The Erythropoietin Promoter Variant rs1617640 Is Not Associated with Severe Retinopathy of Prematurity, Independent of Treatment with Erythropoietin

Christof Dame, Lina K. Sciesielski, Carolin Rau, Chiara-Aiyleen Badur, Christoph Bührer

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The *erythropoietin* promoter variant rs1617640 is not associated with severe retinopathy of prematurity, independent of treatment with erythropoietin

Christof Dame, MD, Lina K. Sciesielski*, PhD, Carolin Rau*, MD, Chiara-Aiyleen Badur, BSc, Christoph Bührer, MD.

Department of Neonatology, Charité – Universitätsmedizin Berlin

* equally contributing authors

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Corresponding author:

Christof Dame, MD
Department of Neonatology
Charité – Universitätsmedizin Berlin
Augustenburger Platz 1
D-13353 Berlin
Germany

Abstract

This case-control study provides evidence that the erythropoietin (*EPO*) promoter variant s1617640, linked to high intravitreal EPO concentrations and increased risk of diabetic retinopathy, is not associated with severe retinopathy of prematurity (ROP). This finding was observed both in infants with and without recombinant EPO administration.

Introduction

Severe retinopathy of prematurity (ROP) is associated with poor neurodevelopmental outcome.[1] Administration of recombinant erythropoietin (rEPO), elevated intravitreal or high systemic endogenous EPO on day 14 have been implicated in increasing the risk of severe ROP (stage ≥3°).[2-4] While transgenic mouse models showed a proangiogenic role of EPO in the proliferative phase of retinopathy,[5-7] exogenous EPO protected the retina from vessel loss during the initiation period of ROP in developing mice.[8]

In adults with diabetes, the erythropoietin (*EPO*) gene variant rs1617640 has been associated with severe proliferative retinal vasculopathy and 7-fold increased intravitreal EPO protein concentrations.[8] The T risk-allele introduces a transcription factor-binding motif in the 5' promoter that experimentally induces *EPO* transcription.[8] For the first time, we analyzed the association between the rs1617640 *EPO* variant and severe ROP in very preterm infants.

Patients and Methods

This retrospective case-control study (1:2 allocation) considered all very low birth weight (VLBW) infants with ROP ≥3° (with/without plus disease) treated in our institution within an 11-year time-period. A total of 2,056 VLBW infants were eligible. Among them, ROP stage 0°/1° was found in 1,815 infants, while 106 infants were diagnosed with ROP stage ≥3°. After reviewing the clinical records as well as the accessibility and quality of DNA specimens for molecular diagnostics, 72 out of the 106 infants with severe ROP were included. Control infants with ROP 0°/1° (n=141) were as tightly matched to the cases as possible by sequentially

matching birth weight (first), gestational age (second), and sex (third). The study cohort of years 1997 to 2009 was chosen because a subgroup was routinely treated with rEPO (250 IU/kg 3x/week i.v./s.c., initiated on day 5 or later once enteral iron supplementation was possible) to prevent red blood cell (RBC) transfusions. Treatment was continued over the observation period of this study (42 d) and was completed at discharge. This allowed evaluating the hypothesis that infants harboring the rs1617640 *EPO* promoter variant might exhibit an additional or increased risk for ROP ≥3°, if additionally treated with rEPO. Approval for the study was given by the Institutional Review Board (EA2/051/09, extended ROP_02_11).

Genomic DNA was isolated from left-over blood spots on filter paper cards of the newborn screening by using the *Nucleo Spin Tissue* Kit (Macherey-Nagel). A 394 bp PCR product of the *EPO* gene promoter (NCBI No. NM_007933.15, *nt* 38349923 to *nt* 38350316) was amplified using the primer set *EPO*SNPfw 5'-GTCCATTGTGCAGGACACAC-3' and *EPO*SNPre 5'-AAGGATCTTCCTGCCTTG-3'. If necessary, the amplicon was gel-purified using a gel extraction kit (Qiagen) or directly treated with 0.32 U Shrimp Alkaline Phosphatase and 3.6 U Exonuclease I (New England Biolabs). The sequencing PCR reactions were performed with the BigDye® Terminator Sequencing kit (Applied Biosystems). The PCR products were sequenced in a 16-capillary 3130*xl* Genetic Analyzer (ABI PRISM® 3130; Applied Biosystems). The SNP variant was determined using the Chromas 2.3 software (Technelysium).

