European Journal of Ophthalmology / Vol. 18 no. 1, 2008 / pp. 21-26

Evaluation of transforming growth factor and vascular endothelial growth factor polymorphisms in Taiwan Chinese patients with pterygium

D.-T. BAU[1,2], C.-C. CHIANG[3,4], Y.-Y. TSAI[3], C.-C. LEE[1], Y. TSAI[2], C.-C. LIN[5], C.-H. TSAI[6], F.-J. TSAI[1,2,6]

[1]Department of Medical Research, China Medical University Hospital
[2]Graduate Institute of Chinese Medical Science, China Medical University
[3]Department of Ophthalmology, China Medical University Hospital
[4]Institute of Medical and Molecular Toxicology, Chung-Shan Medical University, Taichung
[5]Department of Family Medicine, China Medical University Hospital
[6]Department of Biotechnology and Bioinformatics, Asia University, Taichung, Taiwan - ROC

PURPOSE. *Pterygium is an invasive and highly vascularized growth, thought to arise from activated and proliferating limbal epithelial stem cells. Epidemiologic studies have found the increase of active angiogenic and epithelial growth factors in pterygia, and implicated that these molecules could be involved directly or indirectly in the pathogenesis of pterygia as causative factors. The aim of this study was to investigate the association of polymorphisms of transforming growth factor (TGF) and vascular endothelial growth factor (VEGF) with pterygium.*

METHODS. *A total of 133 pterygium patients and 105 volunteers without pterygium were enrolled in this study. Polymerase chain reaction based restriction fragment length polymorphism analysis was used to resolve the TGF-β1-509 and VEGF-460 genotypes.*

RESULTS. *There was no significant difference in the allele frequency or genotype of TGF-β1-509 or VEGF-460 between total pterygium and the control group. No interaction between TGF-β1-509 and VEGF-460 was found either.*

CONCLUSIONS. *These results indicate that TGF-β1-509 and VEGF-460 polymorphisms were not highly associated with the pathology of pterygium. However, it may still be worthwhile to continue to search for angiogenic gene polymorphisms in order to predict the development of pterygium. (Eur J Ophthalmol 2008; 18: 21-6)*

KEY WORDS. *Pterygium, Transforming growth factor, Vascular endothelial growth factor, Polymorphism*

Accepted: August 2, 2007

INTRODUCTION

The pterygium has been described as an abnormal mass of fibrovascular tissue that extends from the conjunctiva onto the cornea (1). The growth of pterygium has been closely associated with the presence of vascular growth factors (1-3), but the precise etiology remains to be determined. A variety of growth factors have been reported to occur in pterygium tissue (3-5), and also immunologic phenomena might play a role (6). Excised pterygia contained lymphocytic infiltration, mainly T cells, which have been reported to produce various angiogenic factors (1). Pterygium fibroblasts express potent angiogenic factors such as basic fibroblast

 1120-6721/021-06$15.00/0

growth factor (b-FGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and tumor necrosis factor α (TNF-α) (1, 2).

TGF-β is a multifunctional cytokine involved in pro- and anti-inflammatory pathways and is expressed in several cell types. TGF-β has three isoforms, TGF-β1, TGF-β2, and TGF-β3, of which TGF-β1 is the predominant isoform found in blood. The *TGF-β1* gene is expressed in endothelial, hematopoietic, and connective-tissue (7). The functional impact of the *TGF-β1* gene polymorphism as well as the TGF-β1 level might contribute to pathogenesis of inflammatory diseases.

VEGF has been shown to play a role in normal and pathologic angiogenesis, including connective tissue growth factor secretion (8) and corneal neovascularization (9). The role of VEGF in angiogenesis or pterygium is under investigation. There is increasing evidence for an expression of VEGF in various vascular tissues in healthy human adults that normally do not show angiogenesis, such as healthy corneas (10). It is reported that pterygial tissues contain increased VEGF levels (11).

The role of TGF-β1 and VEGF polymorphisms in the development of pterygium remains unclear. In this study, using the PCR-RFLP polymorphism method, we aimed at evaluating whether TGF-β1 or VEGF polymorphisms were useful markers for predicting susceptibility to pterygium, and whether the two genes had any interaction on pterygium risk. This is the first survey in this respect.

