Research and Reports in Neonatology

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ORIGINAL RESEARCH

VEGF 936C>T is predictive of threshold retinopathy of prematurity in Japanese infants with a 30-week gestational age or less

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This article was published in the following Dove Press journal:
Research and Reports in Neonatology
2 March 2011
Number of times this article has been viewed

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Abstract: Vascular endothelial growth factor (VEGF) contributes to the development of retinopathy of prematurity (ROP). We investigated the association of ROP with *VEGF* genetic polymorphisms and its clinical parameters in Japanese people. Sixty-seven infants with a gestational age of 30 weeks or less were enrolled and classified into the threshold ROP group (infants with Stage 3 ROP in zone I or II, five continuous or eight total clock hours of the retina and the presence of plus disease, n = 30) and the nonthreshold ROP group (n = 37). The *VEGF* genotypes of −1498T>C, −1154G>A, −634C>G, −7C>T, 936C>T, and 1612G>A were determined. *VEGF* 936C>T polymorphism and 11 clinical parameters were significantly different between the two ROP groups by univariate analysis. A logistic regression analysis with adjustments for gestational age and birth weight showed that the heterozygous or homozygous carrier state of the T alleles of *VEGF* 936C>T polymorphism (odds ratio 5.12; 95% confidence interval: 1.25–20.92; $P = 0.023$) and duration of oxygen administration (odds ratio 1.05; 95% confidence interval: 1.00–1.10; $P = 0.042$) were independent risk factors of threshold ROP. *VEGF* 936C>T polymorphism may predict threshold ROP in Japanese infants with a gestational age of 30 weeks or less.

Keywords: retinopathy of prematurity, vascular endothelial growth factor, gene polymorphism, premature infant

Introduction

In spite of improvements in perinatal care, retinopathy of prematurity (ROP) remains a major complication among premature infants. ROP can cause retinal detachment resulting in visual loss or permanent blindness. ROP is a multifactorial disease, and several studies have identified risk factors such as short gestational age, low birth weight, excessive oxygen supplementation, blood transfusion, and recombinant erythropoietin exposure.[1–8] However, there is no reliable method for predicting whether ROP will regress spontaneously or progress in spite of adequate treatment.

ROP is characterized by incomplete and abnormal neovascularization of the retina in premature infants. It is known that vascular endothelial growth factor (VEGF), a major mediator of vascular permeability and angiogenesis, is necessary for vascular growth in the neonatal retina.[9,10] Increased VEGF synthesis and high levels of VEGF have been observed in the hypoxic retina of an animal model,[11,12] whereas other experiments have shown that inhibition of VEGF prevented retinal ischemia-associated neovascularization.[13,14] Furthermore, the importance of VEGF in the development of ROP has been supported by data from several clinical studies.[15–18]

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DOI: 10.2147/RRN.S17243

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The *VEGF* gene (gene symbol: VEGFA, gene ID: 7422) is located on chromosome 6q21.3, and consists of eight exons and seven introns.[19] Various polymorphisms of the *VEGF* gene have been identified that influence the expression levels of VEGF protein.[20–24] It has been recently reported that genetic polymorphism in the *VEGF* gene promoter may influence the progression of ROP.[25–28] However, there are no data about the correlation between *VEGF* gene polymorphisms and risk of ROP in the Japanese population.

In the present study, we investigated the association of ROP with *VEGF* genetic polymorphisms and clinical (maternal, perinatal, neonatal) parameters in the Japanese population by using univariate and multivariate analyses.

Methods and materials

Patients

Sixty-seven infants with an estimated 30 weeks' gestational age or less were enrolled in this study. They included 52 single infants, six pairs of twins, and one pair of triplets. All infants were treated at the neonatal intensive care unit in Kobe University Hospital. The study was approved by the ethics committee of the Kobe University Graduate School of Medicine, and written informed consent was obtained from the patients' parents.

