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LABORATORY INVESTIGATION

Evaluation of *NLRP1* Gene Polymorphisms in Vogt-Koyanagi-Harada Disease

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Abstract

Purpose: Polymorphisms of the NACHT [neuronal apoptosis inhibitory protein (NAIP), CIITA, HET-E, TP1] and leucine-rich repeat protein 1 (*NLRP1*) gene are reported to be associated with susceptibility to vitiligo and several autoimmune diseases. Vogt-Koyanagi-Harada (VKH) disease is an autoimmune disorder affecting melanocytes in the skin, eyes, inner ear, and meninges. In this study, genetic associations between VKH disease and single-nucleotide polymorphisms (SNPs) surrounding the *NLRP1* gene were investigated.

Methods: Six SNPs (rs6502867, rs925597, rs3926687, rs2733359, rs878329, and rs4790796) near the *NLRP1* gene, including noncoding regions, were sequenced by a direct method to genotype 167 Japanese patients with VKH disease and 187 healthy Japanese volunteers.

Results: None of the six SNPs in the *NLRP1* region were significantly associated with disease susceptibility or the ocular, neurological, and dermatological manifestations of VKH.

Conclusions: Although skin manifestations are clinically similar between vitiligo and VKH disease, the genetic and immunological mechanisms of these two diseases may be different. **Jpn J Ophthalmol** 2011;55:57–61 © Japanese Ophthalmological Society 2011

Keywords: *NALP1*, single-nucleotide polymorphism, uveitis, Vogt-Koyanagi-Harada disease

Introduction

Autoimmune and autoinflammatory diseases involve interactions between genetic risk factors and environmental triggers. Recently, it was reported that two common NACHT [NAIP (neuronal apoptosis inhibitory protein), CIITA, HET-E, and TP1] variants, leucine-rich repeat domain and pyrin domain-containing protein 1 (*NLRP1*), appear to contribute independently to a generalized risk of vitiligo and other autoimmune diseases, including autoimmune thyroid diseases such as Graves' disease and autoimmune hypothyroidism, rheumatoid arthritis, psoriasis, adult-onset autoimmune diabetes mellitus, pernicious anemia, Addison's disease, and systemic lupus erythematosus.[1,2] The *NLRP1* gene is located on chromosome 17p13 and is a key regulator of the innate immune system.[3] There are disease-associated single-nucleotide polymorphisms (SNPs) located within the *NLRP1* structural gene that are tagged by SNP rs6502867, and numerous other SNPs within a 65-kb linkage disequilibrium (LD) block in the extended promoter region of *NLRP1*.[4]

Vogt-Koyanagi-Harada (VKH) disease is one of the most frequent forms of endogenous uveitis in Japan.[5,6] VKH disease is an autoimmune disease affecting melanocytes of the skin, eyes, inner ear, and meninges.[7] In its early stage, it is characterized by a bilateral ocular inflammation of

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the pigmented areas, usually accompanied by cerebrospinal fluid pleocytosis.[8,9] In its later stages, it is characterized by depigmentation of the skin, hair, and ocular fundus (sunset glow fundus).[8,9] Previous experimental studies showed that melanocyte-specific proteins induced an autoimmune disease in dogs that resembled human VKH disease.[7,10] Also, lymphocytes collected from VKH patients react with peptides derived from the tyrosinase family proteins, such as tyrosinase, tyrosinase related protein (TRP)1, and TRP2.[7] Although the etiology of VKH disease remains unknown, genetic factors may play an important role in disease susceptibility, as indicated by an established association between VKH disease and specific human leukocyte antigen (HLA)-DRB1 alleles.[11,12]

Vitiligo is an autoimmune disease characterized by variable numbers of depigmented macules, often symmetrically placed, which frequently affect the skin in exposed areas of the body and around orifices.[13,14] Although the precise etiology of this disease is unknown, an autoimmune component is strongly suggested by immune infiltrates and skin-homing autoreactive T cells that target melanocyte-specific antigens, such as melan-A/MART1, gp100, tyrosinase, TRP1, and TRP2.[15–19] However, the association between HLA and vitiligo is still unknown. The clinical signs of vitiligo vulgaris are strikingly similar to the vitiligo seen in VKH patients, and it has been argued that vitiligo and VKH disease are different clinical expressions of the same disease, rather than two different diseases.[20]

In the present study, we hypothesized that a common allelic variation may exist in these two similar diseases. We investigated SNPs around the *NLRP1* gene in VKH patients and compared the results with those of healthy controls to determine whether SNPs around the *NLRP1* gene are associated with the presence of VKH disease.

Materials and Methods

We recruited 167 VKH patients (72 men and 95 women; Table 1) and 187 healthy volunteers for this study. All patients and control subjects were Japanese. Patients were diagnosed according to the "Revised Diagnostic Criteria for VKH Disease"[8,9] at the Uveitis Survey Clinic of Hokkaido University Hospital and Yokohama City University Hospital. All patients and control subjects were informed of the study's purpose and provided informed consent. The study was approved by the ethics committee at each participating institute.

DNA was prepared from peripheral-blood specimens using a QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan). Six SNPs (rs6502867, rs925597, rs3926687, rs2733359, rs878329, and rs4790796) around the *NLRP1* gene region were examined (Fig. 1). The six SNPs selected were previously reported as being significantly associated with vitiligo.[4] Each SNP was amplified by a standard polymerase chain reaction (PCR) procedure (Table 2). After purification using ExoSAP-IT (USB, Cleveland, OH, USA), the PCR products were sequenced with BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) using either sense or antisense primers (Table 2). The BigDye XTerminator Purification Kit was used to purify the DNA from the sequencing reactions. The sequencing reactions were analyzed using an ABI3130 sequencer (Applied Biosystems).

