

# Expression of WW domain–containing oxidoreductase WWOX in pterygium

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**Purpose:** Pterygium was traditionally regarded as a degenerative disease, but certain characteristics suggest that pterygium is probably premalignant tissue. The human *WWOX* gene, encoding the WW domain containing oxidoreductase (WWOX, FOR, or WOX1), is a candidate tumor suppressor gene. In this study, we investigated the *WWOX* gene and protein expression in pterygium.

**Methods:** Pterygium tissues were obtained from patients (n=16, primary=8, recurrent=8) who received surgical excisions. Each tissue sample was further divided into head and body regions. The *WWOX* gene and protein expression were examined with immunohistochemistry, western blot, and quantitative PCR. For comparison, normal superior temporal bulbar conjunctivas were used as controls.

**Results:** Compared to the controls, upregulation of WWOX and its Tyr33 phosphorylation was observed in the head region of all pterygium specimens. In the head and body of the pterygium specimens, WWOX expression was significantly higher than in the controls. In addition, WWOX expression was stronger in recurrent pterygia than in primary pterygia.

**Conclusions:** Increased WWOX expression, especially in the head region, is probably due to the invasiveness of the pterygium. Our results indicate that WWOX may play a role in pterygium progression and recurrence.

Pterygium is wing-shaped fibrovascular conjunctival tissue encroaching from the bulbar conjunctiva, across the limbus, and onto the cornea. The ultraviolet (UV) radiation of sunlight is a major environmental factor in the development of pterygium [1]. Traditionally, pterygium was considered a degenerative lesion with elastotic degeneration. However, today several tumor-like characteristics, such as the propensity to invade normal tissue, high recurrence rates following resection, and coexistence with secondary premalignant lesions, suggest that pterygium is probably premalignant tissue [2]. Spandidos et al. found that 60% of pterygia exhibited genetic changes that are common in tumor and premalignant cells [3]. Our and previous studies also showed that the pathogenesis of pterygium is closely linked to the *p53* gene mutation [4-7]. Thus, pterygium is considered the result of uncontrolled cellular proliferation, like a tumor.

The *WW domain containing oxidoreductase (WWOX, FOR, or WOX1)* gene is a candidate tumor suppressor gene in chromosome 16q23.3–24.1. It was determined that the *WWOX* gene spanned the common fragile site region FRA16D [8-11].

Abnormalities affecting WWOX at the genomic and expression level have been reported in numerous neoplasia and cancer-derived cell lines including breast, ovarian, esophageal, lung, stomach, liver, pancreas, and hematological malignancies [12-21]. However, in ophthalmology, the role of the *WWOX* gene and the WWOX protein in physiologic, pathological apoptosis, and carcinogenesis remained unknown. To date, no studies of ocular surface disorders regarding WWOX have been conducted. In this study, we investigated the expression of WWOX (WOX1 and its active form, phosphorylated WOX1) in pterygium to provide a molecular basis for better understanding of the pathogenesis in pterygium.

## METHODS

**Ethics statement:** All research protocols were approved by the National Cheng Kung University Hospital (NCKUH) ethics committee and were performed in accordance with the tenets of the World Medical Association's Declaration of Helsinki. This study adhered to the ARVO statement on human subjects.

**Surgical tissue specimens:** Resected specimens (n=16), eight primary and eight recurrent, were obtained from 16 patients who underwent pterygium excision with fibrin glue–assisted conjunctival autografting. Normal conjunctiva was obtained from the superior temporal part while the conjunctival

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TABLE 1. DEMOGRAPHY OF PTERYGIUM SPECIMENS.

