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Original Article

Relationship between the 5'UTR of vascular endothelial growth factor polymorphism and retinopathy of prematurity in Chinese premature newborns

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Abstract: Objective: To investigate the relationship between vascular endothelial growth factor (VEGF) gene polymorphisms and Retinopathy of prematurity (ROP) development in preterm infants of China Han ethnic population. Methods: VEGF gene promoter polymorphisms (-165C/T and -141A/C) were studied in 54 neonates with ROP but not requiring treatment (regressive ROP group), 48 neonates with ROP that requires cryotherapy/photocoagulation (severe ROP group), and in a control group of 62 preterm infants without ROP. Genotyping for VEGF gene promoter was performed by polymerase chain reaction (PCR) and gene sequencing. Results: In this study, -165C/T genotype was found to be associated with severe ROP (P=0.012<0.05), but not with regressive ROP. For the dominant genetic model, subjects carrying the -165T allele had a decreased risk of ROP compared to those non-carrying the -165C allele in both regressive ROP regressive group and severe ROP group, and especially in severe ROP group (in regressive ROP group: OR=2.069, 95% CI=0.986-4.340; in severe ROP group: OR=3.677, 95% CI=1.339-10.099). For the allelic model, comparing the A allele to the C allele, the -165C/T site also showed a decreased risk of ROP in both regressive ROP group and severe ROP group (in regressive ROP group: OR=2.395, 95% CI=1.112-5.157; in severe ROP group: OR=4.258, 95% CI=1.518-11.945). In -141A/C genotypes and alleles were found not to be associated with severe ROP or regressive ROP. Linkage disequilibrium analysis revealed -165C/T and -141A/C were a strong linkage disequilibrium, and haplotype analysis revealed that $T_{-165}C_{-141}$ haplotype was associated with resistance to severe ROP but $C_{-165}A_{-141}$ haplotype was associated with risk to severe ROP. No statistically significant differences were observed in regressive ROP group. Conclusion: VEGF -165C/T SNP is associated with ROP especial in severe ROP. VEGF -141A/C SNP is not associated with ROP. $T_{-165}C_{-141}$ and $C_{-165}A_{-141}$ haplotypes may have a role in severe ROP.

Keywords: Vascular endothelial growth factor, gene polymorphism, retinopathy of prematurity

Introduction

Retinopathy of prematurity (ROP), firstly described by Terry in 1942 [1], is a significant cause of childhood blindness in the world. ROP is a proliferative vascular disorder of the retina that can lead to visual impairment or complete vision loss in premature infants [2]. The incidence of ROP in developing country increase progressively as advances in neonatal intensive care improved the survival rate of premature infants particularly those of extremely low birth weight over past decades. In 16 tertiary class-one hospital of China, the incidence rate of ROP was 11.27% in 2010 [3].

Gestational age, birth weight, male gender, supplemental oxygen, ethnicity, anemia and genetic susceptibility have been considered as risk factors for ROP [4, 5]. Although low birth weight and short gestational age have been consistently shown the most important predisposing factors to be associated with ROP, it is unclear why ROP in a subset of infants with low birth weight progresses to a severe stage despite timely intervention, whereas in other infants with similar clinical characteristics ROP regresses spontaneously. Interestingly, this unpredictability of ROP could be caused by genetic influence. A total of 63 monozygotic and 137 dizygotic twin pairs were identified and ana-

Table 1. Demographic characteristics of ROP infants and controls

	Control group (n=62)	ROP regressive group (n=54)	*P* value[a]	ROP severe group (n=48)	*P* value[b]
Male:Female	35:27	30:24	0.923	27:21	0.983
GA (week)	28.79±1.31	28.46±1.16	0.159	28.31±1.09	0.108
BW (kg)	1.23±0.14	1.21±0.12	0.477	1.20±0.12	0.267
Apgar 1 min	7.95±1.21	7.84±1.26	0.552	7.67±1.17	0.217
Apgar 5 min	8.91±0.99	8.75±0.97	0.384	8.68±0.92	0.215