Statistical Analysis

For statistical analyses, the Hardy-Weinberg equilibrium was tested for each SNP. Genotype frequency differences between the case and control genotypes were assessed by χ-squared test. The calculation of LD and pairwise LD (D' value) between SNPs in the *NALP1* region and the haplotypes were carried out with Haploview software, version 3.32 (http://www.broad.mit.edu/mpg/haploview/). The maximum likelihood estimates of haplotype frequencies were estimated by pairs of genotypes using expectation-maximization algorithms.

Table 1. Characteristics of VKH patients

	%	
Male	43.1	72/167
Female	56.9	95/167
Bilateral ocular involvement	100	167/167
Diffuse choroiditis	100	160/160
Sunset glow fundus	69.4	111/160
Depigmented scars	30.1	47/156
Neurological auditory findings	89.1	147/165
Meningismus (headache, fever, etc)	78.5	124/158
Tinnitus	53.6	75/140
Cerebrospinal fluid pleocytosis	70.4	95/135
Integumentary findings	22.7	37/163
Alopecia	6.7	11/163
Poliosis	17.8	29/163
Vitiligo	10.4	17/163

VKH, Vogt-Koyanagi-Harada.

Results

Allele frequencies for the six SNPs around the *NLRP1* gene in both the patient and control groups were within the

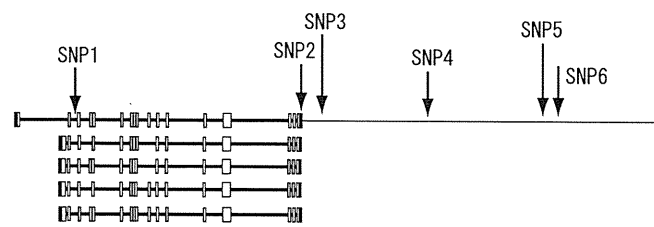

Figure 1. Structure of the *NALP1* gene showing five transcript isoforms and the location of six single-nucleotide polymorphisms (*SNPs*). The black exons indicate the untranslated regions and the white ones the coding regions.

Table 2. Polymerase chain reaction primers used for the *NALP1* SNPs

SNP	No.	Position	Primers	Product size (bp)	Primer sequence
rs6502867	SNP1	5361052	TTTGAATCCAGGTCTGAAGC	462	Forward
			GCACAATGGTGTCTTCACAG		
rs925597	SNP2	5428872	GACTCTCCGAGCAGTTGTGT	340	Reverse
			TCCACGAAAGAGGAAGTCTG		
rs3926687	SNP3	5433160	GAAGGGGTTTTTATCCCTGA	420	Forward
			ACTTGGGGGAAATGAATTGT		
rs2733359	SNP4	5461471	GGCAGAGGACGTTACTCTCA	495	Forward
			ATGCTGCACTGAGCACATTA		
rs878329	SNP5	5493974	GAATCCACTCAACTCCCTCA	309	Reverse
			ACTTACTGGGCCTGGGATAG		
rs4790796	SNP6	5496043	ATGCATTTCCATGTTTGCTT	449	Forward
			AGCCAAATCTCATTTGCATT		

SNP, single-nucleotide polymorphism

Table 3. Genotype frequencies in VKH patients and healthy controls

SNP		VKH patients (n = 167)	%	Healthy controls (n = 187)	%	Odds ratio (95% CI)	HapMap data	P value
rs6502867	T/T	158	95.2	173	93.0	1.48 (0.60–8.67)		0.39
	C/T	8	4.8	12	6.5	0.73 (0.29–8.60)		0.51
	C/C	0	0.0	1	5.4	N/A		0.34
	C	8	2.4	14	3.8	0.63 (0.26–8.65)	3.3	0.30
rs925597	C/C	112	67.1	129	69.4	0.90 (0.57–7.34)		0.64
	C/T	48	28.7	51	27.4	1.07 (0.67–7.47)		0.78
	T/T	7	4.2	6	3.2	1.31 (0.43–10.04)		0.63
	T	62	18.6	63	16.9	1.12 (0.76–7.40)	N/A	0.57
rs3926687	A/G	16	9.6	13	7.0	1.42 (0.66–8.65)		0.37
	G/G	151	90.4	174	93.0	0.71 (0.33–7.73)		0.37
	A	16	4.8	13	3.5	1.40 (0.66–8.59)	5.0	0.38
rs2733359	C/C	7	4.2	6	3.2	1.31 (0.43–10.04)		0.63
	C/T	53	31.7	60	32.3	0.98 (0.62–7.40)		0.92
	T/T	107	64.1	120	64.5	0.98 (0.63–7.34)		0.93
	C	67	20.1	72	19.4	1.05 (0.72–7.34)	20.6	0.81
rs878329	C/C	8	4.8	5	2.7	1.83 (0.59–11.48)		0.29
	C/G	54	32.3	55	29.4	1.15 (0.73–7.50)		0.55
	G/G	105	62.9	127	67.9	0.80 (0.52–7.37)		0.32
	C	70	21.0	65	17.4	1.26 (0.87–7.49)	21.7	0.23
rs4790796	C/C	105	62.9	124	66.3	0.86 (0.56–7.34)		0.50
	T/C	54	32.3	58	31.0	1.06 (0.68–7.44)		0.79
	T/T	8	4.8	5	2.7	1.83 (0.59–11.48)		0.29
	T	70	21.0	68	18.2	1.19 (0.82–7.43)	22.2	0.35

CI, confidence interval.

Hardy-Weinberg equilibrium. The allelic frequency of each SNP in both groups was nearly equal, and no association was detected when compared independently (odds ratio, 1.05–1.40) (Table 3). Table 3 also shows SNP data for international HapMap Japanese subjects obtained with Haploview. A stratified analysis was then performed based on the presence of diffuse choroiditis, sunset glow fundus, nummular chorioretinal depigmented spots, neurological auditory involvement, meningismus, tinnitus, cerebrospinal fluid pleocytosis, or integumentary findings. No evidence of an association with clinical manifestations was found in VKH disease (data not shown). We calculated pairwise D′ values for all SNP pairs in the *NLRP1* gene (Fig. 2). Except for rs6502867, all pairwise D′ values in the *NLRP1* gene were nearly 1, indicating that five of the SNPs were highly associated with each other and that the promoter region of *NLRP1* was contained within a single LD block. This is the same result that was obtained previously in a Caucasian population, although no VKH patients were included in that study.[4] The haplotype analysis showed that the *NLRP1* gene was not associated with VKH disease in our Japanese cohort (data not shown).