Characteristic	Primary	Recurrent	P
Number of eyes	8	8	
Number of patients	8	8	
Gender (% male)	75	75	
Age <sup>a</sup> (years)	60.0±14.0	65.1±9.8	0.345 <sup>b</sup>

<sup>a</sup>Mean ± SD; <sup>b</sup>t test

autograft was harvested. Written informed consent was obtained from each patient. The pterygia specimens were divided into head and body regions. For immunohistochemistry, the specimens (n=8/group) were fixed in formaldehyde, embedded in paraffin, cut into 5 µm sections, and dried overnight at 37 °C. Tissue sections were deparaffinized in xylene, rehydrated through ethanol, and washed in PBS (IX; 150 mM NaCl, 20 mM KCl, 10 mM NaPO<sub>4</sub>, 5 mM KPO<sub>4</sub>, pH 7.4). PBS was also used for all subsequent washes.

**Antibodies and immunohistochemical analysis:** Antibodies against the N-terminal region between the first and second WW domains of WWOX were used for staining [22,23]. Specific species antibodies were also used against the unique COOH termini of human WOX1 [24], and Tyr33 phosphorylation in the first WW domain [25]. The pterygium tissue sections, using these antibodies, were incubated overnight at 4 °C in 2% bovine serum albumin-Tris-buffered saline (BSA-TBS). Sections were extensively washed in PBS before abiotinylated anti-mouse immunoglobulins were added (Vector Laboratories, Burlingame, CA). The primary and recurrent specimens were stained in the same condition. The sections were rinsed and incubated with horseradish peroxidase-conjugated streptavidin (Dako Inc., Carpinteria, CA). The immunoreactivity was revealed by adding 3-amino-9-ethyl-carbazole (Sigma, St. Louis, MO). Assessment of the expression of WOX1 and other indicated family proteins in each human tissue section was as follows: 0, negative (0%); +, focal positive (<5% of sample areas); ++, moderate positive (5–50% sample areas); and +++, diffuse positive (>50% sample areas) [22]. The staining was mainly performed by our research assistant, who also took photos for each sample, and Dr. Yi-Hsun Huang analyzed all the images thereafter.

**Western blot analysis:** Approximately 50 µg total protein was separated in a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk for 1 h at room temperature. After being probed with a primary antibody (p-WOX and WOX1 286–299, provided by Prof. Chang’s laboratory) at 4 °C overnight, the membrane was incubated with a secondary

anti-rabbit IgG antibody (Molecular Probes) for 1 h at room temperature. WWOX expression was normalized to tissue expression of β-actin protein. The signal was detected using an enhanced chemiluminescence reagent (Millipore, Billerica, MA), and the blots were exposed to X-ray film (Fujifilm Medical, Stamford, CT). The experiment was performed in triplicate.

**Real-time quantitative PCR (RT–PCR):** PCR was performed with the ABI Prism 7500 Real-Time PCR System using SYBR Green. The PCR program was as follows: 30 s at 95 °C, followed by 50 cycles of 10 s at 95 °C, 30 s of 60 °C. The 12.5 µl Tag reaction mix included WWOX-specific primers (forward primer, 5'- TCG AAT TCT ATG GCA GCT CTG CGC TAT GCG-3'; reverse primer, 5'- TGC TCG AGC TTA CAA TCT TGG ATG CAA GTA-3') and 1 µl standard plasmid or tissue. The gene expression was normalized based on the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level. GAPDH primers were: Forward: 5'-ACC ACA GTC CAT GCC ATC-3', and reverse: 5'-CAG GTT TCT CCA GGC GGC-3'. The program performed melt curve analysis automatically after the last PCR cycle. All amplifications were performed in triplicate using 1 µl of the first strain cDNA per reaction. The threshold cycle was determined from the three experiments using the GAPDH gene transcription as the reference for normalization. The parameters were then analyzed, and the original concentration of the template was calculated.

**Statistical analysis:** ANOVA was used to compare WWOX expression between different regions in the pterygium and conjunctiva. P values below 0.05 were considered statistically significant.

## RESULTS

**Immunohistochemistry:** The demography of pterygium specimens is summarized in Table 1, and there were no differences between groups. WWOX expression in normal conjunctiva and pterygium (n=8/group) was confirmed with immunohistochemistry. Generally, the 46 kDa WOX1 is the major species found in the extracts of human tissues, organs, and cell lines.