Notes: GA gestational age, BW birth weight. The t test was performed. a: ROP regressive group vs Control group. b: ROP severe group vs Control group.

lyzed for risk of ROP. Data on gestational age, birth weight, gender, respiratory distress syndrome, retinopathy of prematurity, bronchopulmonary dysplasia, duration of ventilation and supplemental oxygen use, and length of stay were comparable between monozygotic and dizygotic twins. After controlling for known and unknown nongenetic factors, genetic factors accounted for 70.1% of the variance in liability for retinopathy of prematurity [6]. Potential candidate genes that have been evaluated known pathophysiologic mediators involved in the progression of ROP [7]. These have included primarily mediators of angiogenesis in the developing retina, with a particular focus on vascular endothelial growth factor (VEGF) [8], the Norrie disease gene (NDP) [9], Frizzled-4(FZD-4) [10], and Insulin-like growth factor 1 (IGF-1) [11].

Vascular endothelial growth factor (VEGF) gene is located on chromosome 6p21.3 and consists of eight exons exhibiting alternate splicing to form a family of proteins [12]. VEGF is important in physiological growth of retinal vessels, and the presence of it is necessary for normal retinal angiogenesis in utero. Recently, a number of experimental and clinical data suggested that VEGF is a major mediator of angiogenesis with the significant role in the pathogenesis of ROP [13]. The retinal synthesis of VEGF decreases in the first phase of ROP, and the inadequate retinal oxygenation triggers abnormal angiogenesis and acts as a survival factor for newly formed abnormal retinal vessels in the second phase of ROP. The 5'UTR of VEGF polymorphisms have been reported to influence promoter activity and responsiveness. In the past few decades, many polymorphisms have been identified in the 5'UTR of VEGF gene region, and a number of studies have investigated the association of these polymorphisms with ROP [14, 15]. In this study, we examined the two 5'UTR of VEGF polymorphisms [-165C/T (rs79469752) and -141A/C (rs28357093)] with ROP risk in Han preterm infants from North China.

Materials and methods

Study population

Subjects in this study were infants receiving obstetrical care at the Bayi Children's Hospital, which was recruited sequentially between Jan 2011 and Dec 2013. All subjects were unrelated ethnic Han Chinese neonates in Beijing and its surrounding regions. The entry criteria were: (1) preterm birth at 24-32 weeks gestational age, (2) birth weight ≤1500 g, and (3) a need for mechanical ventilation or non-invasive respiratory support (nasal CPAP) during the first 3 days of life. In clinical follow-up, they were divided into 3 groups, preterm infants with no ROP (control group, n=62), preterm infants with ROP but not requiring treatment (regressive group, n=54) and preterm infants with ROP that requires cryotherapy/photocoagulation (severe group, n=48). The ophthalmic examination record was reported according to international ROP classification system [16]. Both groups matched with Baseline demographic data like gestational age (GA), birth weight (BW), 1-minute Apgar scores, 5-minute Apgar scores, and sex.

For each participant, the neonatal peripheral blood sample was collected into tubes containing EDTA immediately after delivery. We stored the whole blood samples at 4°C upon collection. Then, all whole blood samples were stored at -80°C until genomic DNA extraction. This study was performed with the approval of the Ethical Committee of General Hospital of Beijing PLA and was conducted according to