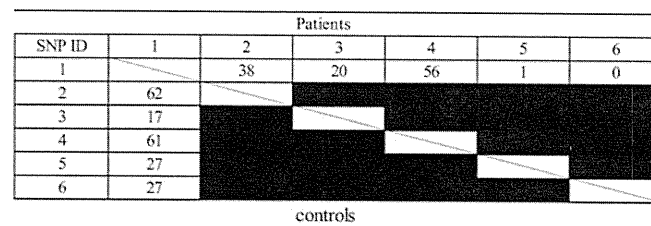

Figure 2. The D′ scores of the six disease-associated SNPs studied across the *NALP1* haplotype. *Black cells* indicate D′ > 0.8.

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Discussion

We compared genotype frequencies and the LD pattern of the six SNPs between the studied subjects and the international HapMap Japanese subjects using Haploview. The genotyping results and LD pattern of the subjects of this study were similar to other Japanese populations genotyped across the *NLRP1* gene. These facts further support the accuracy of the genotyping of all six SNPs in the current studied subjects.

In the present study, we analyzed polymorphisms of a new candidate gene, *NLRP1,* in Japanese patients with VKH disease and found no association between *NLRP1* and VKH disease in the individuals included. A previous study showed that the *NLRP1* gene, located at 17p13, had a significant but moderate association with vitiligo and vitiligo-associated autoimmune diseases.[4] Further analyses led to the conclusion that two independent variants of the *NLRP1* region are associated with an increased risk of vitiligo-associated autoimmune/inflammatory diseases. One (rs6502867) is situated on the *NLRP1* gene, and the other is located upstream in a region involving the gene's promoter.[4] The functional significance of these genetic variants is still unknown. According to the previous study, SNPs in the promoter region rather than nonsynonymous SNPs in the *NLRP1* gene itself are associated with the vitiligo. Jin et al.[4] investigated 15 nonsynonymous SNPs in the *NLRP1* region. Of the 15 SNPs, only rs12150220 was associated with vitiligo, although that region contains no known peptide motifs. We did not investigate rs12150220, but a perfect LD was established between rs12150220 and SNP3 by using the Japanese HapMap database, though the nearest SNP, SNP2, is not available in the database. This result means that rs12150220 is also not associated with VKH disease. NLRP1 is a nucleotide-binding oligomerization domain protein-like receptor, a family of pattern recognition receptors for microorganisms, apoptotic fragments, and cell debris.[21] NLRP1 is also part of cytoplasmic complexes called inflammasomes that regulate the activation of caspases, which in turn convert proinflammatory cytokines into their active forms.[22]

There is some evidence of peripheral tolerance to melanocyte-specific antigens and it has been suggested that ignorance and anergy may play a role.[23,24] However, the mechanism of tolerance induction and the level of persistence are unclear. One possible corollary of melanocyte-specific autoimmunity may be the induction of T cells capable of mediating tumor rejection, a supposition supported by the development of vitiligo in melanoma patients who were successfully treated with interleukin-2.[25] As a consequence of the absence of a basic understanding of vitiligo, patients should be treated with systemic immunosuppressive therapy.[26] Vitiligo is limited to a visible loss of pigmentation and differs from VKH disease clinically by the lack of obvious inflammation of other organs.[26] Although a few studies support the local infiltration of T cells,[27] biopsies of vitiliginous skin rarely show evidence of inflammation.

VKH disease is an autoimmune disease that affects melanocytes in different organs. Kitaichi et al.[28] reported that in their experiments lymphocyte subsets in the aqueous humor and cerebral spinal fluid in humans were identical. Moorthy et al.[29] showed that activated T cells were elevated and attacked melanocytes in the ocular choroidal tissue in patients with active-phase VKH disease.[29] Yamaki et al.[7,10] performed antigen-specific T-cell assays and showed that peptide fragments from tyrosinase family proteins (tyrosinase, TRP1, and TRP2) caused T cells collected from VKH patients to proliferate. Systemic inflammation is the main outcome of VKH disease, and patients should be treated with systemic pulse or high-dose corticosteroid therapy. Prompt therapy reduces the time period that systemic corticosteroids are needed.[30] Vitiligo is a late manifestation of VKH disease, and the frequency of vitiligo in Japanese VKH disease patients is approximately 20%.[29] Considering the data relating to vitiligo, it is important to compare the SNPs analyses around the *NLRP1* gene of VKH disease patients with and without skin lesions. As we previously reported, prompt systemic corticosteroid therapy can successfully cure VKH disease with few side effects.[30,31] Since the patients are treated with high-dose corticosteroids early on at our clinic, few patients with late manifestations of VKH disease (alopecia, poliosis, and vitiligo) were enrolled in the present study. Thus, it remains unclear whether the SNPs around the *NLRP1* gene are associated with the skin lesions of VKH disease. Further studies in other clinics and countries would be helpful. Geographically, VKH disease occurs most commonly in East, Southeast, and South Asia, and in Native American and Spanish populations, particularly those who carry either the *HLA-DR4* or *HLA-DRw53* gene.[12,32] However, HLA cannot completely explain the genetic risk factor of VKH disease. Thus, several candidate genes have been tested for an association with VKH disease, including *TYR*, *TYPR1*, *TYRP2*, *IFN-γ*, *CTLA4*, *SUMO*, *PDCD1*, *MCP-1*, and *PTPN22*.[11,33–38] Most of these studies found associations of only marginal significance, and the results of several studies could not replicated by subsequent studies.

