Original Article
EphB4 expression in pterygium is associated with microvessel density

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Abstract: Objective: Angiogenesis is a key component of the pathogenesis process of pterygium, which is the growth of fibrovascular tissue on the cornea. It has been reported that EphB4, a receptor tyrosine kinase of the ephrin-Eph system, plays important role in vascular development during embryogenesis and tumor angiogenesis and is potentially involved in ocular angiogenesis. The aim of this study is to investigate the role of EphB4 in pterygia. Methods: Fifteen pterygium samples and their paired upper bulbar conjunctiva were evaluated for expression of EphB4 and CD31 by using immunohistochemical staining. The expression level of the mRNA of EphB4 gene in 7 pterygia and matched upper bulbar conjunctiva was evaluated by using a quantitative real-time RT-PCR analysis. Microvessel density (MVD) was assessed with antibody that targets CD31. Results: EphB4 protein was high expressed in the epithelium and stroma of pterygia compared to those in upper bulbar conjunctiva. Immunohistochemical staining showed that pterygia presented with statistically significant higher average count of microvessel compared to normal conjunctivae (28.24 ± 6.79 vs 11.09 ± 2.96 per high power field, $P < 0.001$). MVD values in stroma of the pterygium and normal conjunctiva presented a significant correlation with EphB4 staining ($P < 0.001$). Compared with autologous upper bulbar conjunctiva grafts, the expression of the EphB4 mRNA was increased in pterygia in 4 paired samples, including those 3 recurrent ones. Conclusion: The expression of EphB4 in pterygium was significantly related with the increased MVD and may be involved in angiogenesis. EphB4 protein is a potential target for treatment of pterygium.

Keywords: Pterygium, EphB4, MVD, angiogenesis

Introduction

Pterygium is a common ocular surface lesion characterized by wing-shaped, fibrovascular conjunctival outgrowth that invades the clear cornea. The induced astigmatism and the migration of pterygium centrally into the visual axis may result in visual impairment [1-3]. The standard treatment for pterygium is surgical removal, sometimes with adjunct treatments. However, its recurrence cannot be totally avoided [4-7]. Previous studies suggested several interrelated factors contributing to the etiology of pterygium [8]. With the finding of up-regulations of tumor-related genes in the epithelium of pterygium, pterygium was more considered as an ultraviolet (UV)-related uncontrolled cell proliferation [9-17]. Many tumor-related genes, especially those angiogenic factors, such as vascular endothelial growth factor (VEGF), were found to be overexpressed in pterygium.

Eph (erythropoietin producing hepatocellular carcinoma) receptor tyrosine kinases and their membrane bound ligands, the ephrins, the largest family of tyrosine kinases, are known for their role in coordinating cell movements and positioning mainly in embryonic patterning, axon path-finding and vascular remodeling. The Eph receptors can be divided into two families, EphA and EphB, depending on the similarity within each group of the extracellular domain sequences and on the affinity for binding either ephrins of type A or of type B. The ephrinB2 and its receptor EphB4 have been shown to play an important role in angiogenesis both in tumor and in ocular disease [18-21]. The ligand, ephrinB2, as was expressed on arterial endothelial cells [19]. The receptor EphB4, was once considered expressing only on venous endothelial cells. However, more recent immunohistochemical staining rejected the venule-specific view [22]. Its expressing along both venules and

arterioles has been detected in various adult tissues. To investigate the role of expression of EphB4 in pterygium and its relations with microvessel density, we set out to do this study.

Materials and methods

Patients and samples

All the procedures were carried out at the Department of Ophthalmology and Pathology, Jinling Hospital, Nanjing University School of Medicine. The research protocols were approved by the medical ethic committee, which follows the Helsinki declaration and amended principles. General consent forms were signed by the patients before any procedures were performed. Fifteen patients, 4 male and 11 female, with age ranged from 40-72 (mean 61.67 years), were included in the study. Among those, 10 had primary pterygium and 5 had recurrent pterygium. With local topical anesthesia, pterygium and paired normal bulbar conjunctival specimens were obtained during pterygium excision with free conjunctival autograft transfer. Among the 15 pairs of samples (pterygia vs. matched normal conjunctivae), 8 of them received immunohistochemical staining only. The other 7 (4 primary pterygia and 3 recurrent pterygia) were large enough to be further divided for both immunohistochemical staining and RT-PCR analysis.