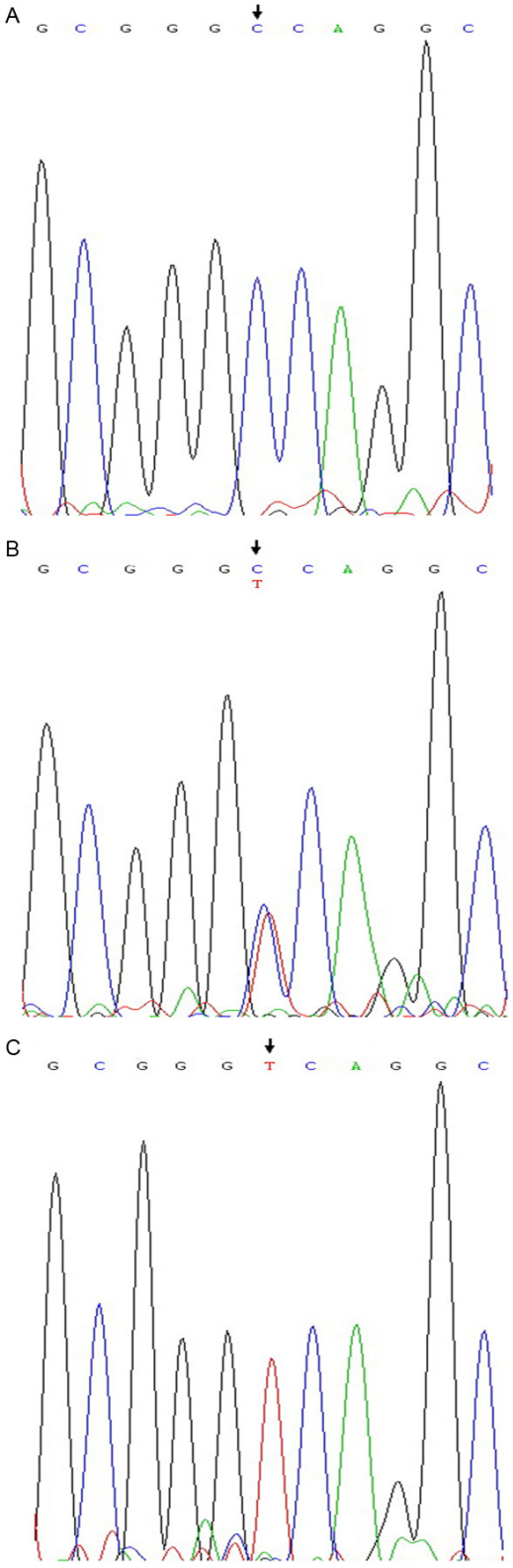

Figure 1. Sequence chromatograms of VEGF -165C/T genotype. A. C/C homozygous genotype. B. C/T heterozygous genotype. C. T/T homozygous genotype. Arrows indicate the positions of altered nucleotides.

the principles expressed in the Helsinki Declaration. At recruitment, written informed consent was obtained from all participants' guardians.

Laboratory tests

DNA extraction and PCR amplification: The genomic DNA of the neonate was purified from total blood using the QIAamp® DNA Blood Mini Kit (QIAGEN, Valencia, USA) according to manufacturer instructions. The primers for the promoter of VEGF genes designed by Primer 5 software. The PCR mixture (total volume of 50 μL) consisted of 100 ng DNA, 1× Pyrobest buffer II, 0.4 μM deoxyribonucleotide triphosphates (dNTPs), 0.4 μM of each primer, and 1.25 U Pyrobest DNA Polymerase (Takara BioTech, Dalian, China). The amplification reaction was carried out the following conditions: an initial melting step of 2 min at 95°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 57°C and 1 min at 72°C with a final elongation of 7 min at 72°C. The PCR products were identified by agarose gel electrophoresis. PCR products were sequenced using the Big Dye Terminator Cycle Ready Reaction Kit V.1.0 (Applied Biosystems, Foster City, CA) on an ABI 3130 automated sequencer (Applied Biosystems, Foster City, CA) and analyzed with Chromas software (http://www.technelysium.com.au/chromas.html).