In conclusion, our results suggest that vitiligo and VKH disease exhibit clinically similar skin manifestations but that the genetic contribution and mechanism of these two diseases may be different. Further molecular genetic studies are needed to detect novel genetic loci and predisposing genes in order to elucidate the genetic mechanisms underlying VKH disease.

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Tissue factor expression in human pterygium

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Purpose: A pterygium shows tumor-like characteristics, such as proliferation, invasion, and epithelial–mesenchymal transition (EMT). Previous reports suggest that tissue factor (TF) expression is closely related to the EMT of tumor cells, and subsequent tumor development. In this study, we analyzed the expression and immunolocalization of TF in pterygial and normal conjunctival tissues of humans.

Methods: Eight pterygia and three normal bulbar conjunctivas, surgically removed, were used in this study. Formalin-fixed, paraffin-embedded tissues were submitted for immunohistochemical analysis with anti-TF antibody. Double staining immunohistochemistry was performed to assess TF and alpha-smooth muscle actin (α-SMA) or epidermal growth factor receptor (EGFR) expression in the pterygia.

Results: Immunoreactivity for TF was detected in all pterygial tissues examined. TF immunoreactivity was localized in the cytoplasm of basal, suprabasal, and superficial epithelial cells. The number of TF-immunopositive cells in pterygial epithelial cells was significantly higher than in normal conjunctival epithelial cells ($p<0.001$). TF immunoreactivity was detected in α-SMA-positive or -negative pterygial epithelial cells. EGFR immunoreactivity was detected in pterygial epithelium, which was colocalized with TF.

Conclusions: These results suggest that TF plays a potential role in the pathogenesis and development of a pterygium, and that TF expression might be involved through EMT-dependent and -independent pathways.

A pterygium represents an epithelial and fibrovascular configuration on the ocular surface adjoining the conjunctiva. The pterygium invades the cornea forming a wing-like shape, causing visual loss. Pathologically, a pterygium is a proliferative, invasive, and highly vascularized tissue [1]. Furthermore, there are transformed cells in pterygial tissue, which is one of the characteristics of a tumor phenotype [2]. Kase et al. [3,4] demonstrated that proliferation activity is high in the pterygial epithelium compared to that in the normal conjunctiva.

The phenomenon of epithelial cells changing their phenotype to fibroblastic cells after morphogenic pressure from injured tissue is called epithelial–mesenchymal transition (EMT) [5,6]. To develop highly invasive characteristics, epithelial tumor cells change their morphology and function, whereby they transiently acquire markers of mesenchymal differentiation (e.g., alpha-smooth muscle actin (α-SMA)), and lose some of their epithelial features (e.g., E-cadherin) [7]. Moreover, blockade of E-cadherin in cultured cancer cells similarly leads to changes in cell shape reminiscent of EMT, and this transition gave rise to cells with a highly metastatic phenotype. It has been demonstrated that E-cadherin immunoreactivity is involved in α-SMA-positive pterygial epithelial cells [4,8], suggesting that EMT plays a key role in the pathogenesis of pterygium.

Tissue factor (TF) is a transmembrane protein that interacts with coagulation factor VIIa, whereby it initiates blood coagulation. This interaction also triggers intracellular signals, which are primarily mediated by G protein–coupled protease-activated receptors in concert with adhesion molecules and several other factors [9]. TF is regulated by oncogenic and differentiation pathways and it functions in tumor initiation, tumor growth, angiogenesis, and metastasis [9-11]. Indeed, it has been demonstrated that epithelial tumor cells, expressing high levels of TF regulated by the differentiation pathway, have mesenchymal characteristics [9]. These results suggest that TF expression is closely related to the EMT of tumor cells, and subsequent tumor development.

The aim of this study was to analyze the expression and immunolocalization of TF in pterygial and conjunctival tissues in humans.

METHODS

Preparation of human tissues: Eight patients with primary nasal pterygia who underwent surgical excision were enrolled in this study. Normal bulbar conjunctival tissues were obtained from three patients during cataract surgery. The