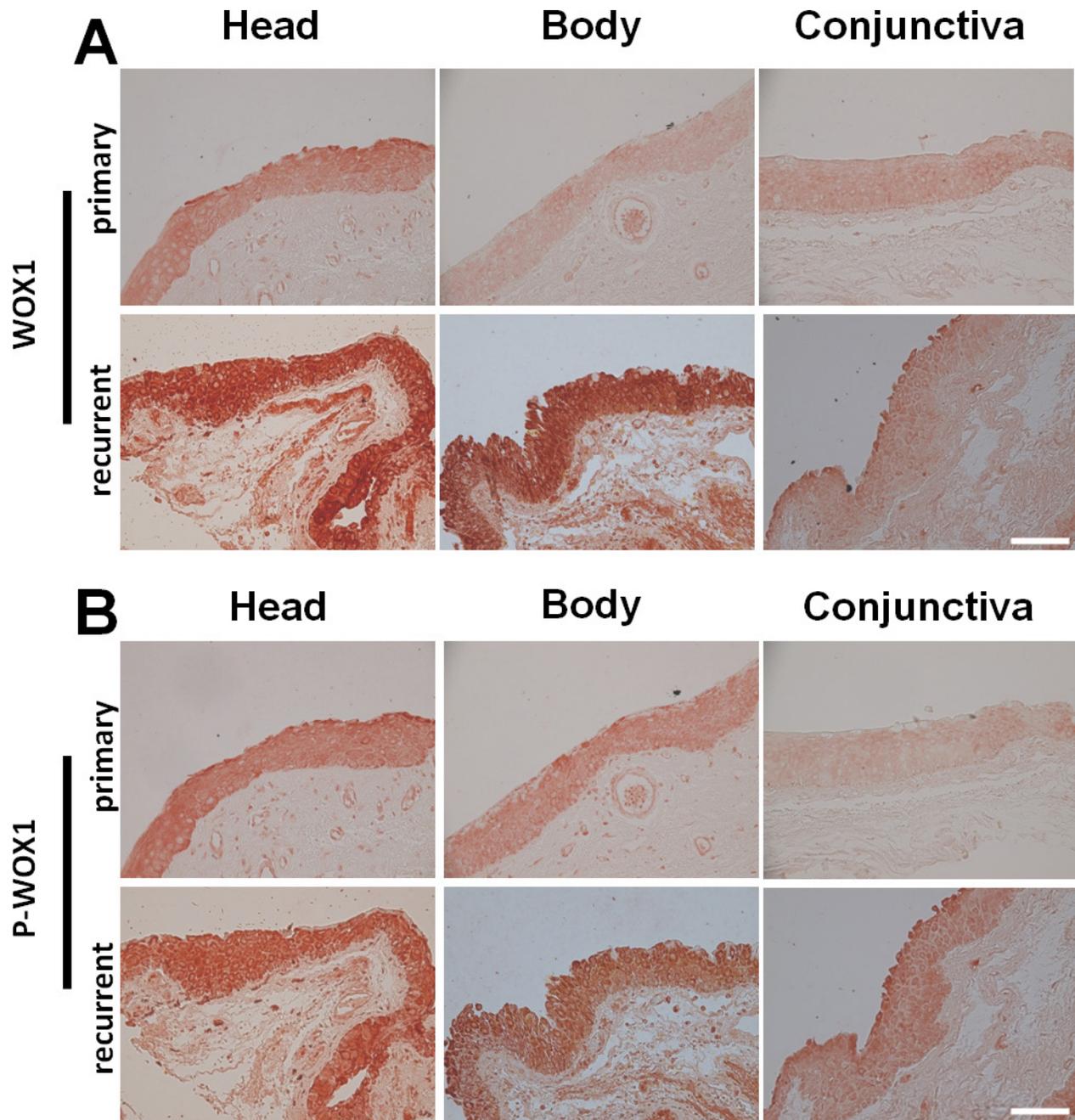


Figure 1. WWOX expression in primary and recurrent pterygium. Immunohistochemical staining of WWOX expression in the head and body of primary and recurrent pterygium and the conjunctiva (n=8/group). **A:** WOX1 and **B:** p-WOX1 are highly expressed in the epithelial cells of the pterygium head, whereas they were less expressed in the body and the conjunctiva. WWOX expression is stronger in recurrent pterygia than in primary pterygia. Magnification: 400X. Bar=25  $\mu$ m.

By using specific antibodies, we found that WOX1 and other family proteins are expressed in the cytoplasm of epithelial cells, and the expression is increased in intensity toward the superficial layers (Figure 1A). Furthermore, cells from recurrent pterygium tissues displayed strong cytoplasmic staining for WWOX compared to that in primary pterygium

(Figure 1A). In all pterygia specimens, including primary and recurrent, WWOX1 expression was significantly upregulated ( $p < 0.05$ ) in the head and body regions compared with the normal conjunctiva (Figure 2). As for the active form, since p-WOX1 plays an important role in the development of breast and prostate cancers before reaching metastasis [26], we also