Statistical analysis

Continuous variables were expressed as the mean ± standard deviation (SD) and compared with student's *t*-test. The fitness to Hardy-Weinberg equilibrium was tested using the random-permutation procedure implemented in the Arlequin package (http://lgb.unige.ch/arlequin). Case-control analyses were done with X^2 statistics or Fisher's exact test. We used both a dominant and an allelic model to assess ROP neonates and control neonates. Odds ratios (ORs) and 95% confidence intervals (CIs) were used to measure the strength of association. An association was considered significant at a *P* value of <0.05, and all statistical tests were two-sided. These analyses were performed using SPSS software (version 15.0; SPSS Inc.). The associations between the VEGF haplotypes and ROP risk were analyzed. The D' value and r^2 for the studied two SNPs were calculated with the SHEsis software [17].

Table 2. Distribution of the -165C/T genotypes in ROP infants and controls

Groups	Cases (n)	Genotypes		
		TT	CT	CC
Control group	62	2 (3.2%)	17 (27.4%)	43 (69.4%)
ROP regressive group[a]	54	0 (0.0%)	8 (14.8%)	46 (85.2%)
ROP severe group[b]	48	0 (0.0%)	4 (8.3%)	44 (91.7%)

Notes: a: ROP regressive group vs Control group, *P*=0.078, Fisher's exact test. b: ROP severe group vs Control group, *P*=0.012, Fisher's exact test.

Table 3. Relative risk estimate on the basis of the distribution of the -165C/T genotypes and alleles in ROP infants and controls

	Control group (%) (n=62)	ROP regressive group (%) (n=54)	*P* value[a]	Crude OR (95% CI)	ROP severe group (%) (n=54)	*P* value[b]	Crude OR (95% CI)
Genotypes							
Carrying T (TT+CT)	19 (30.6%)	8 (14.8%)	0.044	0.394 (0.156-0.992)	4 (8.3%)	0.004	0.206 (0.065-0.654)
Not-carrying T (CC)	43 (69.4%)	46 (85.2%)			44 (91.7%)		
Alleles							
T allele	22 (17.7%)	8 (7.4%)	0.019	0.371 (0.158-0.872)	4 (4.2%)	0.002	0.202 (0.067-0.607)
C allele	102 (82.3%)	100 (92.6%)			92 (95.8%)		

Notes: OR, odds ratio; 95% CI, 95% confidence interval. The chi-square test was performed. a: ROP regressive group vs Control group. b: ROP severe group vs Control group.

Results

Demographic characteristics

All subjects were of Chinese Han descent. The baseline characteristics of the study population, including 54 regressive ROP infants, 48 severe ROP infants, and 62 control infants. There were no significant difference among ROP patients and controls in term of sex, gestational age, birth weight, Apgar 1 min, and Apgar 5 min (**Table 1**).

Distribution of genotypic and allelic frequencies

-165C/T (rs79469752): In -165C/T polymorphism, the frequencies of C/C, C/T and T/T genotypes (**Figure 1**) among ROP severe group neonates varied significantly from those among control group (**Table 2**, P=0.012<0.05), but no difference between ROP regressive group and control group (**Table 2**, P=0.078>0.05).

To investigate genotype effects, we used both a dominant model (frequency of the T/T plus C/T genotypes vs frequency of the C/C genotype) and allelic model (frequency of the T allele vs frequency of the C allele). For the dominant genetic model, subjects carrying the -165T allele (T/T+C/T genotypes) had a decreased risk of ROP compared to those non-carrying the -165T allele (C/C genotype) in both ROP regressive group and ROP severe group, and especially in ROP severe group (**Table 3**, in ROP regressive group: OR=0.394, 95% CI=0.156-0.992, *P*=0.044<0.05; in ROP severe group: OR=0.206, 95% CI=0.065-0.654, *P*=0.004<0.01). For the allelic model, comparing the C allele to the T allele, the -165C/T site also showed a decreased risk of ROP in both ROP regressive group and ROP severe group (**Table 3**, in ROP regressive group: OR=0.371, 95% CI=0.158-0.872, *P*=0.019<0.05; in ROP severe group: OR=0.202, 95% CI=0.067-0.607, *P*=0.002<0.01).

