

Lack of association of the VEGF gene promoter (–634 G→C and –460 C→T) polymorphism and the risk of advanced retinopathy of prematurity

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Abstract

Background Recently, it has been reported that genetic polymorphism (–634 G→C and –460 C→T) in the promoter region of the vascular endothelial growth factor (VEGF) gene can influence the progression of retinopathy of prematurity (ROP). In order to evaluate its general applicability as a screening procedure in clinics and to replicate the above result, we have undertaken the following study.

Methods We have analyzed a cohort of 61 patients with advanced ROP (stage 4 and 5) along with 61 normal controls for the VEGF gene promoter polymorphism. For this purpose, blood samples were collected from each patient and leukocyte DNA was isolated. Genomic DNA was amplified by the polymerase chain reaction (PCR) method with two pairs of primers designed to amplify separately the promoter region (containing –634 G→C and –460 C→T polymorphism) of the VEGF gene. The amplified product was subjected to restriction enzyme digestion. The base change in the restriction site was further confirmed by a BigDye terminator cycle sequencing of the amplified product.

Results Our analysis suggests that there is no significant difference in allelic frequency of the VEGF gene between normal subjects and patients with advanced ROP in our cohort.

Conclusion Our results do not support the association of the VEGF gene promoter polymorphism and the risk of advanced ROP. In order to adapt this method for the

identification of high-risk infants in clinics in the future, a large-scale study involving a mixed ethnically diverse population is much needed.

Keywords Retinopathy of prematurity · Polymorphism · Promoter · Genetic testing

Introduction

Retinopathy of prematurity (ROP) is a vascular vitreoretinopathy and affects infants with a short gestational age and low birth weight. The condition can cause abnormal vessel growth in the vitreous that can lead to hemorrhage, fibrovascular tissue change, and retinal detachment that ultimately results in blindness. Morphologically the condition is similar to familial exudative vitreoretinopathy (FEVR) which occurs in full-term infants. Although some degree of abnormal vascularization is found in all low birth weight infants, the formation of scar tissue and subsequent retinal detachment occurs only in a small portion of these infants. Despite the recent advances in diagnosis and treatment, ROP remains a leading cause of blindness in children in developed countries around the world [1].

The pathogenesis of advanced ROP (stage 4 and 5) and its etiology is currently not known. In the past, many causative factors such as length of time exposed to supplemental oxygen, excessive light exposure, and hypoxia have been suggested, but evidence for these in recent years is not compelling [2]. Recently it has been suggested that vascular endothelial growth factor (VEGF) may play a causative role in the development of ROP [3], and it is known that VEGF is important in physiological growth of retinal vessels. However, VEGF is also found to be present in human subjects with nonproliferative diabetic retinopathy [4].

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Although low birth weight and short gestational age have been consistently shown 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 [5]. However, at present there is no reliable method of predicting which premature infant will develop retinal detachment and blindness. It has been recently reported [6, 7] that genetic polymorphism in the VEGF gene promoter may influence the progression of ROP. In order to validate its general applicability as well as its reproducibility, we have undertaken the following study in a cohort of patients.

Materials and methods

Patients and controls

We have selected two groups of individuals (patients and controls). The patient group consisted of 61 premature babies with advanced ROP, including three pairs of dizygotic and two pairs of monozygotic twins, and in the control group there were 61 unrelated randomly selected full-term normal adult subjects. In the patient set there were 63% males and 37% females (Caucasians). The control population of adults also consisted of Caucasians. All patients had clinical findings typical of advanced ROP (stage 4 and 5) including time of onset and tempo whereas normal patients showed no sign of ROP. Patients with a family history of FEVR, Norrie's disease, bleeding disorder, or ocular findings considered atypical for ROP were excluded from the study. The birth weight and gestational age of premature babies ranged from 600 to 1,300 g (mean: 882 g) and 23 to 30 weeks (mean: 26 weeks), respectively. All patients (parents) were informed of the purpose of the study and it was approved by the Institutional Review Board of Oakland University.

Sample collection and polymerase chain reaction (PCR) amplification

Blood samples were collected from each patient and leukocyte DNA was isolated. Genomic DNA was amplified by the PCR method with two pairs of primers designed to amplify separately the promoter region (containing -634 G→C and -460 C→T polymorphism) of the VEGF gene [8]. Briefly, the primers were annealed at 55–60°C for 1 min and extended at 72°C for 2 min with 32 cycles using the FailSafe PCR buffer G system supplied by Epicenter (Madison, WI, USA).