Immunohistochemical staining

Tissue segments of 15 pterygia and matched normal conjunctivae were fixed by cold 10% formalin in 0.2 mol/L phosphate buffer, pH 7.3, for 3 hours and processed for paraffin embedding. Sections of 4 μm were cut, mounted on glass, and dried at 37°C overnight. All sections were then deparaffinized and rehydrated with a gradient of ethanol concentrations. The sections were autoclaved in 10 mmol/L citrate buffer (pH 6.0) at 120°C for antigen retrieval, then cooled to 30°C and washed in phosphate buffered saline (PBS, pH 7.3). This buffer was used for all subsequent washes. Nonspecific sites were blocked with 10% normal calf serum in PBS for 10 minutes. Goat antihuman EphB4 polyclonal antibodies (R&D Systems, Minneapolis, U.S.) at a dilution of 1:20 and mouse anti-human CD31 monoclonal antibodies (Novocastra; Tebu, Le Perray en Yvelines, France) at a dilution of 1:100 were used as the primary anti-

body. The incubation was at 4°C overnight, followed by washing with PBS. The sections were incubated with secondary antibody (Dako, Ely, U.K.) for 30 minutes at room temperature. Signals were developed with 3, 3'-diaminobenzidine for 5 minutes and counterstained with hematoxylin. Negative controls were obtained by leaving out primary antibody. EphB4 protein expression in colon cancer tissue was used as a positive control. Sections of paraffin-embedded colon cancer samples were collected from the Jinling Hospital, Nanjing University School of Medicine, after written informed consent had been obtained.

Evaluation of staining for EphB4

The immunostained slides were assessed by two pathologists independently, with all information for possible identification masked. In each case, at least 3 representative high power fields (×40) were evaluated. The intensity of staining for EphB4 was scored (0 = negative, 1 = weak, 2 = moderate, 3 = intense), and the number of EphB4 positive cells among epithelial, stromal, and vascular endothelial cells was also scored (0 = negative, 1 = less than 10% of cells, 2 = 10-50% of cells, 3 = more than 50% of cells).

MVD determination

MVD was also assessed by two pathologists in a double-blind format. Any stained endothelial cells or endothelial cell clusters clearly separated from connective tissue elements were considered as a single microvessel and large vessels with thick muscular walls were excluded from the count. The entire tissue section was scanned at low magnification (×10) to find the area that showed the most intense neovascularization, and then the three most highly vascularized hotspots in each case were selected (×40 fields, 0.65 mm² per field). Results were expressed as the highest number of microvessels identified in the hotspot within a microscopic field and the average number of the three counts was recorded.