PATIENTS AND METHODS

Patients

A total of 133 pterygium patients (70 male and 63 female) were enrolled in the study with ages ranging from 35 to 90 years (mean, 65.3 years). A total of 105 volunteers aged 50 years or more without pterygium were enrolled as the control group. There were 65 men and 40 women in the control group (ages ranged from 50 to 83 years with an average of 64.5 years). Both pterygium and control groups were outpatients in our eye department and had similar general ocular disease, e.g., senile cataract, dry eye, presbyopia. This study was carried out with approval from the Human Study Committee of the China Medical University Hospital. Informed consent was obtained from all individuals who participated in this study.

Genotyping methods

Genomic DNA was prepared from peripheral blood by use of a DNA Extractor WB kit (Wako, Japan). Polymerase chain reactions (PCR) were carried out in a total volume of 25 µL containing genomic DNA, 2 to 6 pmol of each primer, 1X Taq polymerase buffer (1.5 mM $MgCl_2$), and 0.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA, USA). The primers for *TGF-β1*-509 were forward 5'-GGA GAG CAA TTC TTA CAG GTG-3' and reverse 5'-TAG GAG AAG GAG GGT CTG TC-3'. The primers for *VEGF*-460 were forward 5'-TGT GCG TGT GGG GTT GAG CG-3' and reverse 5'-TAC GTG CGG ACA GGG CCT GA-3'. PCR amplification was performed in a programmable thermal cycle GeneAmp PCR System 9700 (Perkin Elmer). Cycling conditions for PCR were set as follows: one cycle at 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s (*TGF-β1*-509) or 60 s (*VEGF*-460), and one final cycle for extension at 72 °C for 10 min. The PCR products were further digested with *DdeI* (*TGF-β1*-509) and *BstUI* (*VEGF*-460), and then loaded into a 3% agarose gel containing ethidium bromide for further electrophoresis and analyses.

Statistical analyses

To ensure that the controls used were representative of the general population and to exclude the possibility of genotyping error, deviation of the genotype frequencies of *TGF-β1*-509 or *VEGF*-460 SNP in the control subjects from those expected under the Hardy-Weinberg equilibrium was assessed using the goodness-of-fit test. On the basis of the observed frequencies of the selected polymorphisms, we estimated global haplotype frequencies by using the expectation-maximization algorithm (12). Pearson χ^2 test or Fisher exact test (when the expected number in any cell was less than five) was used to compare the distribution of the *TGF-β1*-509 or *VEGF*-460 genotypes between cases and controls. Data were recognized as significant when the statistical p value is less than 0.05.

RESULTS

There were no significant differences between control and pterygium patient groups in age and sex. The frequency of the alleles for the *TGF-β1*-509 and *VEGF*-460 between pterygium patient and control groups is shown in Table I.

The distribution of the *TGF-β1*-509 and *VEGF*-460 genotypes in controls was in Hardy-Weinberg equilibrium. Genotypes containing the C allele at *TGF-β1*-509 in pterygium patient group were of the same proportion compared to the control group (42.1% and 44.8%, respectively), which means that the T or C allele at *TGF-β1*-509 was not significantly associated with pterygium risk (p>0.05). Similarly, the T or C allele at *VEGF*-460 was not differently distributed in the pterygium patients and control groups (p>0.05).

The frequency of the genotypes of *TGF-β1*-509 and *VEGF*-460 polymorphisms in the pterygium patient and control groups is shown in Table II. The crude odds ratios (ORs) for heterozygotes, homozygotes, a combination of hetero- and homozygotes for *TGF-β1*-509 C-allele were 1.08, 0.94, and 0.98 (95% CI = 0.60–1.94, 0.35–1.60, 0.57–1.71), respectively, using *TGF-β1*-509 T-allele homozygotes as the reference group. Thus, neither hetero- nor homozygous of *TGF-β1*-509 C-allele seemed to be high risk genotypes for pterygium (Tab. II). Similarly in the case of *VEGF*, the ORs for heterozygotes, homozygotes, a combination of hetero- and homozygotes for *VEGF*-460 C-allele were 1.02, 2.89, and 1.09 (95% CI = 0.61–1.72, 0.58–14.51, 0.65–1.82), respectively, using T-allele homozygotes as the reference group. The data showed that neither hetero- nor homozygotes of C-allele of *VEGF*-460 seemed to be risk or protective genotypes, taking homozygous T-allele as reference, for pterygium (Tab. II).

When investigating the gene–gene interaction, there was still no significant difference (x=4.15, df=5, p>0.05) between pterygium patient and control groups (Tab. III).

