

Increased expression of nestin in human pterygial epithelium

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Abstract

• **AIM:** To investigate the distribution of nestin-positive cells in pterygium, as well as the relationship between nestin-positive cells and proliferative cells in the pathogenesis of pterygium.

• **METHODS:** Nine pterygium specimens and 5 normal conjunctiva specimens were investigated. All explanted specimens were immediately immersed in 5-Ethynyl-2'-deoxyuridine, and were subjected to hematoxylin and eosin staining, as well as immunostaining to detect nestin.

• **RESULTS:** Small sub-populations of nestin-expressing cells in both normal and pterygial conjunctiva epithelium were found. These were located at the superficial layer of the epithelium, and were significantly increased ($P=0.007$) and spread out in the pterygial conjunctiva epithelium, even though these cells were mitotically quiescent.

• **CONCLUSION:** In pterygium, more nestin-positive cells were present at the superficial layer of the epithelium. With growing scientific evidence that nestin plays an important role in defining various specialized cell types, such as stem cells, cancer cells and angiogenic cells, further investigations on the roles of nestin-expressing cells in pterygium may help to uncover the mechanisms of initiation, development and the prognosis of this disease.

• **KEYWORDS:** pterygium; nestin; proliferation; distribution; human

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INTRODUCTION

Pterygium is one of the most commonly occurring eye diseases leading to astigmatism and vision loss. It is widely regarded to be a degenerative condition arising from benign growth of the conjunctiva, characterized by fibro-vascularization, conjunctiva invasion and elastic degeneration of collagen. Some studies have shown that pterygium share some similarities to cancer, whereby active cell proliferation occurs with minimal apoptosis^[1,2]. Besides the expression of tumor related proteins^[3], pterygium also displays tumor-like properties, such as invasion of cornea, high recurrence after surgical excision, and co-existence with secondary premalignant lesions^[2,4,5].

Nestin is an intermediate filament protein that was previously believed to be exclusively associated with neuro-progenitor cells^[6]. However, there is now increasing scientific evidence suggesting that nestin is also an important molecular marker of various other types of stem/progenitor cells, including bone marrow mesenchymal stem cells^[7], endothelial progenitor cells^[8], and hair follicle stem cells^[9]. Nestin-positive cells in bone marrow are adjacent to hematopoietic stem cells, and form a maintenance niche for these cells within bone marrow^[10]. Besides normal healthy cells, more than ten different types of tumor cells such as

pancreatic cancer cells, gastrointestinal stromal tumor cells, breast cancer cells and malignant melanoma cells were also demonstrated to be nestin-positive^[6]. Therefore, nestin is also a cancer cell marker. Proliferation, migration and immaturity are the common features of cell types demonstrated to express nestin.

Nestin-positive cells that play an important role in the pathogenesis of pterygium exhibit the characteristic features of immaturity, proliferation and migration. Nestin is expressed by a few types of ophthalmic cells, including ciliary body epithelial cells^[11] and limbal epithelial stem cells^[4]. Nevertheless, these were all based on animal studies, with the former being detected on rat normal ciliary body under the stimulation of insulin and FGF2^[11], while the latter was detected on *in vitro* expanded normal limbal epithelial cells from rabbits. Currently, there is a lack of data on nestin expression within human ophthalmic tissues, the distribution of nestin-positive cells in pterygium, as well as the relationship between nestin-positive cells and proliferative cells in the pathogenesis of pterygium. To answer these questions, the distribution of nestin-positive and 5-Ethynyl-2'-deoxyuridine (EdU)-stained cells in human pterygium tissues were investigated in this study.

SUBJECTS AND METHODS

Subjects All pterygium specimens were harvested from the head, which meant the pterygium invaded the cornea, by surgical removal after verbally informed consent was obtained from patients. The experimental group included 9 cases of primary pterygium (4 males and 5 females), with donor age ranging from 48-67 years (average age of 56 years). The control group comprised 5 normal bulbar conjunctiva specimens from cadavers (2 males and 3 females), with donor ages ranging from 51-65 (average age of 57). This study was carried out with the approval of the Human Research Ethics Committee at Xiangya Hospital (Document No. 201212029) and adhered to the tenets of the Declaration of Helsinki.

