WNT signaling pathway were most commonly found, namely the genes, *NDP*, *FZD4*, and *LRP*5.[44,46–48]. Mutations in these genes can lead to the condition, familial exudative vitreoretinopathy (FEVR), which shares some findings with ROP but occurs in full term infants. FEVR can have great variability in expression even within family members with the same gene variant. Therefore, it remains unknown if premature infants with severe ROP and gene variants found in FEVR had FEVR but happened to be born prematurely (verbal communication with Kim Drenser MD PhD, September 2014).

Neonatal Research Network

The Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) Neonatal Research Network (NRN) comprises NICUs in tertiary academic medical centers that receive federal funding to do clinical trials and observational studies in neonates in order to identify best practices and improve outcomes. The NRN developed a large cohort to study the association of cytokines with morbidities and neurodevelopmental outcomes in extremely low birth weight infants.[53] The strengths of this study group include: inclusion of infants within a narrow range of prematurity, all of extremely low birth weight (born <1000 g) and managed in NRN centers; large number of infants (approximately 1000); short duration of enrollment (1998 to 2001); and same definition of severe ROP being threshold disease when the risk of retinal detachment or blindness approached 50%.[54] Genomic DNA was extracted from infant blood spot samples obtained for the cytokines

study that had been stored in an anonymized DNA biorepository by the Eunice Kennedy Shriver NICHD NRN. 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 very low and low birth weights (Table 1).[54] Another difference between the replication and discovery cohorts was that the duration of enrollment was longer for the replication cohort and included infants who were managed under guidelines of the Cryotherapy for ROP study (infants enrolled approximately from 1998 through 2003)[40] and the Early Treatment of ROP study (infants enrolled from 2004 to 2013)[41] in which severe ROP was defined as type 1 ROP, having a 15% risk of a poor outcome. Therefore, there were differences between the discovery and replication cohorts. However, it was not possible to enroll new patients into a cohort similar to the discovery cohort for ethical reasons because new recommendations to consider treatment for ROP at a less severe level are now standard of care.[41]

The discovery cohort included 145 candidate genes reported important in genetic studies as well as in experimental studies of ROP. Candidates included pathways in neurodevelopment, angiogenesis, inflammatory and oxidative signaling pathways.

Tag SNPs are SNPs that are in tight linkage disequilibrium with neighboring SNPs. This means that recombination rarely occurs in the regions of Tag SNPs, so that identification of the genotype of the Tag SNP makes it likely that the neighboring SNP will be predictable. Using Tag SNPs allows the investigator to make educated guesses about a larger number of SNPs in a single gene. For the NRN candidate gene ROP study, tagging SNPs were chosen for genotyping using HapMap provided by public domain using the following criteria: minor allele frequency greater than 10%, r^2 value of at least 0.8 and Tag SNP tagged for at least six other SNPs. To represent the entire variation within a gene additional SNPs approximately every 3 to 5000 base pairs were included. Whole genome amplified DNA from stored blood spot samples was genotyped with the Illumina GoldenGate platform for 1614 TagSNPs of the candidate genes. Data cleaning and analysis were performed, and SNPs were removed that had low genotyping pass rate (greater than 10% missing and/or were not in Hardy-Weinberg equilibrium (HWE) in infants without ROP (HWE $P < 3\ 3\ 10^{-5}$ based on P=0.05/1614 SNPs). All infants had been examined by credentialed ophthalmologists for ROP zone and stage in at least one examination prior to death or discharge. For both replication and discovery cohorts, severe ROP was diagnosed as ROP warranting treatment. Therefore, there the classification of severe ROP was different between the discovery cohort, which diagnosed severe ROP as the more advanced threshold disease, and the replication cohort, in which some infants were treated for threshold disease and others for the less severe type 1 ROP.

Initially, significant epidemiologic risk factors were performed for each analysis using logistic regression. Three different analyses were performed, each done independently from the other. To determine the risk of visual morbidity, which occurs in many premature infants with ROP who do not develop severe treatment-warranted ROP, two analyses were performed: any ROP vs. no ROP and severe vs. non-severe ROP. This study was the largest candidate gene study to date and was unique in probing for variants associated with the most

severe phenotype, threshold ROP. This third analysis addressed this: severe vs. non-severe and no ROP.

In the analysis assessing any ROP vs. no ROP, only days of ventilation within the first 28 postnatal days was significantly associated with ROP after stepwise regression analysis and control for multiple variables. In the analysis assessing severe vs. non-severe ROP, only occurrence of seizures was associated with severe ROP and in the analysis of no and non-severe ROP vs. severe ROP, both occurrence of seizure and ventilation days were significant. It is noted that neither gestational age nor birth weight remained significant in the statistical analysis, potentially because all infants were born extremely premature. Besides significant epidemiologic factors, Eigenvector values were used as covariables to control for continental ancestry. These values had been calculated as part of the NRN's analyses toward a genome wide scan of 800 infants from the discovery cohort in the candidate gene study.[55]

Stepwise regression analysis for genetic variants associated with ROP vs. no ROP revealed no significant SNPs after controlling for significant epidemiologic factors, Eigenvector values, and multiple comparisons. However, in the analyses comparing severe vs. non severe ROP or severe vs. no and nonsevere ROP, two SNPs in the gene encoding brain-derived neurotrophic factor (*BDNF*) were significantly associated with severe ROP: rs7934165 and rs2049046 ($P=3x10^{-5}$ and $6x10^{-5}$, respectively for second analysis, severe vs. non severe ROP) and rs7934165 and rs2049046 ($P=2x10^{-5}$ and $3x10^{-5}$, respectively for the third analysis, severe vs. no and nonsevere ROP).