The first ophthalmic examination was performed at 3–4 weeks of age, and was followed up until 40 weeks postconceptual age. ROP degree was classified according to the International Committee for Classification of Retinopathy of Prematurity.[29] Threshold disease of ROP is considered to be present when Stage 3 ROP is present in either zone I or zone II, with at least five continuous or eight total clock hours of disease, and the presence of plus disease.[30] For the purpose of this study, the 67 infants were divided into two groups, ie, a threshold ROP group and a nonthreshold ROP group. The threshold ROP group consisted of 30 infants and the nonthreshold ROP group included 37 infants.

Maternal and perinatal variables documented included maternal age, gravidity, parity, maternal exposure to antenatal steroids, maternal smoking, delivery route, pregnancy-induced hypertension, premature rupture of membranes, threatened premature labor, nonreassuring fetal state, pathological chorioamnionitis, pathological funisitis, and placental infarction.

The variables recorded at birth included gestational age (based on the date of the last menstrual period), birth weight, gender, intrauterine growth restriction, multiple births, and APGAR scores at 1 minute and 5 minutes. Variables recorded after birth included the number of surfactant administrations, administration of catecholamines, glucose-insulin infusion, duration of mechanical ventilation, duration of oxygen treatment, neonatal jaundice requiring phototherapy, septicemia (positive blood culture), hypoglycemia, patent ductus arteriosus, intraventricular hemorrhage, chronic lung disease, apnea, dexamethasone exposure, administration of recombinant erythropoietin, transfusion, and exchange transfusion. Chronic lung disease was diagnosed when the infants required oxygen therapy at 36 weeks' corrected postnatal gestational age.[31] The laboratory findings at birth included the first count of white blood cells, hemoglobin level, creatine kinase level, C-reactive protein, and IgM. A serum C-reactive protein concentration of >1.0 mg/dL was defined as significantly elevated. A serum IgM concentration of >20 mg/dL at birth was regarded as clinically significant.

VEGF genotyping

Genomic DNA was extracted from the buccal mucosa or umbilical cord using a DNeasy Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's directions. Using the public National Center for Biotechnology Information Single Nucleotide Polymorphism database and available literature, we selected six *VEGF* candidate polymorphisms, ie, −1498T>C (rs833061) and −1154G>A (rs1570360) in the promoter region, −634C>G (rs2010963) and −7C>T (rs25648) in the 5' UTR, and 936C>T (rs3025039) and 1612G>A (rs10434) in the 3' UTR. Each polymorphism showed a minor allele frequency of at least 0.10 in the Japanese population,[23,32–35] and all polymorphisms were investigated in terms of their association with various types of diseases or clinical situations.[22,36–39] The six *VEGF* genotypes were evaluated using TaqMan^R minor groove binding probe-based polymerase chain reaction, and confirmed by direct sequencing, as described previously.[40]

Statistical analysis

Values were expressed as means ± standard deviations. Statistical analysis was performed using the SPSS statistical package (version 14.0; SPSS Inc., Chicago, IL). The statistical significance of differences between the mean values was calculated for unpaired samples using a Student's *t*-test or Welch's *t*-test, provided that the samples were normally distributed. If this was not the case, a Mann–Whitney *U* test was applied. In the categorical univariate analysis, the dependent variable was the threshold or nonthreshold ROP

as defined earlier. Some independent variables were classified into two or three categories. The Fisher's exact test was used to evaluate the relationship between these independent variables and ROP. The significant factors in the univariate analysis were then included in further analyses, including a stepwise logistic regression model, with adjustments for gestational age and birth weight. In all analyses, *P* values less than 0.05 (two-tailed) were considered to be statistically significant.

Results

Univariate and multivariate analyses were performed in the 67 patients, some of whom had missing data. The mean gestational age of preterm infants in the threshold ROP group was significantly shorter than that in the nonthreshold ROP group (26.8 ± 1.8 [range 23.4–29.7] weeks versus 28.7 ± 1.6 [range 25.7–30.9]) weeks. The average birth weights of the preterm infants were also significantly different between the threshold and nonthreshold ROP group (875 ± 258 and 1052 ± 221 g, respectively). APGAR scores at 1 minute and 5 minutes, duration of artificial ventilation, and duration of oxygen administration significantly affected the development of threshold ROP as well. Pregnancy-induced hypertension, number of surfactant administrations, chronic lung disease, transfusion, and septicemia were significant risk factors of developing threshold ROP by univariate analysis of categorized factors. The risk of threshold ROP was not significantly influenced by other tested factors (Table 1).