Quantitative real-time reverse transcriptase-polymerase chain reaction

Total RNA isolated from 7 pterygia and matched normal conjunctivae were subjected to RT-PCR analysis. It was performed with a SYBR Green®
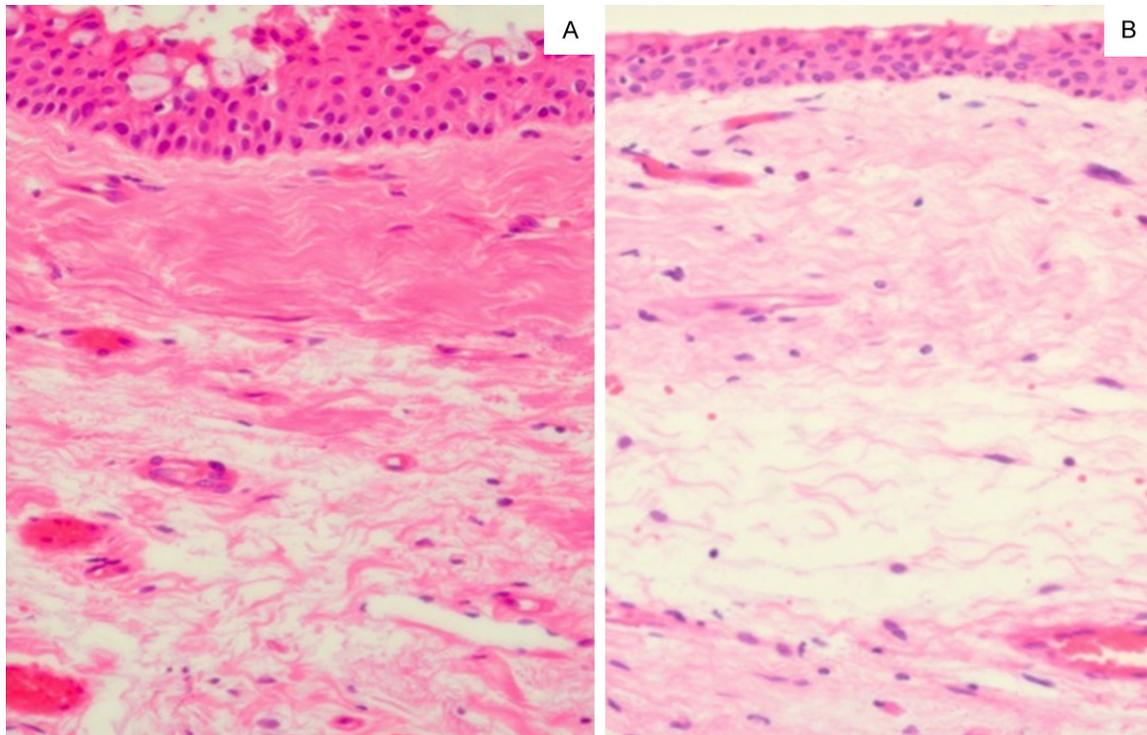
Figure 1. Hematoxylin and eosin staining and pathological diagnosis of pterygium (A) and normal conjunctiva (B).

Table 1. Average EphB4 intensity scores and count scores for EphB4-positive cells in the epithelium, stroma and vascular endothelium of pterygial and conjunctival specimens

EphB4 immunostaining	Pterygium	Conjunctiva	P value
Average EphB4 intensity scores in the epithelium	2.80 ± 0.41	2.00 ± 0.93	P = 0.01*
Average count scores for EphB4-positive cells in the epithelium	2.87 ± 0.35	2.40 ± 0.63	P = 0.008*
Average EphB4 intensity scores in the stroma	2.33 ± 0.72	1.46 ± 0.64	P = 0.004*
Average count scores for EphB4-positive cells in the stroma	2.13 ± 0.74	1.60 ± 0.74	P = 0.023*
Average EphB4 intensity scores in the vascular endothelium	2.67 ± 0.49	2.33 ± 0.49	P = 0.059
Average count scores for EphB4-positive cells in the vascular endothelium	2.87 ± 0.35	2.73 ± 0.46	P = 0.157

*P-value was less than 0.05.

Realtime PCR Master Mix kit (TOYOBO CO., LTD, Japan), using the ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, U.S.).

The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The sense primer is 5'-GAGGGGTGATGTGGGGAGTA-3' and the anti-sense primer is 5'-GAGCTTCCCGTTCAGCTCAG-3'. Specific primers used for PCR amplification of EphB4 genes were as follows: the sense primer is 5'-TCCTGCAAGGAGACCTTCAC-3' and the anti-sense primer is 5'-CAGAGGCCTCGCAACT-ACAT-5'. The reaction mixture consisted of 10 µl 2× SYBR PCR master mix; 1.0 µl 1.0 µmol/l sense primer; 1.0 µl 1.0 µmol/l antisense primer; 1.0 µl cDNA template, and distilled water for a total volume of 20 µl. The PCR cycling conditions were used as follows: 4 min at 95°C, followed by 40 amplification cycles of denaturation at 95°C for 15 s, annealing and elongation at 60°C for 1 min.