TABLE I - ALLELE FREQUENCIES FOR TGF-509 AND VEGF-460 POLYMORPHISMS IN THE PTERYGIUM AND CONTROL GROUPS

Allele	Cases (%), n=266	Controls (%), n=210	p value
TGF-509			0.626
Allele T	154 (57.9)	116 (55.2)	
Allele C	112 (42.1)	94 (44.8)	
VEGF-460			0.573
Allele T	189 (71.1)	155 (73.8)	
Allele C	77 (28.9)	55 (26.2)	

TABLE II - ASSOCIATION OF TGF-509 AND VEGF-460 POLYMORPHISMS AND PTERYGIUM RISK

Genotype	Cases (%), n=133	Controls (%), n=105	Crude OR (95% CI)
TGF-509			
T/T	41 (30.8)	32 (30.5)	1.00
T/C	72 (54.1)	52 (49.5)	1.08 (0.60–1.94)
C/C	20 (15.1)	21 (20.0)	0.94 (0.35–1.60)
VEGF-460			
T/T	63 (47.4)	52 (49.5)	1.00
T/C	63 (47.4)	51 (48.6)	1.02 (0.61–1.72)
C/C	7 (5.2)	2 (1.9)	2.89 (0.58–14.51)

TABLE III - DISTRIBUTION OF COMBINATION OF TGF-509 AND VEGF-460 POLYMORPHISMS IN THE PTERYGIUM AND CONTROL GROUPS

Genotype	Cases (%)	Controls (%)	p value
TGF/VEGF			>0.05
TT/TT	14 (10.5)	17 (16.2)	
TT/TC or CC	27 (20.3)	15 (14.3)	
TC/TT	39 (29.3)	26 (24.8)	
TC/TC or CC	33 (24.8)	26 (24.8)	
CC/TT	10 (7.5)	9 (8.6)	
CC/TC or CC	10 (7.5)	12 (11.4)	

DISCUSSION

In this paper, TGF-*β1*, which is thought to play an important role in bone turnover (13), was firstly investigated its role in pterygium development. Pterygia represent an invasive and highly vascularized growth, thought to arise from activated and proliferating limbal epithelial stem cells (14). Although the pathogenesis is poorly understood, the formation and progression of pterygia are known to depend on neovascularization (5, 15). In this context, the homeostasis between angiogenic stimulators and angiogenic inhibitors may control the overall angiogenesis (16). Very likely, the development of pterygia is the result of an unbalanced angiogenic stimulator-to-inhibitor ratio. In this study, the two proteins important to the angiogenic effects of pterygium development, TGF-β1 and VEGF, were investigated.

It has been reported that TGF-β can enhance epithelial-to-mesenchymal transition, downregulate cellular adhesion molecules, augment expression of metalloproteases, increase tumor cell motility and tumor angiogenesis, and cause local and systemic immunosuppression, leading tumors to an invasive and metastatic status (17-19). The T allele at -509 of *TGF-β1* was associated with an elevated TGF-β1 level (20). The *TGF-β1* gene may be a candidate locus for susceptibility to numerous diseases. Recently, *TGF-β1* gene polymorphisms have been reported to be associated with increased risk of several human diseases, including endometriosis (21), breast cancer (22), cervical carcinoma (23), systemic sclerosis (24), rheumatoid arthritis (25), and diabetic retinopathy (26). In contrast, some literature reported that there was no obvious association of the *TGF-β1* gene polymorphisms with other diseases, such as melanoma (27), Alzheimer disease (28), tuberculosis (29), and periodontitis (30). In this study, there was no difference between individuals with pterygium and normal control groups in their *TGF-β1*-509 allele frequencies and genotypes (Tabs. I and II).

VEGF, a homodimeric, heavily glycosylated, 46–48 kDa protein, is an important mediator of angiogenesis during embryogenesis, tissue remodeling, and wound healing, as well as in malignant and other inflammatory diseases of the adult (31). In the eye, VEGF protein has been detected in normal vascularized tissues: the conjunctiva, iris, retina, and the choroid–retinal pigment epithelium complex (32). Recently, mounting evidence has shown that VEGF is notably increased in pterygia, and the suggestion is that VEGF could be involved directly or indirectly in the pathogenesis of pterygia (5, 11). A study in type 1 and 2 diabetics demonstrated an association of the -460 C-allele with retinopathy (33). A haplotype analysis of the polymorphic human *VEGF* promoter has shown that one or both of +405 and –460 polymorphisms is of functional importance (34). In this study, although there was no significant difference in the *VEGF* T-460C allele frequency or genotype between pterygium patient and control groups (Tabs. I and II), we cannot exclude the involvement of *VEGF* polymorphisms in pterygium. A nonsignificant 2.89-fold pterygium risk was associated with –460 CC homozygotes (Tab. II).