The VEGF polymorphisms −1498T>C, −1154G>A, −634C>G, −7C>T, 936C>T, and 1612G>A were found at an allele frequency of 45/128, 27/128, 79/128, 21/128, 25/128, and 14/128, respectively. The distribution of the *VEGF* 936C>T polymorphism showed a significant statistical difference between the threshold ROP and nonthreshold ROP groups. However, there was no difference in distribution of the other polymorphisms for the *VEGF* gene between the threshold and nonthreshold ROP groups (Table 2).

Amongst the 12 significant risk factors in the univariate analysis, gestational age and birth weight appeared to be important for ROP. To prevent the possible effects of confounding factors, a subsequent multivariate logistic analysis was performed with an adjustment for gestational age and birth weight. In addition, six patients with missing data were excluded from the following multivariate analysis to improve accuracy. The numbers of surfactant administrations and septicemia were excluded from the multivariate analysis because no infants without surfactant administration and all infants with septicemia developed threshold ROP, and the 95% confidence interval (CI) was wide. The multivariate logistic regression analysis showed that duration of oxygen administration (odds ratio 1.05; 95% CI: 1.00–1.10; $P = 0.042$) and the heterozygous or homozygous carrier state of T alleles of *VEGF* 936C>T polymorphism (odds ratio 5.12; 95% CI: 1.25–20.92; $P = 0.023$) were independent risk factors for the development of threshold ROP (Table 3). These factors allowed the diagnosis of ROP with 80.3% confidence.

Discussion

In the present study, we found that *VEGF* 936C>T polymorphism and duration of oxygen administration were independent risk factors for threshold ROP using a multivariate logistic analysis with adjustment for gestational age and birth weight.

The importance of VEGF in the development of ROP has been reported in several previous studies. Sonmez et al demonstrated that intravitreous levels of VEGF were elevated in Stage 4 ROP. This suggests that VEGF is highly active in Stage 4 ROP, and is more likely to play a key role in the maintenance of neovascularization.[18] Another study investigated 38 cases of Stage 5 ROP at vitrectomy, and revealed increased VEGF immunoreactivity in the vascularized regions of fibrovascular membranes.[17] Meanwhile, two studies have reported about the systemic VEGF levels in infants; one described that the plasma VEGF level was similar in both infants with and without ROP,[41] and the other reported that serum VEGF level in infants with ROP requiring treatment was significantly lower than that in infants without ROP.[28] From these studies, the correlation between the development of ROP and systemic VEGF level remains unclear. It is probable that pathogenic retinal angiogenesis in ROP is mostly driven by local VEGF synthesis.

Recently, various genetic polymorphisms in the *VEGF* gene have been identified that influence the levels of VEGF expression. Our results showed that there was a significant difference in distribution of the 936C>T genotype in premature Japanese infants with threshold ROP. While this finding is intriguing, our present pilot study was limited by small sample size. A further study with a larger sample size could clarify the VEGF genotype-specific effect on threshold ROP.

The 936C>T polymorphism is located in the 3' UTR of the *VEGF* gene which is an important regulatory site controlling mRNA stability and translation under certain conditions.[42–44]

Table 1 Univariate analysis of categorized and noncategorized factors associated with threshold ROP