-141A/C (rs28357093): Although the frequencies of the C/C, A/C, and A/A genotypes (**Figure 2**) among ROP regressive group and ROP severe group were different from the control group in -141A/C polymorphism, there were still no significant difference between them (**Table 4**, *P*=0.570>0.05, *P*=0.440>0.05, respectively). And it is also no significant differences between ROP regressive group and controls, as well as ROP severe group and control group in the allelic frequencies (**Table 5**, *P*=0.164>0.05, *P*=0.067>0.05, respectively).

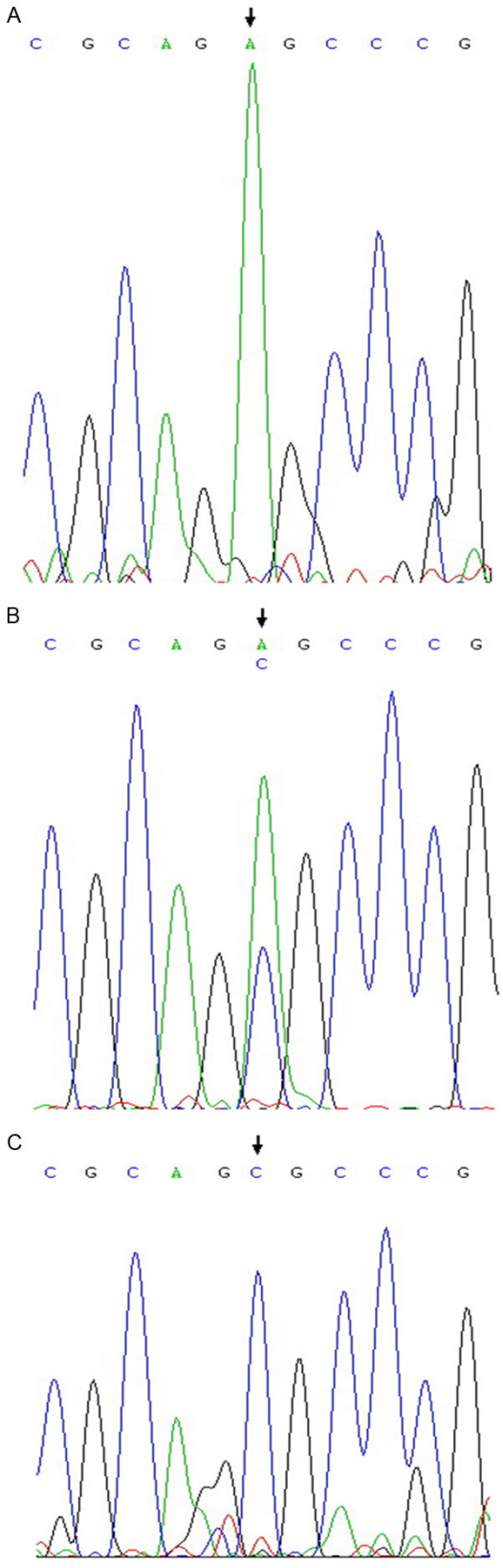

Figure 2. Sequence chromatograms of VEGF -141A/C genotype. A. A/A homozygous genotype. B. A/C heterozygous genotype. C. C/C homozygous genotype. Arrows indicate the positions of altered nucleotides.

VEGF haplotypes and ROP risk: The associations between the VEGF haplotype and ROP risk were analyzed in this study. The polymorphisms at two loci were in strong LD (Linkage disequilibrium) (**Figure 3**, D'=1.0). The estimated haplotype frequencies of the VEGF polymorphisms are shown in **Table 6**. In ROP severe group, T-165C-141 haplotype was associated with resistance to severe ROP (**Table 6**, OR=0.274, 95% CI=0.089-0.842, *P*=0.016<0.05). Meanwhile, C-165A-141 haplotype was associated with risk to severe ROP (**Table 6**, OR=2.592, 95% CI=1.053-6.384, *P*=0.033<0.05). No statistically significant differences were observed in ROP regressive group.