Statistical analysis

MVD count results were analyzed using the paired t-test. Mean EphB4 intensity scores and EphB4-positive cell count scores in the epithelium, stroma and vascular endothelium between the two groups were compared using the Wilcoxon matched-pairs signed-ranks test. Pearson correlation analysis was used to evaluate the relationship between MVD and stromal EphB4 expression. All statistical analyses were performed using the SPSS software (SPSS,
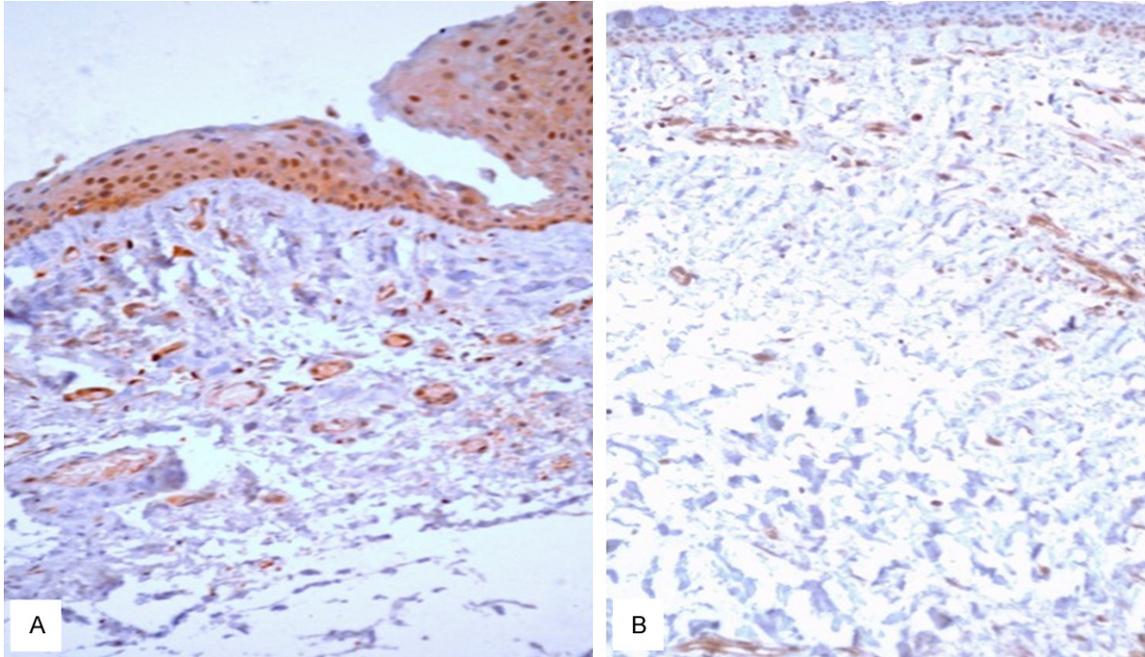
Figure 2. Immunostaining with anti-EphB4 in pterygium specimen (A) and normal conjunctiva (B). EphB4-positive cells showed in the epithelium, stroma and vascular endothelium of pterygial and conjunctival specimens.

Table 2. Microvessel density counts in specimens of pterygium and matching normal conjunctiva immunostained with CD31

Patient	Microvessel Density (MVD)	
	Pterygium	Conjunctiva
1	33.00	17.67
2	26.33	13.33
3	18.00	11.33
4	20.67	6.67
5	22.67	12.00
6	29.00	9.67
7	21.67	8.33
8	30.67	13.00
9	17.67	8.33
10	32.00	14.00
11	33.67	11.00
12	36.33	10.33
13	30.33	8.33
14	31.67	8.33
15	40.00	14.00
Mean ± SD	28.24 ± 6.79	11.09 ± 2.96
P value	P < 0.001	

Chicago, IL, U.S.). For all of the statistical tests, a *P*-value of less than 0.05 was considered statistically significant.