Methods Freshly harvested specimens were immersed in EdU (Cell-light EdU Apollo 643 DNA *in vitro* kit, Cat No. C10310-2; Guangzhou Ribobio Co. Ltd., China) solution at a concentration of 50 μ mol/L in cell culture media and incubated at room temperature for 2 hours. The specimens were then washed with phosphate buffer solution, fixed in 4% paraformaldehyde, dehydrated in a gradient of increasing ethanol concentrations, embedded in paraffin, and cut into 5 μ m sections. All sections were deparaffinized, rehydrated with a gradient of decreasing ethanol concentrations, and then treated with 2mg/mL glycine followed with 0.5% Triton

X-100. Antigen retrieval was achieved by heating the samples in 1mmol/L EDTA buffer (pH 8.0). Non-specific binding was blocked by treatment with goat serum albumin (AR0009, Wuhan Boster Biological Technology Co., Ltd, China) at 4 $^{\circ}$ C for 30 minutes. Anti-human nestin monoclonal antibody (Clone 10C2, MAB5326, Millipore, USA) was diluted at 1:200 and incubated with the specimens at 4 $^{\circ}$ C overnight, followed by staining with FITC-conjugated goat anti-mouse IgG secondary antibody (1:500 dilution, A11001, Invitrogen, USA) at 37 $^{\circ}$ C for 1 hour. After washing 3 times, EdU fluorescence was activated following the manufacturer's instructions. Briefly, specimens were incubated with Apollo staining solution followed with Hoechst 33342 in darkness at room temperature for 30 minutes. All specimens were then covered with anti-fading media and coverslip, and stored at 4 $^{\circ}$ C in darkness. Images were captured with an Olympus confocal microscope (BX61W1-FV1000, Olympus, Tokyo, Japan) and analyzed with AutoQuant X3 (Media Cybernetics, Inc., Washington, USA) and Imaris analysis software (Bitplane Inc., South Windsor, USA). The validity and specificity of the nestin primary antibody was tested on human uterine endometrium specimens. Both an isotype control and a secondary antibody control were used as negative controls. For hematoxylin and eosin staining (HE staining), sections were deparaffinized and rehydrated as described above. The slides were then immersed in hematoxylin for 3 minutes followed by immersion in Eosin for 1 minute. After thorough washing, the slides were covered and observed under a light microscope.

Statistical Analysis Quantitative data were analyzed by a two-sample independent *t*-test (SPSS 13 for windows, SPSS Inc. Chicago, Illinois, USA). A value of $P \leq 0.01$ was considered to be significantly different.

RESULTS

With HE staining, it was observed that pterygium specimens displayed more capillaries than normal conjunctiva epithelium (Figure 1A, B). Upon staining with nestin-specific monoclonal antibody, cells at the superficial epithelium displayed bright green fluorescence (Figure 1C-E). Cells close to the stratumbasale were stained significantly less than the superficial cells. The green fluorescence signal was restricted within the cytoplasm without overlapping with nuclei, because nestin is an intermediate filament protein, which is synthesized and stored in the cytoplasm. The average depth of the nestin-stained area within the pterygial epithelium was 1.7 times as thick as that of normal conjunctiva epithelium ($P=0.007$, Figure 1F). The distribution of nestin-positive cells in pterygium was broader than that of