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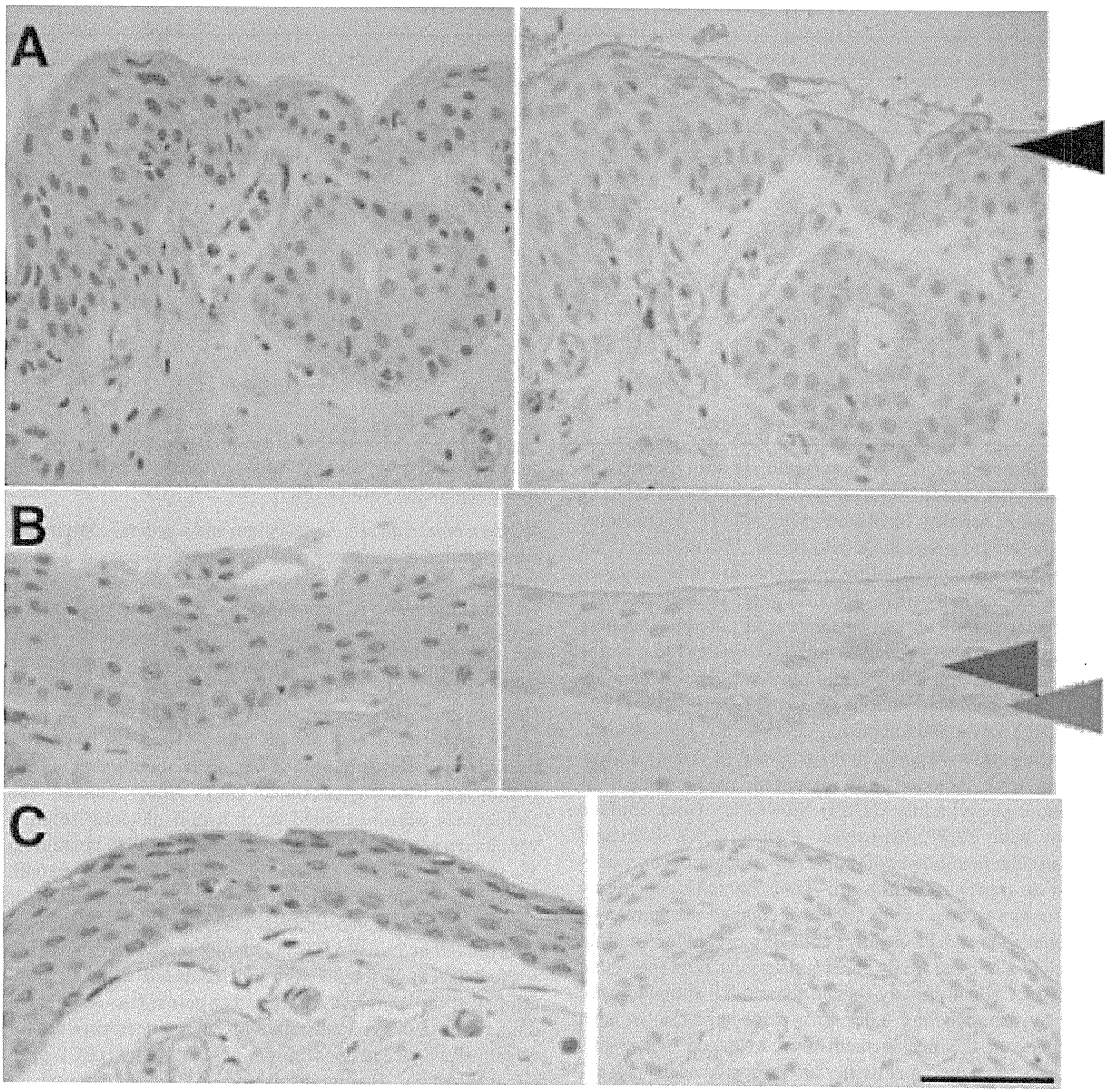

Figure 1. Immunohistochemistry for tissue factor (TF) in a human pterygium and normal conjunctiva. Left panels are H&E staining and right panels are TF immunoreactivity in two representative cases of a pterygium. TF is expressed in the cytoplasm of basal (**B**; red arrow head), suprabasal (**B**; blue arrow head), and superficial cells (**A**; black arrow head). In the normal conjunctiva, however, immunoreactivity for TF is not detected (**C**). The scale bar represents 50 μm.

tissues were then fixed in 4% paraformaldehyde. After fixation, slides were washed in phosphate-buffered saline and processed for paraffin sectioning. Informed consent was obtained according to the Declaration of Helsinki. All human experiments conformed to the requirements of ethics committee in Hokkaido University Graduate School of Medicine.

Immunohistochemistry: Dewaxed paraffin sections were immunostained using the alkaline phosphatase complex method. Formalin-fixed, paraffin-embedded serial tissue sections were cut at a 4 μm thickness and endogenous peroxidase activity was inhibited by immersing the slides in 3% hydrogen peroxide in methanol for 10 min. As a pretreatment, microwave-based antigen retrieval was performed in phosphate-buffered saline (PBS). Then, non-

TABLE 1. THE NUMBER OF TISSUE FACTOR (TF)-IMMUNOPOSITIVE CELLS IN PTERYGIAL EPITHELIAL AND NORMAL CONJUNCTIVAL CELLS.

Pterygia			Normal conjunctivas		
Age	**Gender**	**TF**	**Age**	**Gender**	**TF**
72	M	12.7%	54	M	0%
83	M	54.8%	80	M	0%
75	F	47.7%	82	M	0%
72	M	69.8%			
71	M	69.1%			
68	M	84.3%			
77	M	45.0%			
69	M	54.9%			
	Mean	54.8%		Mean	0%

In the Table, M indicates male and F indicates female.

specific binding of the primary antibody was blocked by incubating the slides in blocking bovine serum for 30 min. The slides were serially incubated with anti-TF monoclonal antibody (1:50; American Diagnostic Inc., Stamford, CT) for 2 h at room temperature, followed by a biotin-conjugated goat anti-mouse IgG. Positive signals were visualized using diaminobendizine as a substrate. In double staining immunohistochemistry, the sections were incubated with the above-mentioned first antibody, followed by the rhodamine-conjugated secondary antibody for 30 min, and FITC-conjugated anti-α-SMA monoclonal antibody (1:50; Abcam, Tokyo, Japan) for 30 min at room temperature. After washing, sections were mounted with mounting media with 4',6-diamino-2-phenylindole (DAPI; SlowFade® Gold antifade reagent with DAPI; Invitrogen, Eugene, OR). Preretinal fibrovascular membranes of proliferative diabetic retinopathy served as positive controls for TF immunohistochemistry [12]. In microscopic observation, we counted the number of epithelial cells and TF-positive cells of pterygium or normal conjunctiva in three fields under high power field (objective lens 40×). Cells positively stained for anti-TF antibody were noted by their labeling index as a percentage (%) in each specimen, and the measurements were averaged. The results regarding TF in pterygial tissues are presented as the mean.

To investigate the hypothesized co-localization of TF and EGFR in pterygium tissues, double staining immunohistochemistry was performed using mouse monoclonal antibody against human TF (1:50; Abcam) and anti-rabbit epidermal growth factor receptor (EGFR) polyclonal antibody (1:100 dilution; Santa Cruz Biotech, Santa Cruz, CA) as the primary antibody. Binding of the primary antibody was localized with the Alexa Fluor® 488 goat anti-mouse antibody (1:100 dilution; Invitrogen, Carlsbad, CA) and Alexa Fluor® 546 goat anti-rabbit secondary antibody (1:200 dilution; Invitrogen) for 30 min, respectively. Finally, sections were mounted with mounting media with DAPI.