Results

High expression of EphB4 in the pterygium compared to paired conjunctiva

The pterygium and paired normal bulbar conjunctival were pathologically confirmed with H & E staining (**Figure 1**). The immunolabeling for EphB4 in the epithelium, stroma and vascular endothelium of pterygium and paired conjunctiva were analyzed (**Table 1**). EphB4 protein was positively stained in cellular membrane and cytoplasm of epithelial cells (**Figure 2A**). In the pterygia tissue, there were higher intensity of EphB4 staining and more EphB4 positive cells, especially stromal and endothelial cells (**Figure 2A**). In normal conjunctiva, EphB4-positive epithelial cells can be detected mainly in the basal and parabasal layer (**Figure 2B**). Endothelial cells also partially stained by EphB4. Stromal cells' staining is weak.

High MVD in pterygium than in paired conjunctivae

More intense angiogenic activity was observed in pterygium than in normal conjunctiva tissue, particularly at the subepithelial area (**Table 2**). Pterygium tissues were more vascularized
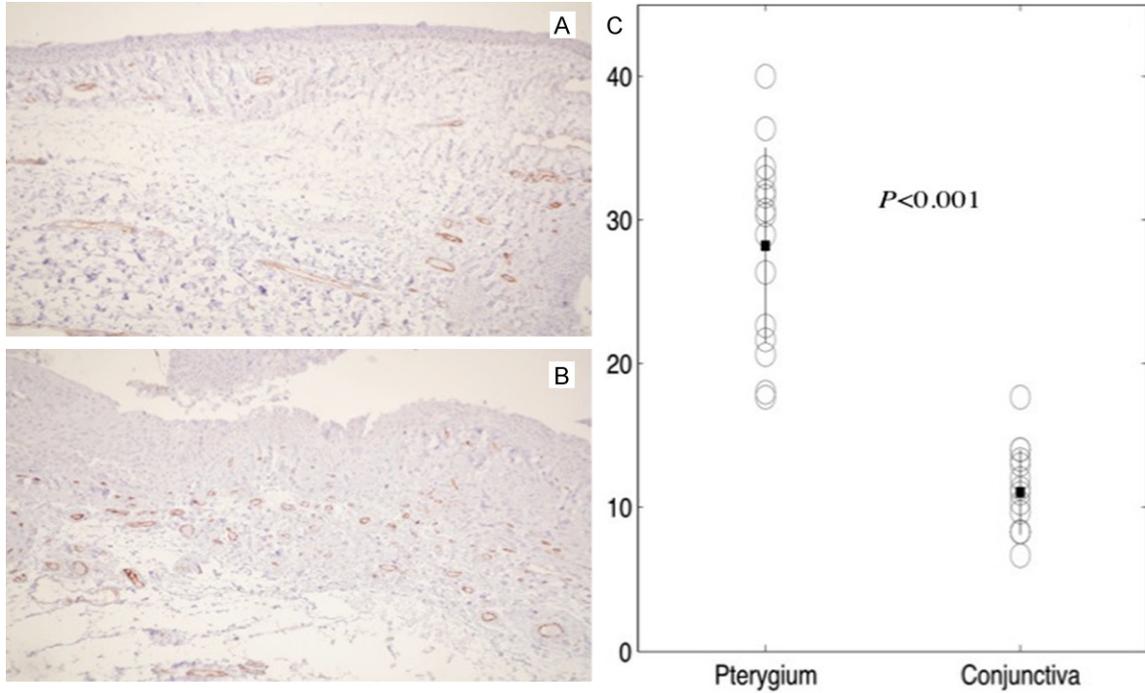
Figure 3. Light micrographs of specimens stained with CD31 in normal conjunctiva (A) and pterygium (B), demonstrating the great counts of MVD in the pterygium tissue (C).
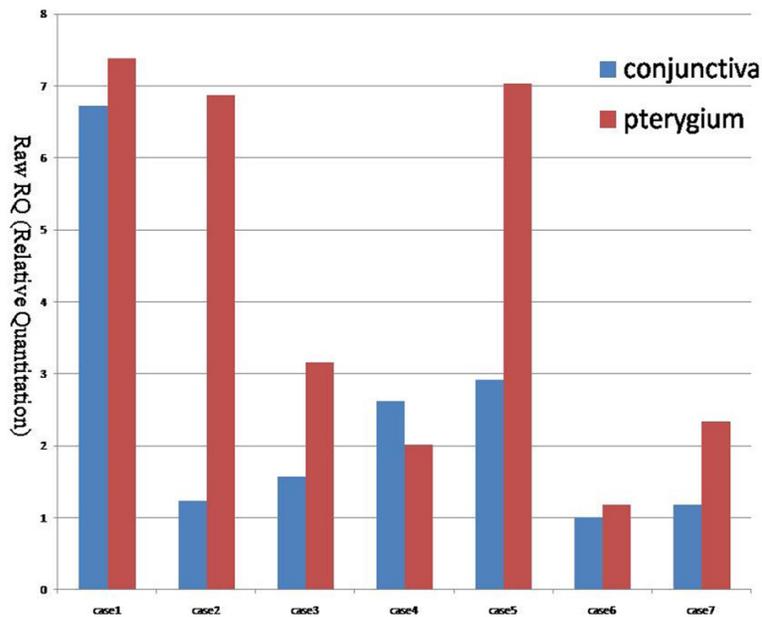