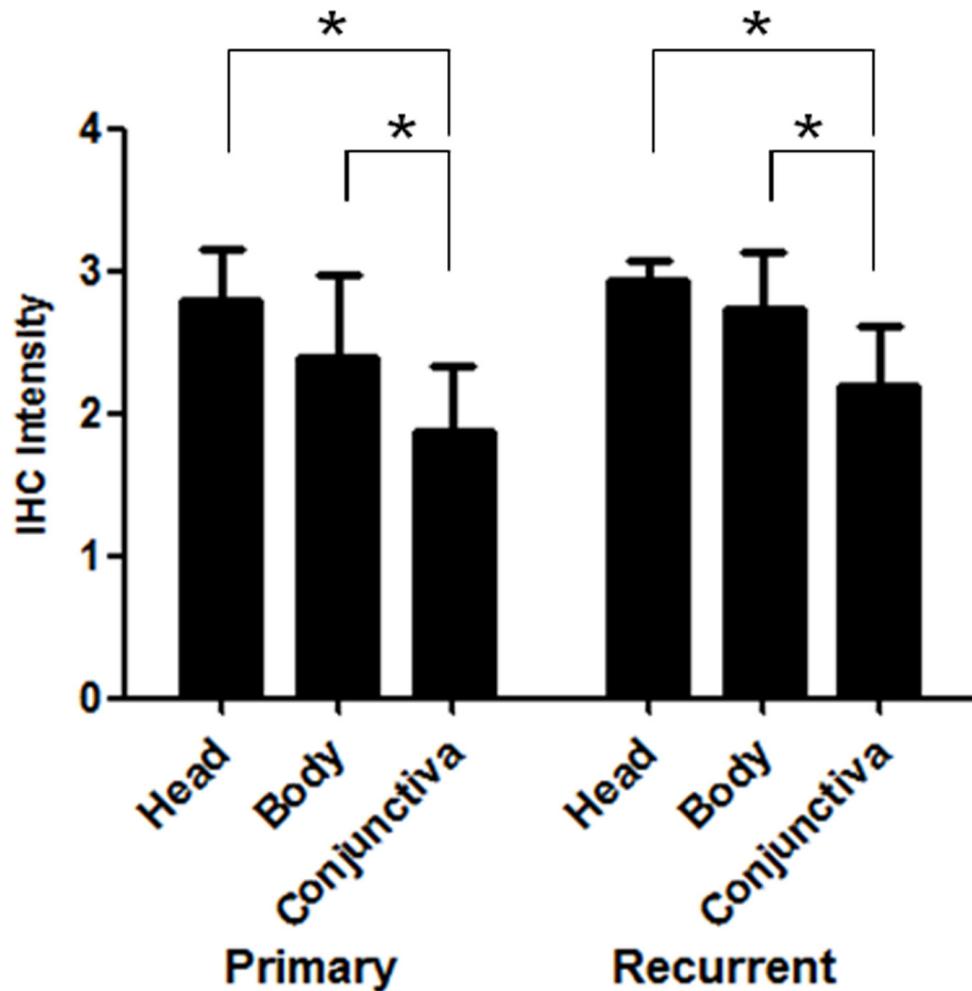


Figure 2. Grading of WWOX expression in primary and recurrent pterygium. Using IHC (n=8/group), WWOX expression was shown to be significantly higher in the head and body of pterygium compared with the conjunctiva. \* p<0.05.

performed immunohistochemistry (IHC) for p-WOX1 (Figure 1B). There was a trend of increased p-WOX1 expression in the head region of the pterygium tissues, but the difference did not reach statistical significance.

*Western blot and real-time quantitative PCR analysis:* Primary pterygium samples were collected, and WWOX protein expression was analyzed with western blots from three independent experiments. WOX1 and p-WOX1 expression was upregulated compared to normal conjunctiva (Figure 3A), and WOX1 expression was significantly higher (p<0.05) in the pterygium compared to the conjunctiva. The average primary pterygium WWOX mRNA expression, after it was normalized to that of GAPDH, was significantly higher in the head and body regions compared to the conjunctiva (Figure 3B).

## DISCUSSION

Previous reports have described WWOX changes based on expression with immunohistochemistry, western blot, and RT-PCR. The present study is the first to analyze WWOX expression in pterygium and normal conjunctiva, using the methods described. In this study, we have determined for the first time that the tumor suppressor WOX1 protein was upregulated in pterygium, whereas the protein is normally present in the conjunctiva. In the 16 cases, WOX1 expression was highly present in the head and body regions of primary (n=8) and recurrent (n=8) pterygium. In contrast, the WOX1 levels were low in the conjunctiva. Upregulated WWOX expression in the pterygium suggested that WWOX was involved in the process of pterygium formation. Interestingly,