Variable[a]	Nonthreshold ROP (n = 37)	Threshold ROP (n = 30)	*P* value[a]
Maternal and perinatal variables			
Maternal age (years)	32.7 ± 5.1	31.2 ± 4.4[b]	0.211
Gravidity	2.4 ± 1.5	2.2 ± 1.3	0.479
Parity	0.8 ± 1.0	0.6 ± 0.7	0.534
Maternal smoking (no/yes)	27/9[b]	27/2[b]	0.094
Pregnancy-induced hypertension (no/yes)	28/9	29/1	*0.019*
Threatened premature delivery (no/yes)	24/13	12/18	0.052
Premature rupture of membranes (no/yes)	30/7	21/9	0.390
Antenatal steroids (no/yes)	18/18[b]	18/12	0.464
Nonreassuring fetal state (no/yes)	32/5	27/3	0.722
Pathological funisitis (no/yes)	30/3[b]	22/6[b]	0.279
Pathological chorioamnionitis (no/yes)	20/13[b]	15/14[b]	0.609
Placental infarction (no/yes)	27/6[b]	24/4[b]	0.741
Variables at birth			
Gender (male/female)	21/16	15/15	0.628
Gestational age (weeks)	28.7 ± 1.6	26.8 ± 1.8	*<0.001*
Birth weight (g)	1052 ± 221	875 ± 258	*0.004*
Intrauterine growth retardation (no/yes)	22/15	22/8	0.304
APGAR score at 1 min	6.6 ± 2.0	4.8 ± 2.3	*0.002*
5 min	8.1 ± 1.5	6.9 ± 2.1	*0.004*
Delivery route (cesarean/vaginal)	33/4	26/4	1.000
Multiple birth (no/yes)	29/8	23/7	1.000
Variables after birth			
Number of surfactant administration (0/1/≥2)	7/16/14	0/21/9	*0.013*
Duration of artificial ventilation (days)	37.2 ± 22.2[b]	59.3 ± 19.3[b]	*<0.001*
Duration of oxygen administration (days)	47.6 ± 20.6	76.9 ± 26.6	*<0.001*
Chronic lung disease (no/yes)	24/13	8/22	*<0.003*
Dexamethasone exposure (no/yes)	36/1	26/3[b]	0.312
Patent ductus arteriosus (no/yes)	27/10	17/13	0.200
Catecholamine administration (no/yes)	3/34	0/30	0.247
Glucose-insulin infusion (no/yes)	33/4	21/9	0.065
Transfusion (no/yes)	31/5[b]	14/16	*0.001*
Exchange blood transfusion (no/yes)	36/0[b]	27/3	0.089
Erythropoietin administration (no/yes)	3/34	0/30	0.247
Neonatal jaundice requiring phototherapy (no/yes)	1/36	0/30	1.000
Hypoglycemia (no/yes)	30/7	28/2	0.172
Apnea (no/yes)	8/29	13/17	0.069
Intraventricular hemorrhage (no/yes)	34/3	27/3	1.000
Septicemia, positive blood culture (no/yes)	37/0	24/6	*0.006*
Laboratory findings at birth			
White blood cells (×100/μL)	73.8 ± 58.3	92.0 ± 66.5	0.273
Hemoglobin (g/dL)	15.9 ± 2.2	14.9 ± 2.1	0.079
C-reactive protein (no/yes)	36/1	29/0[b]	1.000
Creatine kinase (IU/L)	321 ± 144	301 ± 208[b]	0.321
IgM (no/yes)	35/2	28/1[b]	1.000

Notes: Values for noncategorized factors were expressed as means ± standard deviations. [a]Variables with $P < 0.05$ in the univariate analysis are indicated in italics; [b]These patients had missing data.

Abbreviation: ROP, retinopathy of prematurity.

To date, it has been reported that the 936C>T polymorphism affects plasma VEGF level, and adult carriers of a 936 T allele have significantly reduced levels of plasma VEGF.[22,45,46] However, no data are available on the genetic effects on VEGF level in immature eyes. Meanwhile, the computationally predicted potential binding sites for miR-210, miR-517a, miR-517b, and miR-517c included the 936C>T polymorphic site in the VEGF mRNA 3' UTR.[47] Although the roles of these microRNAs remain obscure, it has been reported that miR-210 is upregulated in various cell

Table 2 Genotype distribution for the VEGF polymorphism in threshold and nonthreshold ROP