Figrue 4. Detection of EphB4 expression in pterygium and conjunctive tissues by a quantitative RT-PCR.

expression of EphB4 and MDV was analyzed. MVD values in stroma presented significant correlation with EphB4 staining in the pterygium and normal conjunctiva ($P < 0.001$).

Expression of the EphB4 transcript in pterygium and paired conjunctivae

Expression of the EphB4 transcript was measured using quantitative real-time RT-PCR in 7 pterygium specimens and its paired normal conjunctivae. The values of EphB4 mRNA expression were normalized by dividing the amount of the investigated RNA by the amount of GAPDH mRNA. High expression of EphB4 was detected in 4 pterygium samples including 3 recurrent pterygia. There is no difference of expression in 3 samples (**Figure 4**). The q-PCR reaction products were subjected to 10% agarose gel to confirm (**Figure 5**).

(28.24 ± 6.79 per high power field) than normal conjunctivae (11.09 ± 2.96 per high power field) (**Figure 3**). The difference was statistically significant ($P < 0.001$). The relation between
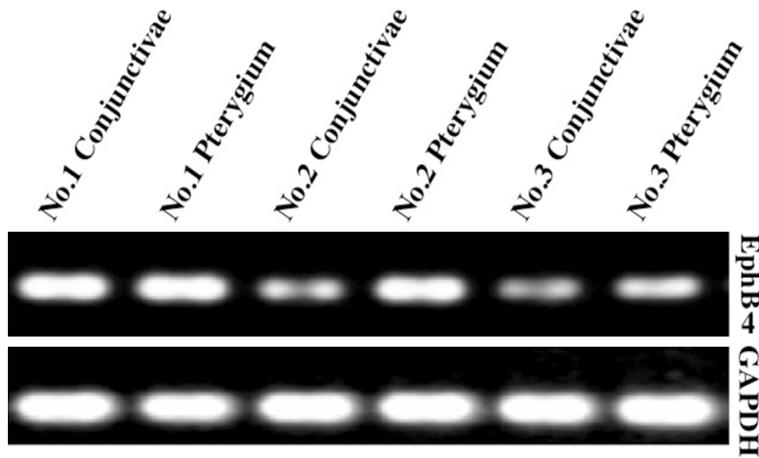
Figure 5. RT-PCR amplification of EphB4 and house keeping gene GAPDH in pterygium and normal conjunctiva.