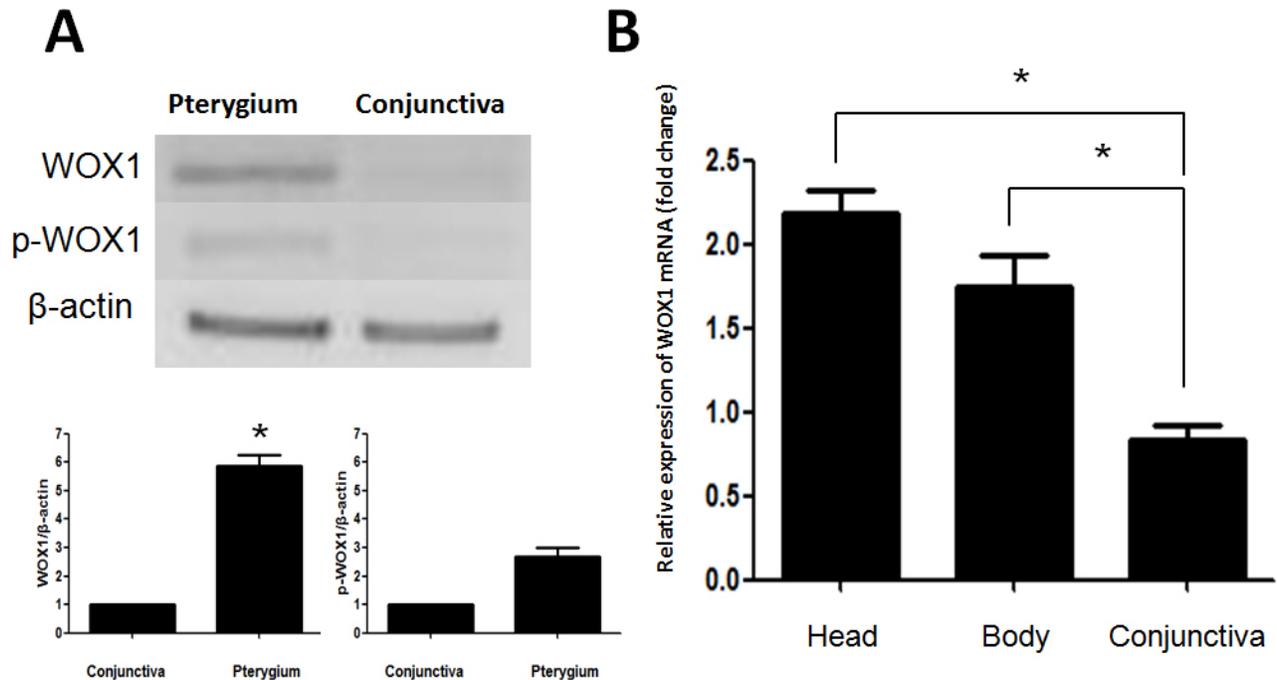


Figure 3. Western blot and RT-PCR analysis of WWOX expression in pterygium. **A:** Western blot analysis of WWOX. Pterygium and conjunctiva lysates (n=8/group) were analyzed with western blot with antibodies against WOX and p-WOX1. Pterygium and conjunctiva lysates (n=8/group) were analyzed with western blot with antibodies against WOX and p-WOX1. The expression levels were normalized with  $\beta$ -actin. Western blots from three independent experiments were analyzed with densitometry. The indicated fold changes represent the density relative to the conjunctiva, \*  $p < 0.05$  compared with the conjunctiva. **B:** The mRNA expression amounts of WOX1 (n=8/group), after normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were significantly higher in the head and body of the pterygium tissue compared with the conjunctiva. \*  $p < 0.05$ .

WOX1 labeling and Tyr 33 phosphorylation also showed a positive correlation with pterygium status; that is, WOX1 expression was highly present in recurrent pterygium and lower in primary pterygium.

We were interested in the relationship between the *WWOX* gene and pterygium due to its chromosomal location and potential tumor suppressor activity. The *WWOX* gene encompasses FRA16D, the second most actively expressed fragile site in humans and a region prone to breakage with an increased risk of carcinogen-induced damage [8]. Since UV light exposure is a major cause of pterygium [27], the susceptibility of FRA16D to UV light or other carcinogen-induced damage [22] may explain the high frequency of *WWOX* gene changes in pterygium, which further implies the possibility of pterygium as a growth disorder especially after UV light exposure. This unique feature of *WOX1* makes it an interesting and potential investigation target for pterygium.