Discussion

VEGF has been identified as the key factor driving the development and growth of blood vessels. Several receptors and growth factors work together to new vessel growth. Of these growth factors, VEGF is essential for proper physiological angiogenesis [18]. Oxygen tension and VEGF play a key role in retinal blood vessel growth. Much of the evidence for the role of VEGF in ROP has been acquired through studies in animal models of oxygen-induced retinopathy (OIR) [19]. Recent evidence have supported an important role for genetics in determining risk for ROP [20]. VEGF gene contains a very long (1038 bp) 5'UTR which is characterized by a high G+C content (83%) upstream to the translation initiation site. The 5'UTR of VEGF has an important role in the expression of VEGF, which potentiates expression by enhancing either transcription or translation [21]. Many researchers believe that single nucleotide polymorphisms (SNPs) in 5'UTR of VEGF are related to the changes of protein expression during ROP and hence the susceptibility to develop ROP [22]. Kwinta et al. evaluated the correlation between VEGF -460T/C and 405G/C polymorphisms with risk of ROP. The infants were also divided into 3 groups; preterm infants with no ROP, infants with ROP but not requires treatment and preterm infants with ROP that requires laser or cryotherapy. They found that the polymorphic allele -460T/C was significantly more frequent in ROP newborns that required treatment as compared to the no ROP group. VEGF serum concentrations in the patients ascribed to different groups depending on the 405G/C or -460T/C polymorphisms were similar [14]. Cooke et al. found that carriage of the

Table 4. Distribution of the -141A/C genotypes in ROP infants and controls

Groups	Cases (n)	Genotypes		
		CC	AC	AA
Control group	62	2 (3.2%)	13 (21.0%)	47 (75.8%)
ROP regressive group[a]	54	0 (0.0%)	10 (18.5%)	44 (81.5%)
ROP severe group[b]	48	0 (0.0%)	7 (14.6%)	41 (85.4%)

Notes: a: ROP regressive group vs Control group, *P*=0.570, Fisher's exact test. b: ROP severe group vs Control group, *P*=0.440, Fisher's exact test.

Table 5. Relative risk estimate on the basis of the distribution of the -141A/C genotypes and alleles in ROP infants and controls

	Control group (%) (n=62)	ROP regressive group (%) (n=54)	*P* value[a]	ROP severe group (%) (n=54)	*P* value[b]
C allele	19 (15.3%)	10 (9.3%)	0.164	7 (7.3%)	0.067
A allele	105 (84.7%)	98 (90.7%)		89 (92.7%)	

NOtes: The chi-square test was performed. a: ROP regressive group vs Control group. b: ROP severe group vs Control group.

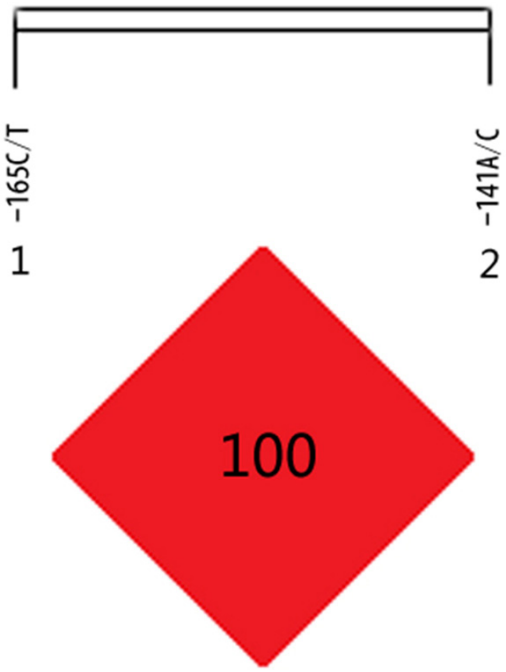

Figure 3. The haplotype analysis of two polymorphisms. The linkage disequilibrium was analyzed using SHEsis software (http://analysis.bio-x.cn/myAnalysis.php). The color scheme was: white (D=0), pink (0<D<1) and red (D=1).