Results

Infants with ROP ≥3° and ROP 0/1° were tightly matched by birth weight, gestational age, and sex (Table 1): The median birth weight of cases was 707 g (IQR 599-801 g) and of controls 725 g (IQR 636-800 g), the median gestational age of cases was 24+6/7 (IQR 24+3/7-26+2/7) and of controls 25+4/7 (IQR 24+6/7-26+4/7) showing their stratification as patients with highest risk for severe ROP. Indices of neonatal morbidity (duration of mechanical ventilation, postnatal steroids, rates of intraventricular hemorrhage) were more prevalent in

infants with ROP ≥3° (Table 1). In contrast, the homozygous variant TT of the *EPO* promoter variant rs1617640 was equally frequent in VLBW infants with ROP ≥3° and ROP 0/1°, and there was no association between the T risk-allele and severe ROP (Table 2). Although the number of infants who received RBC transfusions was almost equal in both groups, ROP ≥3° was significantly associated with earlier initiation, higher number, and bigger total volume of transfusions (Table 1). Stratification according to rEPO treatment did not indicate a higher incidence of ROP ≥3° (Table 1), also not in rEPO-treated infants harboring the T risk-allele (neither homo- nor heterozygously) in the *EPO* promoter (Table 2).

Discussion

In conclusion, the rs1617640 *EPO* promoter variant is not associated with a higher risk of ROP ≥3° in VLBW infants. This finding has a more general implication concerning the function of EPO in proliferative vasculopathy. Of at least 11 single nucleotide polymorphisms (SNP) identified in the *EPO* gene, only the rs1617640 variant has been examined functionally. This *EPO* promoter variant increases transcription in reporter gene assays and in a mouse model of oxygen-induced retinal neovascularization.[8] Therefore, the *EPO* rs1617640 variant gained much attention and was subsequently analyzed in cohort studies that significantly varied regarding the number of patients, the ethnicity, the type of diabetes, and its association with proliferative diabetic retinopathy (PDR), end-stage renal disease (ESRD), and diabetic microvascular complications.[8-14] A meta-analysis on the association of the *EPO* rs1617640 variant with PDR and ESRD (a total of 3,162 case and 3,845 control subjects across five separate cohorts of European and European-American ancestry) retained statistical significance,[11] although the association between the EPO rs1617640 variant and PDR was not confirmed in each cohort.[9, 10, 13] Recently, the predominant clinical relevance of the rs1617640 *EPO* variant was verified in adults with diabetic retinopathy and ESRD, who exhibited diabetic microvascular complications.[15] This may indicate that additional risk factors are required in order to turn the function of the rs1617640 *EPO* variant into a mechanism that is harmful for microvessels.

Notably, a very distinct phenotype of the controls (free from both PDR and ESRD after 10-15 years of diabetes) was defined in the initial study reporting on the association and function of the *EPO* rs1617640 variant.[8] By excluding VLBW infants with ROP stage 2° from our study, we also applied a restrictive methodical concept. Moreover, we tightly matched for birth weight, gestational age and sex. This emphasizes our major conclusion that the rs1617640 *EPO* promoter variant is not associated with a higher risk of ROP ≥3°.

In the largest previous candidate gene study, two variants in *BDNF* encoding brain derived neurotrophic factor were associated with severe ROP.[16] In strategies to identify genetic risk factors for severe ROP, one may consider that in the sicker infant, inflammation and oxidative stress cause epigenetic modifications. Such modifications may predispose very preterm infants to develop severe ROP or not.[17, 18]

Notably, the extremely low gestational age newborn (ELGAN) study indicated that in infants born between 23 and 27 weeks of gestation the risk for severe ROP was increased when blood EPO concentrations on day 14 were in the top quartile.[3] Thus, the question on the role of exogenous (recombinant) EPO in the development of ROP continues to be of ongoing interest.[2, 19, 20] In neonatal rats, high-dose rEPO given as a single high dose on postnatal day 1 penetrated the blood-eye barrier and accumulated in the eye with a peak concentration measured at 24 h after injection.[21] Although this accumulation was not found after administration of a dose of 500 U/kg[21], it is reassuring that in our study population even infants harboring the T-risk allele of the rs1617640 *EPO* promoter variant homozygously did not display a higher risk of ROP ≥3° if treated with 3 x 250 U/kg rEPO per week over a period of 4-5 weeks (Table 2). Thus, our findings further extend conclusions from two recent meta-analyses consistently finding that early rEPO administration, even given in very high dose for neuroprotection, did not increase the risk of ROP ≥3°.[19, 20]

Almost all studies included in these two meta-analyses considered randomized clinical trials, in which eligibility for enrollment of patients was defined by a broad range of birth weight (from 500 up to 1,250 or 1,500 g, respectively) and of gestational age (up to ≤ 32 weeks). In fact, the incidence of ROP correlates inversely with gestational age and birth weight.[22-24] Of

children born at 24, 25, 26, 27, and 28 weeks, 19.6 %, 8.6 %, 4.6 %, 1.1 % and 0.8 % developed severe ROP, respectively.[23] This is well reflected by the low gestational age and birth weight in our study population (Table 1). Thereby, our study functions as a as highly representative analysis for addressing the question on the combined effects of the rs1617640 *EPO* promoter variant and rEPO treatment.

