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

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In this case-control study, the erythropoietin (*EPO*) promoter variant s1617640, linked to high intravitreal EPO concentrations and increased risk of diabetic retinopathy, was not associated with severe retinopathy of prematurity. This finding was observed both in infants with and without recombinant EPO administration. *(J Pediatr 2018;■■:■■-■■).*

Severe retinopathy of prematurity (ROP) is associated with poor neurodevelopmental outcome.[1] Administration of recombinant erythropoietin ([EPO] rEPO), elevated intravitreal EPO, or high systemic endogenous EPO on day 14 have been implicated in increasing the risk of severe ROP (stage ≥3).[2-4] Although 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.[6]

In adults with diabetes, the *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] The purpose of this study was to analyze the association between the rs1617640 *EPO* variant and severe ROP in very preterm infants.

Methods

This retrospective case-control study (1:2 allocation) evaluated all very low birth weight (VLBW) infants with ROP stage ≥3 (with or without plus disease) treated in our institution within an 11-year time period. A total of 2056 VLBW infants were eligible. Among them, ROP stage 0/1 was found in 1815 infants, and 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 of 106 infants with severe ROP were included. Control infants with ROP stage 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 1997-2009 was chosen because a subgroup was routinely treated with rEPO (250 IU/kg ×3/week intravenously or subcutaneously, initiated on day 5 or later once enteral iron supplementation was possible) to prevent red blood cell transfusions. Treatment was continued over the observation period of this study (42 days) 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 stage ≥3, if additionally treated with rEPO. Approval for the study was given by the Charité—Universitätsmedizin Berlin Institutional Review Board (EA2/051/09, extended ROP_02_11).

Genomic DNA was isolated from leftover blood spots on filter paper cards of the newborn screening by using the Nucleo Spin Tissue Kit (Macherey-Nagel, Düren, Germany). A 394 bp polymerase chain reaction (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, Hilden, Germany) or directly treated with 0.32 U Shrimp Alkaline Phosphatase and 3.6 U Exonuclease I (New England Biolabs, Ipswich, Massachusetts). The sequencing PCR reactions were performed with the BigDye Terminator Sequencing kit (Applied Biosystems, Foster City, California). The PCR products were sequenced in a 16-capillary 3130*xl* Genetic Analyzer (ABI PRISM 3130; Applied Biosystems). The single nucleotide polymorphisms variant was determined using the Chromas 2.3 software (Technelysium, South Brisbane, Australia).

Results

Infants with ROP stage ≥3 and ROP stage 0/1 were matched by birth weight, gestational age, and sex (**Table I**). Indices of neonatal morbidity (duration of mechanical ventilation,

ROP	Retinopathy of prematurity
EPO	Erythropoietin
PCR	Polymerase chain reaction
rEPO	Recombinant EPO
VLBW	Very low birth weight

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Table I. Demographic data and major morbidities in VLBW infants with severe ROP (ROP stage ≥3) and case-control infants (ROP 0/1)

Clinical parameters	ROP stage 0/1 n = 141	ROP stage ≥3 n = 72	OR (95% CI)	*P* value
Sex, female, n (%)	71 (50.4)	28 (38.9)	0.63 (0.35-1.12)	.146
Birth weight (g), median (range)	725 (387-1,470)	707 (410-1,475)	—	.534
Gestational age (wk + d), median (range)	25 + 4 (23 + 3-29 + 6)	24 + 6 (23 + 2-29 + 5)	—	.021
Birthweight percentile ≤10 (n), median (range)	23 (16.3)	7 (9.7)	0.55 (0.22-1.36)	.218
Mechanical ventilation, n (%)	131 (92.9)	70 (97.2)	2.67 (0.57-12.53)	.230
Duration of mechanical ventilation (d), median (range)	17 (1-42)	35 (1-42)	—	<.001
O_2 supplementation, n (%)	136 (96.5)	72 (100.0)	∞ (NaN-∞)	.170
Postnatal steroids, n (%)	22 (15.6)	28 (38.9)	3.44 (1.78-6.64)	<.001
Parenteral feeding (d), median (range)	21 (1-42)	23 (8-42)	-	.233
Intracranial hemorrhage, n (%)	27 (19.1)	33 (45.8)	3.57 (1.91-6.68)	<.001
Patent ductus arteriosus, n (%)	117 (83.0)	57 (79.2)	0.78 (0.38-1.60)	.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)	.053
Initiation of red blood cells transfusion (d), median (range)	4 (1-40)	2 (1-29)	—	.028
Number of red blood cell transfusions (n), median (range)	4 (1-14)	6 (1-13)	—	<.001
Cumulative transfusion volume (mL), median (range)	47 (9-294)	90 (15-195)	—	<.001
Cumulative iron supplementation (mg), median (range)	158 (5-357)	147 (6-228)	—	.008
rEPO treatment, n (%)	89 (63.1)	35 (48.6)	0.55 (0.31-0.98)	.559
Initiation of rEPO (d), median, (range)	10 (5-28)	11 (5-41)	—	.258

NaN, Not a number.
Follow-up data cover a time period of 42 days after birth. Statistical differences were analyzed using the 2-tailed Mann-Whitney U test or 2-tailed Fisher exact probability test for dichotomous traits for which the OR and the 95% CI are stated.

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

Table II. Frequency of *EPO* promoter polymorphism rs161760 in the study groups with stratification according to rEPO treatment

rs1617640	ROP stage 0/1 n = 141	ROP stage ≥3 n = 72	*P* value
TT genotype, n (%)	56 (39.7)	29 (40.3)	
GT genotype, n (%)	63 (44.7)	36 (50.0)	.48
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)	
GT genotype, n (%)	44 (49.4)	15 (42.9)	.49
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)	
GT genotype, n (%)	19 (36.5)	21 (56.8)	.19
GG genotype, n (%)	7 (13.5)	3 (8.1)	

Statistical analysis: Freeman-Halton extension of the Fisher exact probability test for a 2-row by 3-column contingency table was used. The lack of association of the *EPO* promoter polymorphism rs1617640 and severe ROP was also evident in each alternative genetic model (allele, genotype, dominant, or recessive model, respectively) for such analysis (data not shown).

Discussion

In this study, the rs1617640 *EPO* promoter variant was not associated with a higher risk of ROP stage ≥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 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 in the number of patients, the ethnicity, the type of diabetes, and its association with proliferative diabetic retinopathy, end-stage renal disease, and diabetic microvascular complications.[8-14] A meta-analysis of the association of the *EPO* rs1617640 variant with proliferative diabetic retinopathy and end-stage renal disease (a total of 3162 cases and 3845 control subjects across 5 separate cohorts of European and European-American ancestry) showed statistical significance,[11] although the association between the EPO rs1617640 variant and proliferative diabetic retinopathy was not confirmed in each cohort.[9,10,13] Recently, the clinical relevance of the rs1617640 *EPO* variant was verified in adults with diabetic retinopathy and end-stage renal disease, who exhibited diabetic microvascular complications.[15] This may indicate that additional risk factors are required to turn the function of the rs1617640 *EPO* variant into a mechanism that is harmful for microvessels.

Notably, a very distinct phenotype of the control patients with diabetes (free from both proliferative diabetic retinopathy and end-stage renal disease after 10-15 years of diabetes)

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