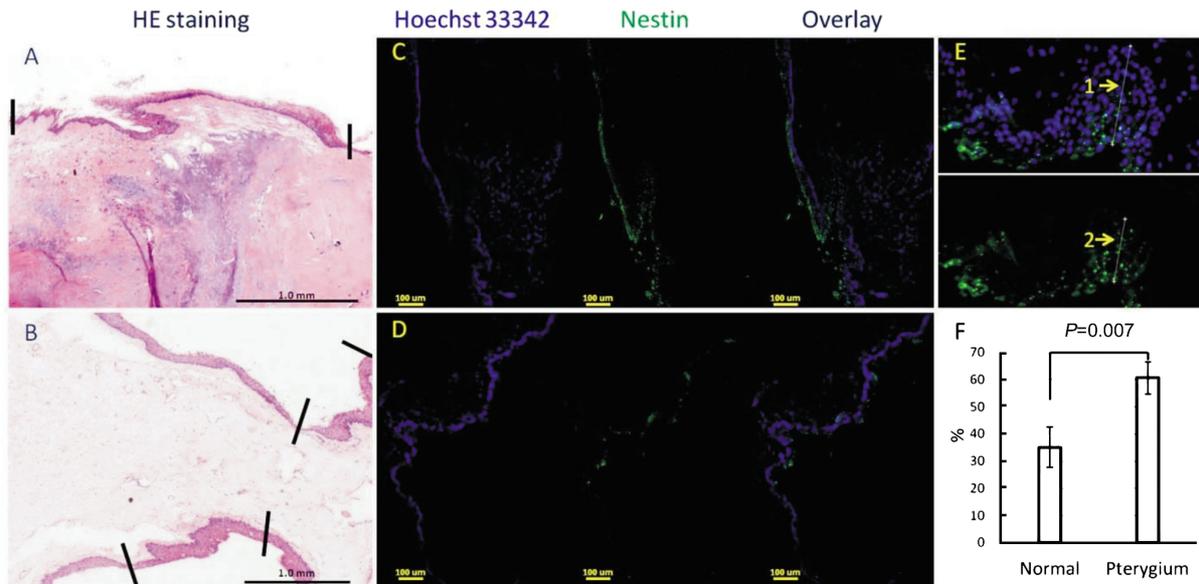


Figure 1 Nestin expression in eye epithelium A: HE stained sections of pterygial epithelium; B: Normal conjunctiva epithelium; C and D: The corresponding fluorescent staining images of Hoechst 33342, nestin as well as the overlay of both Hoechst 33342 and nestin staining for A and B respectively. Nestin staining on pterygium specimens C was significantly brighter and broader than corresponding staining on normal epithelial specimens D. The black lines in A and B indicate the borders of nestin-positive areas observed under microscopy; E: The method of measuring the thickness of fluorescence-stained sections. The '1' represents thickness of Hoechst 33342 stained section, while the '2' represents thickness of nestin-stained section; F: The percentage of thickness of nestin-stained section with respect to thickness of Hoechst 33342 stained section. The percentage of nestin-positive staining area depth=100×1/2. There was significantly thicker nestin-positive area in pterygial epithelium compared to normal epithelium ($P=0.007$). A, B:×40; C, D: ×100; E: ×400.

normal conjunctiva epithelium (Figure 1 A, B).

To explore the relationship between nestin-positive cells and proliferating cells, EdU staining was applied to freshly harvested specimens. EdU staining involves incorporation of the nucleoside analog 5-Ethynyl-2'-deoxyuridine into newly synthesized DNA strands at the S-phase of the cell cycle. Therefore, the Edu signal should overlap with the Hoechst 33342-stained nuclei, but not the nestin-stained cytoplasm. Our observed results were as expected (Figure 2), with EdU-positive cells being detected close to the stratumbasale and capillaries (Figure 2 A, B, white arrow). The EdU-stained cells in pterygial epithelium were interspersed with nestin-positive cells (Figure 2C). Images with different staining combinations indicate the physical distribution of cell subpopulations expressing the different markers (Figure 2C). However, the co-existence of EdU with nestin staining was only observed in 2 out of 9 pterygium specimens and none with normal conjunctiva epithelium specimens.

DISCUSSION

The results showed some interesting data. Firstly, both normal and pterygial human conjunctiva epithelium had sub-populations of nestin-expressing cells, as demonstrated by immunostaining. Similar results were reported in a rabbit model, with approximately 0.4% of all limbal epithelial cells expressing nestin [4]. Nestin was detectable in human limbal

epithelial cells by PCR analysis [12]. These results suggest that nestin-positive cells may represent a minority sub-population of epithelial cells with certain specific functions. As previously discussed, nestin can be generally regarded as a stem cell marker [4,7,9], as well as a cancer marker [6], or even an angiogenesis marker [13] and was also found to be expressed on undifferentiated corneal epithelial cells [14]. It is necessary to further investigate whether nestin-expressing cells in conjunctiva epithelium share similar properties of stem cells or tumor cells, associated with proliferation, migration/invasion and immaturity.

Secondly, pterygial epithelium had a significantly larger sub-population of nestin-positive cells compared to normal conjunctiva epithelium, as indicated by a thicker and broader area stained with green fluorescence. This suggests that an increase in the number of nestin-expressing cells may be related to the development of pterygium. It was previously reported that nestin is transiently expressed by cells during early development *i.e.* eye development [15], but is inducible in adult tissues under pathological conditions, such as scar formation [16], oxygen-induced retinopathy [17] and tumor [18]. Nestin-positive cells may represent immature cells in epithelium, which constitute a niche for maintaining stable epithelial cell numbers in normal conjunctiva. The increase in number of immature cells in pterygial specimens may either