Western blot analysis: A pterygium and a normal conjunctiva were surgically removed, and then were sonicated in lysis buffer (1× RIPA buffer; Cell Signaling Technology, Danvers, MA) with protease inhibitor (Roche, Basel, Switzerland) on ice, and centrifuged at 1,3850× g for 20 min at 4 °C. These are stored in −80 °C until assayed. These samples were electrophoretically separated on SDS–PAGE using a 4% stacking and 10% separating gels. Proteins in gels were electro-transferred (80 V, 90 min, 4 °C) to Hybond-P polyvinylidene difluoride transfer membranes (GE Healthcare, Buckinghamshire, UK). After transfer, the membranes were incubated for 1 h in a blocking solution which consisted of 1% skim milk powder in PBS containing 1% tween (PBST), washed briefly in PBST, then probed with anti-TF monoclonal antibody (1:500; above described) or anti-α-SMA polyclonal antibody (1:500; Abcam, Cambridge, UK) diluted in 5% BSA/TBST. Membranes were extensively washed in PBST for 30 min and incubated with a 1:1000 dilution of the appropriate horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit IgG at room temperature for 60 min. Then placed in chemiluminescent reagent (ECL plus, GE healthcare, Buckinghamshire, UK) and exposed to luminescent image analyzer (Fujifilm, Tokyo, Japan).

Statistical analysis: Student's t-test was used for statistical comparison of the number of TF-immunopositive epithelial cells between pterygium and normal control groups. Differences between the means were considered significant when the probability values were <0.05.

RESULTS

Morphologically, pterygial epithelium consisted of multilayer nuclei showing squamous metaplasia (Figure 1A). Table 1 summarizes the immunohistochemical results of TF in pterygial epithelium. Immunoreactivity for TF was detected

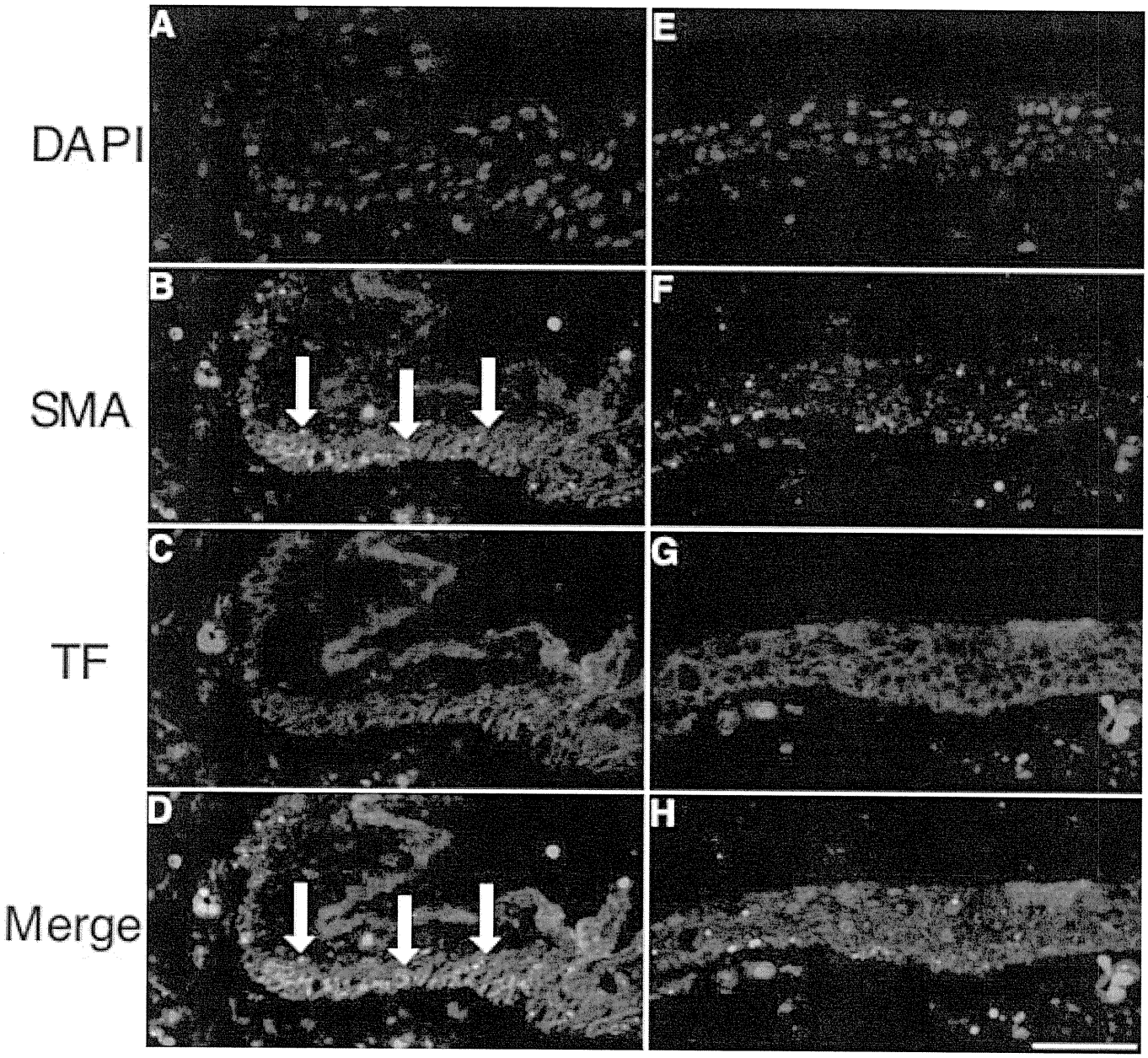

Figure 2. Double staining immunohistochemistry was performed for TF (red) and α-SMA (green) in pterygial tissue. **A-D**: α-SMA immunoreactivity is colocalized with TF-positive areas in pterygial epithelial cells (**D**, arrows). **E-H**: TF immunoreactivity is detected in the other part of epithelial cells negative for α-SMA. The scale bar represents 50 μm.

in all pterygial tissues examined. TF immunoreactivity was localized in the cytoplasm of basal, suprabasal, and superficial epithelial cells, and in subepithelial stroma along with epithelium (Figure 1A,B). In the normal conjunctival epithelium, however, immunoreactivity for TF was not detected (Figure 1C). Microvascular endothelial cells showed a weak immunoreaction for TF in both normal conjunctiva and pterygium. The number of TF-immunopositive cells was significantly higher in pterygial epithelial cells than in normal cells ($p<0.001$; Table 1).