Discussion

Angiogenesis plays an important role in many ocular diseases, such as corneal neovascularization and diabetic retinopathy. More recent studies suggested that it is also involved in the pathogenesis and development of pterygium. Some studies demonstrated higher microvascular density in pterygium stroma than in normal conjunctiva. Other studies have shown that angiogenic factors, such as vascular endothelial growth factor (VEGF) and anti-von-Willebrand factor (vWF), are highly expressed in pterygium [23, 24].

Eph is the largest subfamily of receptor tyrosine kinase, play important roles in development and tumorigenesis. We previously studied the expression of Eph receptors and erphrin ligands in a set of human tumors [25-28]. The Eph/ephrinB family plays an important role in vessel and cardiac development and angiogenesis [29-31]. Kojima et al checked the expression of EphA and EphB receptors and their ligands of Ephrin A and B in mouse corneal [32]. They found that in the vascularized corneas, ephrinB1 was immunolocalized mainly to the keratocytes around the vessels, and ephrinB2, EphB1 and EphB4 were colocalized mainly with CD31 to the vascular endothelial cells. They concluded that the Eph/ephrin family may play a role in the regulation of corneal angiogenesis. They demonstrated that ephrinB1 and EphB1 were expressed in basic fibroblast growth factor induced vascularized corneas in another work [33]. Recently, we focus our study of Eph

on human benign diseases that related to inflammation or angiogenesis [25, 34, 35]. For ocular diseases, it has been suggested over-expression of EphB4/ephrinB2 are associated with ocular angiogenesis in retinopathy of prematurity and corneal neovascularization [21, 32]. A soluble monomeric form of the EphB4 extracellular domain (sEphB4) has been investigated in target therapy for choroidal endothelial cell migration and tube formation and on experimental laser-induced choroidal neovascularlization, and tumor angiogenesis [18, 36]. sEphB4 has the potential therapeutic role in angiogenesis of pterygium. Increasing data have proven that EphB4 and ephrinBs play important roles in angiogenesis of malignant or benign diseases [19, 35, 37-43].

In this study, we compared the expression of EphB4 in pterygium with its paired normal bulbar conjunctival specimens, using immunohistochemical staining and quantitative real-time RT-PCR. The over-expression of EphB4 was found in most cases of the pterygium, especially in recurrent ones. High expression of EphB4 protein was observed in all pterygium tissues. However, in a few cases, EphB4 mRNA was equally expressed in pterygium and paired normal bulbar conjunctival tissues. This can be interpreted that there is a regulation after transcription of EphB4 in pathogenesis of pterygium. Pterygium tissues were more vascularized than normal conjunctivae and there was significant correlation between MVD values and EphB4 expression in pterygium and normal conjunctiva stromal layer (P < 0.001). This result provides solid evidence that overexpression of EphB4 plays an important role in the angiogenesis of pterygium and may be involved in pathogenesis of pterygium. However, this study was hampered by the small sample size, which limited the statistical power. In the treatment surgery for pterygium case, after removing it, a small piece of upper bulbar conjunctiva was transplanted to the nasal sclera bed. In order to keep a comparatively normal upper bulbar conjunctiva for future other surgeries,

we made this piece of conjunctiva graft as small as we could. Only in the cases where the area of the sclera bed was relatively small and the conjunctival graft was comparatively larger, we could obtain paired conjunctival tissues for the study. This constriction limited the chances for us to get a large sample size.

In summary, over-expression of EphB4 plays an important role in the angiogenesis of pterygium. Clinically, inhibition the expression of EphB4 may provide a new strategy for the treatment of pterygium and prevention its recurrence.

Acknowledgements

Disclosure of conflict of interest

None.

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