Restriction analysis and sequencing

The amplified product was subjected to restriction enzyme digestion with either 2–5 units of *BsmF1* enzyme (for -634 G→C) or 2–5 units of *BstU1* (for -460 C→T) at 67°C for 3 h as previously described [8]. The digested DNA fragments were analyzed on a 12% polyacrylamide gel with 1× Tris-Borate-EDTA buffer followed by ethidium bromide staining. The base change in the restriction site was further confirmed by a BigDye terminator cycle sequencing of the amplified product according to the procedure provided by the manufacturer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The significance of allele frequency differences was tested by using a χ^2 test with Yates' correction and confirmed by Fisher's exact test. Although study size estimations were difficult to make because of limited information on the frequencies in the preterm population, the power of Fisher's exact test shows that if the frequency of alleles in the ROP group is increased by 25%, we would have 98% (-634 G→C) and 94% (-460 T→C) chance of detecting this difference with 61 infants in each group.

Results

In order to further understand the relationship between the promoter polymorphism (-634 G→C and -460 C→T) of the VEGF gene and the progression of ROP, we have analyzed 61 advanced ROP patients consisting of three pairs of dizygotic and two pairs of monozygotic twins and 61 normal patients with no sign of ROP. The allelic frequency is presented in Table 1. The restriction digestion pattern (for -634 G→C) suggests that all three genotypes (G/G, G/C, and C/C) were present in patients with advanced ROP in a proportion of 44, 45, and 11%,

Table 1 Allele frequencies of VEGF gene promoter polymorphisms in ROP and normal patients

Sequence	Position	Allele frequency	
		ROP	Normal
G→C	+405 (or -634)	G 0.664	0.672
		C 0.336	0.328
C→T	-460	C 0.418	0.434
		T 0.582	0.566
Birth weight (g)		600–1,300	2,430–3,960
Gestational age (weeks)		23–30	34–40

respectively. Similarly, in normal controls the proportions of G/G, G/C, and C/C genotypes were 46, 43, and 11%, respectively. These three genotypes were further confirmed by selecting three different genotypic individuals and by DNA sequencing of the freshly amplified product. A similar result is obtained with -460 C→T polymorphism. Although admittedly it is a small-scale study, in this cohort of individuals it appears that the C/C genotype is rare, which is consistent with the literature [8]. However, there is no significant statistical difference between ROP patients and the normal controls in the genotypic distribution of promoter polymorphism of the VEGF gene. Both the χ^2 test with Yates' correction and Fisher's exact test gave the same *p* value of 0.8922 (-634 G→C) and 0.897 (-460 C→T). Hence we conclude that there is no association between the progression of ROP and the VEGF gene polymorphism. The 95% confidence intervals for the odds ratio are 0.53, 1.64 (-634 G→C) and 0.62, 1.83 (-460 C→T), which supports the conclusion.

Discussion

Despite advances in our understanding and management of ROP, the disorder is still considered to be a major sight-threatening disease among children. It is unclear at present why ROP in a subset of infants with low birth weight progresses to severe disease despite timely intervention, whereas in other infants with similar clinical characteristics ROP regresses spontaneously. The ability to identify these high-risk infants would certainly permit more aggressive monitoring and surgical intervention of these patients at the early stages of the disease process. Because VEGF is important in physiological growth of retinal vessels and the VEGF gene promoter polymorphism has been reported to produce varying levels of VEGF protein [8], we predicted that the G/G genotype might be more prevalent in ROP than controls. Moreover, an increased level of VEGF has also been reported to be present in stage 4 but not in stage 5 ROP infants. Unfortunately, however, our results do not support the relationship between advanced ROP and the genotypic distribution of the VEGF gene promoter polymorphism. The allelic frequency obtained from this study is very similar to that reported for the normal healthy population [8]. These results are in agreement with the other report [9] but in direct contradiction to the previously published data [6, 7] in which a significant difference in the G/G genotype has been reported. However, because of the

small number of patients enrolled in this study, we cannot directly compare our results with other reports [6, 7] that found an association. In addition, we have not chosen our control group consisting of infants of similar gestational age and birth weight but without ROP because they did well and were not referred in. However, the present study is similar to the other reports with respect to the ethnic background, birth weight, and gestational age of patients but not disease status. Although we cannot draw a strong conclusion, the power calculation in the present report supports our conclusion. In the future, a large-scale multicenter study involving patients of mixed ethnic backgrounds is needed before this method of screening of premature babies to predict the progression of ROP to a severe stage can be adapted into clinical practice.

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