The two BDNF SNPS were genotyped in the replication cohort of 118 related and 426 unrelated infants. Seizure occurrence and ventilation days were significant in the cohort but neither rs7934165 nor rs2049046 was significantly associated with ROP in unrelated or related infants in any of the analyses of the replication cohort. There were enough differences between the discovery and replication cohorts (e.g., differences in severity of ROP and time periods for enrollment of infants) that data could not be pooled. Therefore, a meta-analysis was performed and under a recessive model, rs7934165 increased in associated significance with severe ROP in the analysis comparing no and nonsevere ROP vs. severe ROP ($P=2.9\ x10^{-7}$).

Additional studies are needed before confirming the role of BDNF in ROP. The significant SNPs in *BDNF* were within the intron of the gene on chromosome 11, and so it is still unknown if the SNPs affect protein expression. Still, there is clinical evidence of reduced circulating BDNF levels in severe ROP.[56–58] Blood spot samples of infants from some infants in the discovery cohort in this report were analyzed for cytokines,[56] and reduced serum protein BDNF was found associated with severe ROP.[56] To determine if the intronic variants in *BDNF* affect biologic outcomes, deep sequencing of the gene in future non-biased human studies in conjunction with mechanistic experimental studies in cultured cells and/or animal models can be helpful. The rationale how BDNF might play a role in severe ROP involves at least 2 levels, the role of BDNF in the retina and in the brain. BDNF is an important survival factor for retinal glia and neurons. It is also important in ganglion cell maturation and is reduced during dark rearing in mice.[59] Mice reared in the dark or lacking

the gene encoding melanopsin developed vascular anomalies that could predispose to ROP.[60] Melanopsin is also important to certain forms of ganglion cells.[61] These studies suggest a hypothesis that BDNF may be involved in the maturation of certain ganglion cells that have a role in retinal angiogenesis and that may be affected by stresses associated with premature birth. Clinical studies provided links between ROP risk and time of birth and light exposure.[30] However, the situation may also involve the role of BDNF in the brain and its role in neuroprotection, as there are links between ROP neural development as seen on spectral domain OCT and neurocognitive development.[62] Additional studies through the NRN are planned including the analysis of a genome wide association scan in extremely low birth weight infants.

Summary

ROP is a complex disease in that evidence suggests it is influenced by both genetic and environmental factors. Design in genetic studies is challenging because of changing recommendations for the management of prematurity and ROP, the fact ROP is rare, and the differences in resources that affect management of premature infants throughout the world. In addition, there is a shortage of ophthalmologists with the ability to diagnose and characterize severe ROP. Careful determination of the degree of prematurity is important when evaluating genetic studies. Controlling for significant epidemiologic factors and multiple comparisons is also important consideration when evaluating genetic studies. Future studies using unbiased techniques to assess genetic risk are important as are in-depth study of *BDNF* through deep sequencing and associated mechanistic studies using appropriate experimental models.

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Table 1 ***

Subject Characteristics

	Discovery Cohort			Replication Cohort**		
Characteristic	**No ROP (n=264)**	**Non-Severe ROP (n=467)**	**Severe ROP (n=126)**	**No ROP (n=331)**	**Non-Severe ROP (n=195)**	**Severe ROP (n=14)**
Gestational Age in Weeks (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 in Grams (SD)	823.6 (126.0)	758 (133.0)	697 (125.0)	1242 (356.0)	904 (291.0)	678 (167.0)
Small for Gestational	64 (24.3)	55 (12.1)	6 (4.8)	36 (10.9)	23 (11.8)	3 (21.4)
Age, number (%)						
Male, number (%)	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, number (%)	13 (4.9)	43 (9.4)	25 (20.2)	2 (0.6)	3 (1.5)	3 (23.1)
Antenatal Steroids, number using (%)	218 (82.9)	351 (77.1)	90 (73.2)	312 (94.3)	181 (93.3)	13 (92.9)
Race (self-reported)						
Black, number (%)	136 (51.5)	219 (46.9)	60 (47.6)	32 (9.7)	155 (71.1)	10 (71.4)
White, number (%)	122 (46.2)	240 (51.4)	65 (51.6)	256 (77.3)	19 (8.7)	1 (7.1)
* Other, number (%)	6 (2.2)	8 (1.7)	1 (0.7)	43 (12.9)	44 (20.1)	3 (21.4)
Ethnicity						
Hispanic, number (%)	29 (11.0)	101 (21.6)	24 (19.0)	20 (6.0)	8 (3.7)	1 (7.1)
Non-Hispanic, number (%)	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

*** From Hartnett ME et al. Genetic variants associated with severe retinopathy of prematurity in extremely low birth weight infants. Invest Ophthalmol Vis Sci 2014;55:6194–6203.[54]