VEGF -634G allele in the VEGF gene was considered as risk factor for ROP [15]. Vailati et al. revealed that the C allele of the -634G/C polymorphism is associated with higher VEGFA gene expression in the human retina [23]. But some research found 5'UTR of VEGF polymorphisms were not associated with ROP risk at allelic, co-dominant, dominant and recessive models [24, 25]. Therefore, the different results across studies may result from small sample size and/or genotyping technique. Since the studies included were very limited, it is necessary to validate the association between 5'UTR of VEGF polymorphism and ROP risk in future studies.

In genetics, LD is defined as the nonrandom association, in a given population, between alleles of 2 or more loci. LD between SNPs in the regulatory region of a gene can be used as a method for identifying associations of certain haplotype with sickness or disease in a population. This can be achieved when levels of LD between SNPs within haplotypes are seen to change substantially in a disease or sickness population group when compared to the normal baseline population. In such cases, the relationship between LD, SNPs, and TFBS can be used to identify potential gene regulatory TF (transcription factor) binding changes, which could result in disease or sickness [26]. The LD VEGF SNP pair (-141A/C rs28357093 and -165C/T rs79469752) was found in the past research, the SNP (rs28357093) is located in the TF zinc finger protein (ZFX) response element where the protein is a member of krueppel C2H2-type zinc finger protein family and probably acts as a transcriptional activator, while the SNP (rs79469752) is located in the TFs AP2α, NFIC, PAX5 and ZFX REs. The VEGF SNP (rs28357093) has a common allele (A) that occurs in a less conserved (8 and 19%) nucleotide REs location of the ZFX TFs while the other SNP (rs79469752) has a common allele [G (+strand) or C (-strand)] that occurs in highly conserved (97%) nucleotide RE locations for the AP2α and NFIC TFs, respectively [27]. This is consistent with our findings.

In conclusion, we found that VEGF -165C/T polymorphism was associated with the risk of ROP especial in severe ROP in a Chinese popu-

Table 6. The estimated haplotype frequencies of the VEGF polymorphisms between ROP and controls

Haplotypes	Control group (%)	ROP regressive group (%)[a]	*P* value[b]	Crude OR (95% CI)[b]	ROP severe group (%)[c]	*P* value[d]	Crude OR (95% CI)[d]
$C_{-165}A_{-141}$	103 (83.1%)	98 (90.7%)	0.087	1.998 (0.896-4.457)	89 (92.7%)	0.033	2.592 (1.053-6.384)
$C_{-165}C_{-141}$	4 (3.2%)	2 (1.9%)	0.511	0.566 (0.102-3.153)	3 (3.1%)	0.966	0.968 (0.214-4.430)
$T_{-165}C_{-141}$	17 (13.7%)	8 (7.4%)	0.122	0.504 (0.208-1.218)	4 (4.2%)	0.016	0.274 (0.089-0.842)

Notes: Order of polymorphisms: -165C/T, -141A/C (rs28357093). a: Global Fisher's is 2.289, df=2, *P*=0.239 (frequency <0.03 in both control & case has been dropped). b: ROP regressive group vs Control group. c: Global Fisher's is 5.910, df=2, *P*=0.050 (frequency <0.03 in both control & case has been dropped). d: ROP severe group vs Control group.

lation but not in -141A/C polymorphism. T-165C-141 and C-165A-141 haplotypes may have a role in severe ROP. If confirmed by larger sample sizes studies, our findings of genetic factors contributing to the pathogenesis of ROP may have implications for the screening and treatment of this disorder.

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Disclosure of conflict of interest

None.

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