There was no obvious association of *TGF-β1*-509 or *VEGF*-460 with pterygium in this study; however, not only the relationship between other *TGF-β1* or *VEGF* polymorphisms and pterygium can be analyzed, but also the association of the polymorphisms in other genes can be investigated. Furthermore, we can estimate the gene– environment interaction in addition to gene–gene interaction with a larger population. Since individual ultraviolet exposure and other environmental factors known to be related to pterygium are difficult to be evaluated by questionnaires only, some functional assays are needed so that a comprehensive understanding of the development of pterygium can be accessed. For instance, it is found that there is an association between *GSTM1* null and early onset of pterygium (35), and a GSTM1-antioxidant capacity assay model can provide more direct evidence for a causal relationship between *GSTM* and early onset pterygium.

In conclusion, pterygium seemed not to be associated with *TGF-β1*-509 or *VEGF*-460, and searching for other biomarkers will be useful for screening prediction and gene therapy of pterygium.

ACKNOWLEDGEMENTS

The authors thank all the people enrolled and who donated their blood, and Y.A. Hsu and S.Y. Lin for their technical assistance.

This study was supported by the research grant from China Medical University Hospital DMR-95-097.

Proprietary interest: None.

Reprint requests to:
Fuu-Jen Tsai, MD, PhD or
Yi-Yu Tsai, Md, PhD
Department of Pediatrics
Department of Ophthalmology
Medical Genetics and Medical Research
China Medical University Hospital
2 Yuh-Der Road
Taichung, 404 Taiwan, ROC
yiyutsai@seed.net.tw