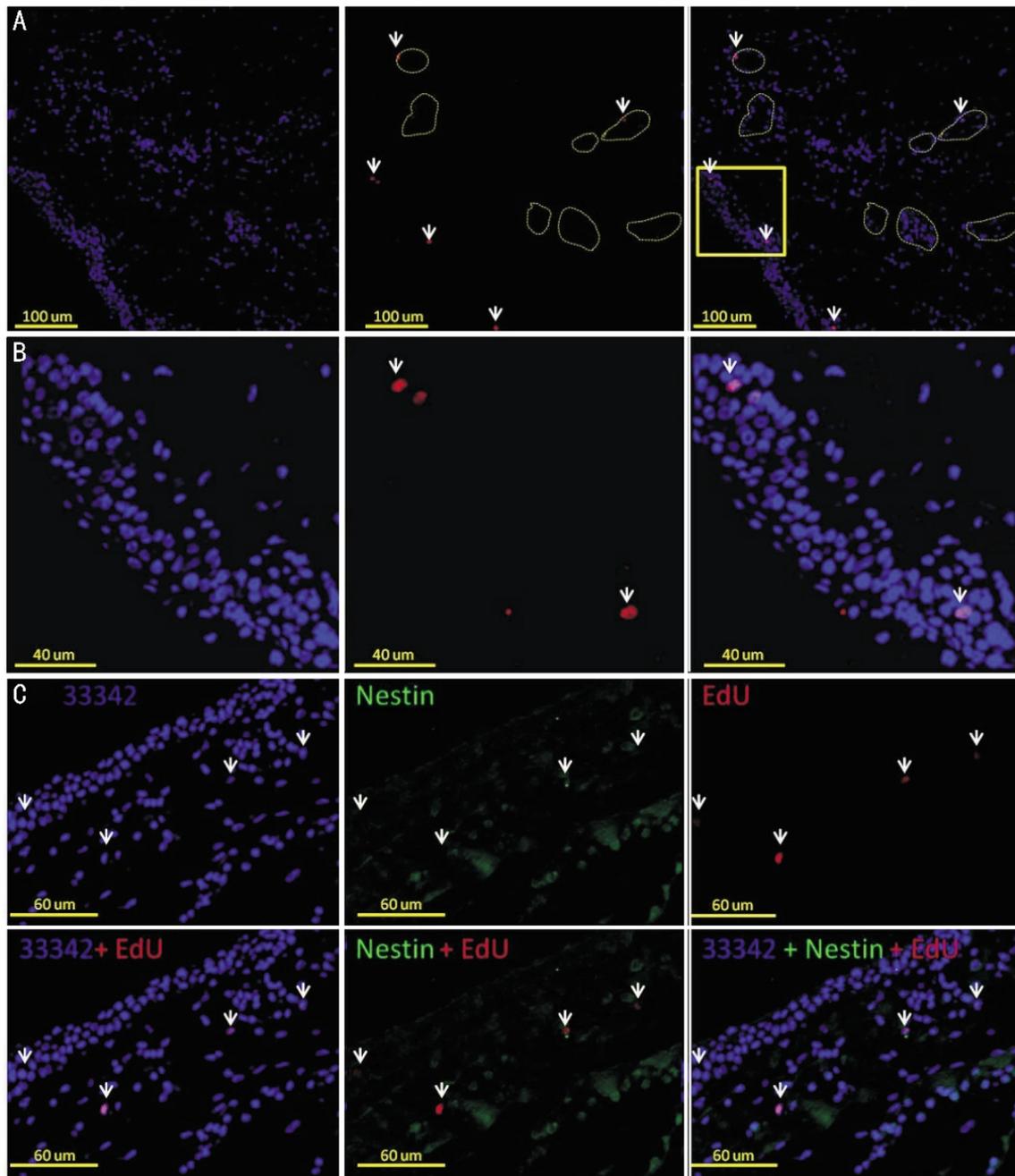


Figure 2 Relationship between EdU –positive and nestin –positive cells in pterygium A: EdU-positive cells are located in close proximity to the stratumbasale of the pterygium epithelium ($\times 200$). Yellow circles represent the capillary area. Arrows indicate EdU-positive cells; B: A blow-up of the EdU-stained areas within the yellow square box inset of A, with and without overlap with the Hoechst 33342-stained nuclei ($\times 400$); C: EdU-positive cells were interspersed with nestin-positive cells ($\times 600$), while nestin-stained cells did not overlap with Hoechst 33342-stained nuclei.

arise from the proliferation of nestin-positive cells or the de-differentiation of normal conjunctiva epithelial cells upon stimulation with ultraviolet irradiation or other pathological stimuli.

To investigate whether nestin-expressing cells can actively proliferate, we utilized double fluorescence staining with EdU to label DNA in mitotic cells. EdU positive cells could readily be identified within pterygial specimens, whereas none could be observed in normal conjunctiva specimens. This is consistent with the hypothesis that pterygium is a

disease involving cell proliferation. Most EdU positive cells were in fact distributed around capillaries and at the basement of the epithelium, which would be the likely location of actively proliferating cells. However, we found that cells expressing both nestin and EdU can only be detected in a few pterygial epithelium specimens. Neither were all nestin-positive cells labeled with EdU, nor were all EdU-labeled cells being surrounded by nestin-positive cells. In fact, nestin-positive cells were mainly localized at the superficial epithelium. Previous studies have also reported

nestin-positive cells to be mitotically quiescent^[11]. These cells could form a niche in close proximity to conjunctiva epithelial stem cells that is responsible for supporting and maintaining stem cells numbers^[7].

In conclusion, this study confirmed a small sub-population of nestin-expressing cells in human conjunctiva epithelium, which is increased in pterygium. Further investigations on nestin-expressing cells in pterygium would likely be helpful in unraveling the mechanism of initiation and development of this disease.

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