Double staining immunohistochemistry involving pterygial tissues was performed for TF and α-SMA expression. α-SMA was expressed in several epithelial cells (Figure 2B,F), where TF immunoreactivity was colocalized (Figure 2C,G). TF immunoreactivity was also detected in α-SMA-negative epithelial cells (Figure 2E-H).

To check the expression of TF and α-SMA by other methods in human pterygium and normal conjunctiva, western blot analysis was performed using anti-TF and α-SMA antibodies. TF and α-SMA protein expression was

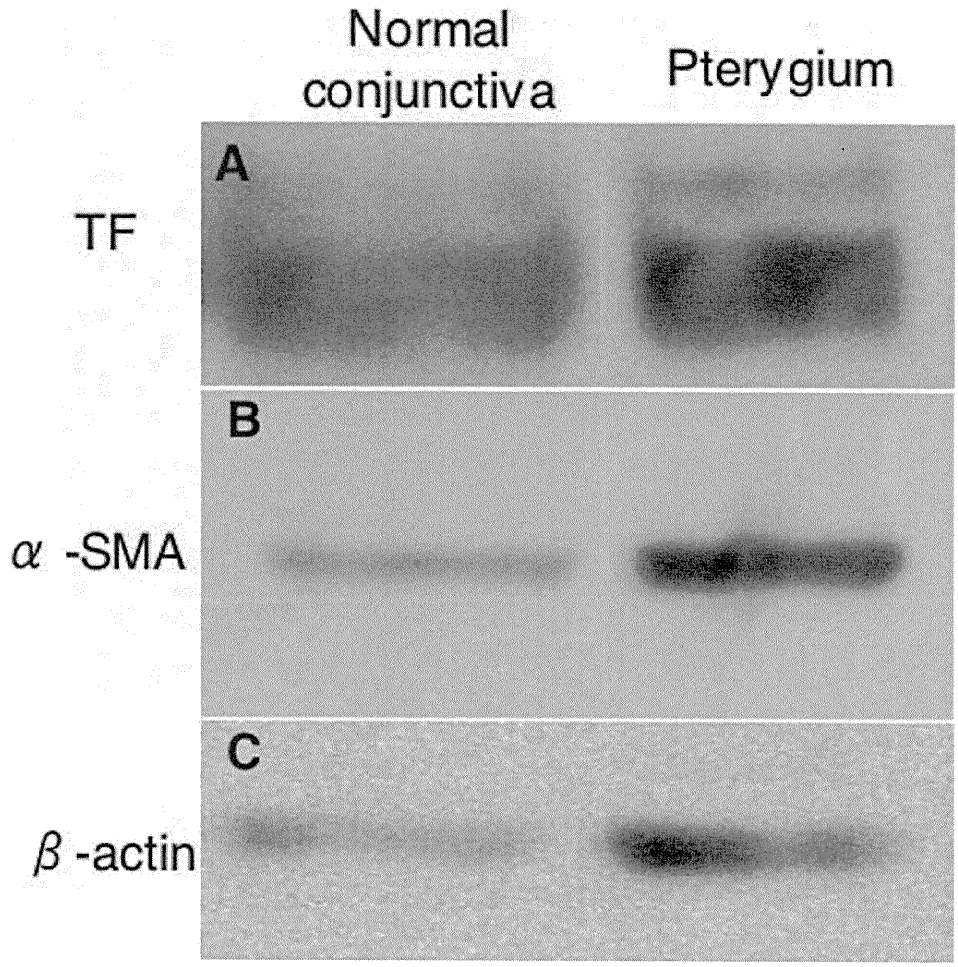

Figure 3. Western blot analysis using anti-TF and α-SMA antibodies. TF (**A**) and α-SMA (**B**) protein expression is clearly detected in both pterygium and normal conjunctival tissue.

clearly detected in both total proteins extracted from pterygium and normal conjunctival tissues (Figure 3).

Double staining immunohistochemistry for TF and EGFR was also performed in pterygial tissue. EGFR immunoreactivity was observed in pterygial epithelial cells, which was colocalized with TF in preferentially basal cells (Figure 4).

DISCUSSION

Pterygium has common biologic features with epithelial tumor, as is proliferative tissue and presence of EMT cells [8]. It has been demonstrated that TF functions in tumor initiation, tumor growth, angiogenesis, and metastasis [9-11]. Therefore, we supposed that TF might play a key role in the pathogenesis of pterygium; however, TF expression has yet to be determined in human pterygium. In this study, we demonstrated that TF protein was expressed in pterygial tissues using immunohistochemistry and western blot. Moreover, TF was mainly immunolocalized in pterygial epithelial cells. As shown in Table 1, the number of TF-positive cells was more than half of that of pterygial epithelial cells. In contrast, TF was not expressed in normal conjunctival epithelium. Microvascular endothelial cells showed a weak immunoreaction for TF in both the normal conjunctiva and pterygium, which was not significant. The result showing a significantly higher expression of TF in pterygial epithelium than the normal conjunctiva suggests that TF plays a role in the pathogenesis and development of a pterygium.