In the developing eye, however, EPO acts not only as angiogenetic factor,[6] it also reduces *in vitro* oxidative stress and experimentally prevents retinal degeneration.[25, 26] These findings and a recent meta-analysis indicating a better neurocognitive outcome after early initiated rEPO for neuroprotection in very preterm infants,[27] speak in favor of reconsidering rEPO for preterm infants. Starting with high-dose rEPO in the initiation phase of ROP, followed by low-dose during the proliferative phase would take into account the experimental evidence of the dual role of EPO in the pathophysiology of ROP.[28]

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Tables

Table 1. Demographic data and major morbidities in VLBW infants with severe ROP (ROP ≥3°) and case-controls (ROP 0/1°) in a 1:2 ratio. Follow-up data cover a time-period of 42 days after birth. Statistical differences were analyzed using the two-tailed Mann-Whitney-U test or two-tailed Fisher's exact probability test for dichotomous traits for which the Odds Ratio (OR) and the 95% confidence interval (95%-CI) are stated. NaN: Not a number

	ROP 0/1° **n = 141**	**ROP ≥3° n = 72**	**OR (95%-CI)**	***p* value**
Sex, female, *n* (%)	71 (50.4)	28 (38.9)	0.63 (0.35-1.12)	0.146
Birth weight (g), *median* (range)	725 (387-1,470)	707 (410-1,475)	-	0.534
Gestational age (weeks + days), *median* (range)	25+4 (23+3 - 29+6)	24+6 (23+2 - 29+5)	-	0.021
Percentile ≤10 (n), *median* (range)	23 (16.3)	7 (9.7)	0.55 (0.22-1.36)	0.218
Mechanical ventilation, *n* (%)	131 (92.9)	70 (97.2)	2.67 (0.57-12.53)	0.230
Duration of mechanical ventilation (d), *median* (range)	17 (1-42)	35 (1-42)	-	<0.001
O_2 supplementation, *n* (%)	136 (96.5)	72 (100.0)	∞ (NaN-∞)	0.170
Postnatal steroids, *n* (%)	22 (15.6)	28 (38.9)	3.44 (1.78-6.64)	<0.001
Parenteral feeding (d), *median* (range)	21 (1-42)	23 (8-42)	-	0.233
Intracranial hemorrhage (IVH/ICH), *n* (%)	27 (19.1)	33 (45.8)	3.57 (1.91-6.68)	<0.001
Patent ductus arteriosus, *n* (%)	117 (83.0)	57 (79.2)	0.78 (0.38-1.60)	0.575
Necrotizing enterocolitis, *n* (%)	9 (6.4)	5 (6.9)	1.09 (0.35-3.40)	1.000
Red blood cell transfusion, *n* (%)	123 (87.2)	69 (95.8)	3.37 (0.96-11.83)	0.053
Initiation of red blood cells transfusion (d), *median* (range)	4 (1-40)	2 (1-29)	-	0.028
Number of red blood cell transfusions (n), *median* (range)	4 (1-14)	6 (1-13)	-	<0.001
Cumulative transfusion volume (ml), *median* (range)	47 (9-294)	90 (15-195)	-	<0.001
Cumulative iron supplementation (mg), *median* (range)	158 (5-357)	147 (6-228)	-	0.008
rEPO treatment, *n* (%)	89 (63.1)	35 (48.6)	0.55 (0.31-0.98)	0.559
Initiation of rEPO (d), *median*, (range)	10 (5-28)	11 (5-41)	-	0.258

Table 2. Frequency of *EPO* promoter polymorphism rs161760 in the study groups with stratification according to rEPO treatment. Statistical analysis: Freeman-Halton extension of the Fisher's exact probability test for a two-rows by three-columns contingency table was used. The lack of association of the *EPO* promoter polymorphism rs161760 and severe ROP was also evident in each alternative genetic model (allele, genotype, dominant, or recessive model, respectively) for such analysis (data not shown).

rs1617640	**ROP 0/1° n = 141**	**ROP ≥3° n = 72**	***p* value**
TT genotype, *n* (%)	56 (39.7)	29 (40.3)	0.48
GT genotype, *n* (%)	63 (44.7)	36 (50.0)	
GG genotype, *n* (%)	22 (15.6)	7 (9.7)	
With rEPO treatment	**n = 89**	**n = 35**	
TT genotype, *n* (%)	30 (33.7)	16 (45.7)	0.49
GT genotype, *n* (%)	44 (49.4)	15 (42.9)	
GG genotype, *n* (%)	15 (16.9)	4 (11.4)	
Without rEPO treatment	**n = 52**	**n = 37**	
TT genotype, *n* (%)	26 (50.0)	13 (35.1)	0.19
GT genotype, *n* (%)	19 (36.5)	21 (56.8)	
GG genotype, *n* (%)	7 (13.5)	3 (8.1)	

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