REFERENCES

1. Hill JC, Maske R. Pathogenesis of pterygium. Eye 1989; 3(Pt 2): 218-26.
2. Kria L, Ohira A, Amemiya T. Immunohistochemical localization of basic fibroblast growth factor, platelet derived growth factor, transforming growth factor-beta and tumor necrosis factor-alpha in the pterygium. Acta Histochem 1996; 98: 195-201.
3. Kria L, Ohira A, Amemiya T. Growth factors in cultured pterygium fibroblasts: immunohistochemical and ELISA analysis. Graefes Arch Clin Exp Ophthalmol 1998; 236: 702-8.
4. Cornand G. Pterygium. Clinical course and treatment. Rev Int Trach Pathol Ocul Trop Subtrop Sante Publique 1989; 66: 31-108.
5. Lee DH, Cho HJ, Kim JT, Choi JS, Joo CK. Expression of vascular endothelial growth factor and inducible nitric oxide synthase in pterygia. Cornea 2001; 20: 738-42.
6. Pinkerton OD, Hokama Y, Shigemura LA. Immunologic basis for the pathogenesis of pterygium. Am J Ophthalmol 1984; 98: 225-8.
7. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. N Engl J Med 2000; 342: 1350-8.
8. Suzuma K, Naruse K, Suzuma I, et al. Vascular endothelial growth factor induces expression of connective tissue growth factor via KDR, Flt1, and phosphatidylinositol 3-kinase-akt-dependent pathways in retinal vascular cells. J Biol Chem 2000; 275: 40725-31.
9. Amano S, Rohan R, Kuroki M, Tolentino M, Adamis AP. Requirement for vascular endothelial growth factor in wound- and inflammation-related corneal neovascularization. Invest Ophthalmol Vis Sci 1998; 39: 18-22.
10. van Setten GB. Vascular endothelial growth factor (VEGF) in normal human corneal epithelium: detection and physiological importance. Acta Ophthalmol Scand 1997; 75: 649-52.
11. Jin J, Guan M, Sima J, et al. Decreased pigment epithelium-derived factor and increased vascular endothelial growth factor levels in pterygia. Cornea 2003; 22: 473-7.
12. Excoffier L, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. Mol Biol Evol 1995; 12: 921-7.
13. Hinke V, Seck T, Clanget C, Scheidt-Nave C, Ziegler R, Pfeilschifter J. Association of transforming growth factor-beta1 (TGFbeta1) T29—> C gene polymorphism with bone mineral density (BMD), changes in BMD, and serum concentrations of TGF-beta1 in a population-based sample of postmenopausal german women. Calcif Tissue Int 2001; 69: 315-20.
14. Dushku N, Reid TW. Immunohistochemical evidence that human pterygia originate from an invasion of vimentin-expressing altered limbal epithelial basal cells. Curr Eye Res 1994; 13: 473-81.
15. Di Girolamo N, Coroneo MT, Wakefield D. Active matrilysin (MMP-7) in human pterygia: potential role in angiogenesis. Invest Ophthalmol Vis Sci 2001; 42: 1963-8.
16. Miller JW. Vascular endothelial growth factor and ocular neovascularization. Am J Pathol 1997; 151: 13-23.
17. Muraoka RS, Dumont N, Ritter CA, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. J Clin Invest 2002; 109: 1551-9.
18. Siegel PM, Massague J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Nat Rev Cancer 2003; 3: 807-21.
19. Tang B, Vu M, Booker T, et al. TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. J Clin Invest 2003; 112: 1116-24.
20. Grainger DJ, Heathcote K, Chiano M, et al. Genetic control of the circulating concentration of transforming growth factor type beta1. Hum Mol Genet 1999; 8: 93-7.
21. Hsieh YY, Chang CC, Tsai FJ, Peng CT, Yeh LS, Lin CC. Polymorphism for transforming growth factor beta 1-509 (TGF-B1-509): association with endometriosis. Biochem Genet 2005; 43: 203-10.
22. Lee KM, Park SK, Hamajima N, et al. Genetic polymorphisms of TGF-beta1 & TNF-beta and breast cancer risk. Breast Cancer Res Treat 2005; 90: 149-55.
23. Stanczuk GA, Tswana SA, Bergstrom S, Sibanda EN. Polymorphism in codons 10 and 25 of the transforming growth factor-beta 1 (TGF-beta1) gene in patients with invasive squamous cell carcinoma of the uterine cervix. Eur J Immunogenet 2002; 29: 417-21.
24. Ohtsuka T, Yamakage A, Yamazaki S. The polymorphism of transforming growth factor-beta1 gene in Japanese patients with systemic sclerosis. Br J Dermatol 2002; 147: 458-63.
25. Sugiura Y, Niimi T, Sato S, et al. Transforming growth factor beta1 gene polymorphism in rheumatoid arthritis. Ann Rheum Dis 2002; 61: 826-8.
26. Beranek M, Kankova K, Benes P, et al. Polymorphism R25P in the gene encoding transforming growth factor-beta (TGF-beta1) is a newly identified risk factor for proliferative diabetic retinopathy. Am J Med Genet 2002; 109: 278-83.
27. Edmunds SC, Kelsell DP, Hungerford JL, Cree IA. Mutational analysis of selected genes in the TGFbeta, Wnt, pRb, and p53 pathways in primary uveal melanoma. Invest Ophthalmol Vis Sci 2002; 43: 2845-51.
28. Araria-Goumidi L, Lambert JC, Mann DM, et al. Association study of three polymorphisms of TGF-beta1 gene with Alzheimer's disease. J Neurol Neurosurg Psychia-

try 2002; 73: 62-4.
29. Niimi T, Sato S, Sugiura Y, et al. Transforming growth factor-beta gene polymorphism in sarcoidosis and tuberculosis patients. Int J Tuberc Lung Dis 2002; 6: 510-5.
30. Holla LI, Fassmann A, Benes P, Halabala T, Znojil V. 5 polymorphisms in the transforming growth factor-beta 1 gene (TGF-beta 1) in adult periodontitis. J Clin Periodontol 2002; 29: 336-41.
31. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003; 9: 669-76.
32. Kim I, Ryan AM, Rohan R, et al. Constitutive expression of VEGF, VEGFR-1, and VEGFR-2 in normal eyes. Invest Ophthalmol Vis Sci 1999; 40: 2115-21.
33. Ray D, Mishra M, Ralph S, Read I, Davies R, Brenchley P. Association of the VEGF gene with proliferative diabetic retinopathy but not proteinuria in diabetes. Diabetes 2004; 53: 861-4.
34. Stevens A, Soden J, Brenchley PE, Ralph S, Ray DW. Haplotype analysis of the polymorphic human vascular endothelial growth factor gene promoter. Cancer Res 2003; 63: 812-6.
35. Tsai YY, Lee H, Tseng SH, et al. Null type of glutathione S-transferase M1 polymorphism is associated with early onset pterygium. Mol Vis 2004; 10: 458-61.