EMT is a major factor in pterygium progression [8]. In this study, protein expression of α-SMA, a classic sign of EMT, was observed in several pterygial epithelial cells, where TF immunoreactivity was colocalized on double staining immunohistochemistry. These results indicate that epithelial cells changing to the mesenchymal phenotype expressed TF. In tumor cells, Milsom et al. [9] demonstrated that E-cadherin modulated TF expression, and this could be alleviated by EMT-like changes. These results suggest that TF expression might be controlled by EMT in pterygium as well.

On the other hand, we found that pterygial epithelial cells, showing a negative results for α-SMA, also expressed TF. This suggests that the expression of TF is regulated not only by E-cadherin and EMT, but also by other TF-related molecules such as epidermal growth factor-receptor (EGFR). We and other colleagues previously demonstrated that E-

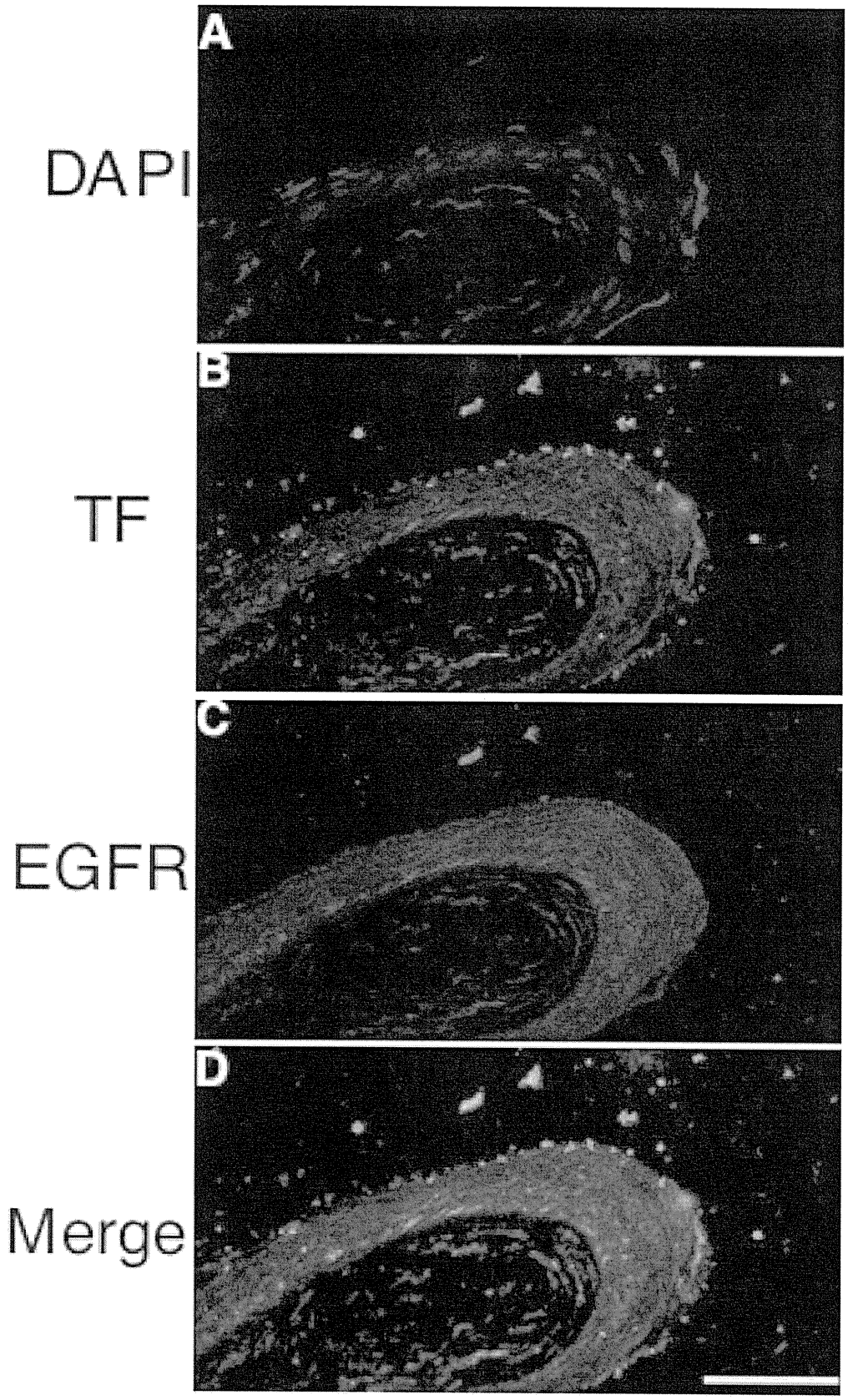

Figure 4. Double staining immunohistochemistry was performed for TF (green) and EGFR (red) in pterygial tissue. Nuclear staining and TF immunoreactivity are shown in **A** and **B**, respectively. **C**, **D**: EGFR immunoreactivity was observed broadly in pterygial epithelial cells. The scale bar represents 50 μm.

cadherin and EGFR immunoreactivity were shown by pterygial epithelial cells [4,8,13], and we immunohistochemically showed colocalization with TF and EGFR. In human squamous cell carcinoma, the activation of EGFR stimulates TF expression, which is modulated by E-cadherin in vitro, and an E-cadherin-neutralizing antibody led to the upregulation of TF expression [9]. Indeed, this induction of TF was completely inhibited by an EGFR inhibitor [9]. These findings suggest that EGFR signaling pathway may also play an important role in the regulation of TF expression.

It has been demonstrated that subsequent EMT and the activation of TF signaling can induce angiogenesis, tumor growth, and invasion [9]. In fact, invasion to the cornea and angiogenesis are characteristics in the pathobiology of a pterygium. Further investigations of the TF signaling pathway in the pterygium are necessary to clarify TF-mediated pterygial progression. Since targeting TF has been considered to be of therapeutic significance in tumor initiation [9], TF may be a therapeutic molecular target to treat pterygia.

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