Variable[a]	Nonthreshold ROP (n = 37)	Threshold ROP (n = 30)	*P* value[a]
VEGF genotype			
−1498T>C (TT/TC/CC)	19/10/5[b]	10/15/5	0.172
−1154G>A (GG/GA/AA)	24/9/1[b]	16/12/2	0.412
−634C>G (CC/CG/GG)	6/16/12[b]	3/15/12	0.699
−7C>T (CC/CT/TT)	52/9/0[b]	19/10/1	0.492
936C>T (CC/CT/TT)	27/7/0[b]	14/14/2	*0.012*
1612G>A (GG/GA/AA)	26/8/0[b]	24/6/0	0.771

Notes: [a]Variables with $P < 0.05$ in the univariate analysis are indicated in italics; [b]These patients had missing data.
Abbreviations: ROP, retinopathy of prematurity; VEGF, vascular endothelial growth factor.

lines under hypoxic conditions.[47–50] Fasanaro et al have also reported that miR-210 upregulation in normoxic conditions increased tubulogenesis and migration in the endothelial cell line, whereas the blockade of miR-210 under hypoxia led to inhibition of tubulogenesis and migration.[48,49] Given the regulatory protein and certain microRNAs that potentially promote mRNA degradation or delay translation bind to the 936C>T polymorphic site in the VEGF mRNA 3'-UTR, recognition of the specific mRNA sequence and secondary structure by these regulatory factors may be disrupted by the polymorphism. It was speculated that *VEGF* 936 T allele was responsible for hindering and delaying mRNA degradation or translational silencing, and subsequently a persistent ocular VEGF level, leading to abnormal neovascularization in immature eyes.

In contrast with our results, Cooke et al described that there was no association between the allele state of *VEGF* 936C>T and risk of ROP. However, we cannot directly compare our results with those of Cooke et al because our study is dissimilar with respect to the patient gestational age, birth weight, and ethnic background.[25] In addition, the association of the *VEGF* −1498T>C and −634G>C polymorphisms with the risk of threshold ROP has also been examined, resulting in inconsistent results across groups.[25,26,28,51] In the present study, there was no association between VEGF −1498T>C or −634G>C polymorphism and risk of threshold ROP in premature Japanese infants. There is a possibility that the 936C>T polymorphism is related to −1498T>C and/or −634G>C polymorphisms, and a further haplotype analysis might provide a rational explanation for these discrepancies.

Some previous studies have suggested septicemia as a risk factor.[52–54] Septicemia may injure developing blood vessels in the retina, induce the release of cytokines or growth factors, and result in threshold ROP. In the present study, all infants with septicemia developed threshold ROP. Septicemia was suggested as a risk factor of ROP. However, the CI was wide, and we did not make adjustments for the multivariate analysis.

In conclusion, *VEGF* 936C>T polymorphism and duration of oxygen administration are significant independent risk factors for threshold ROP, even after gestational age and birth weight are controlled for. Our data suggest that infants with the 936 T allele need to be monitored closely for development of threshold ROP so that they may receive effective photocoagulation therapy *VEGF* 936C>T polymorphism is predictive of the development of threshold ROP in Japanese infants of 30 weeks' gestational age or less, but further investigations with larger populations are required to confirm the associations between *VEGF* polymorphism and ROP.

Table 3 Logistic regression analysis of the risk factors for threshold ROP[a]

Variable[b]	Odds ratio	95% CI	*P* value[b]
Duration of oxygen administration	1.05	1.00–1.10	*0.042*
VEGF 936C>T			
CC	1		
CT, TT	5.12	1.25–20.92	*0.023*

Notes: [a]A logistic regression analysis with adjustment for gestational age and birth weight was performed in 61 preterm infants; [b]Variables with $P < 0.05$ in the univariate analysis are indicated in italics.
Abbreviations: CI, confidence interval; ROP, retinopathy of prematurity; VEGF, vascular endothelial growth factor.

Acknowledgment

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Disclosure

The authors report no conflicts of interest in this work.

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