The pathogenesis of pterygium shares the features of many premalignant tissues, including epithelial proliferation, goblet cell hyperplasia, angiogenesis, and inflammation [2,28]. In this study, compared to the controls, upregulation

of WWOX and its Tyr33 phosphorylation was observed in the head region of all pterygium specimens. Our results indicated that WWOX might be involved in the invasiveness and progression of pterygium. Moreover, higher WWOX expression in recurrent specimens (Figure 2) suggested the possibility of premalignant change. In the literature, WWOX expression was decreased or absent in malignant tissues [12-21]. We surmise the possibility of increased expression in pterygium, especially in the head region, might follow a similar pattern as breast and prostate cancers [26]. Briefly, there was initial upregulation and activation of WOX1 to support the carcinogenesis to premetastatic state, followed by downregulation of WOX1 for cancer progression [26]. Furthermore, Chang et al. found that WOX1 induced apoptosis in a caspase-independent manner [29], and WOX1 can mediate cell death synergistically with p53 [25]. Without WOX1, p53 apoptosis is abolished. Nonetheless, WOX1 can act alone in causing cell death with or without p53 [25]. Since WWOX is mainly expressed in the cytoplasm, we surmise that during pterygium progression, the functional p-WOX1 might not enter the nucleus. Therefore, p53 does not activate, and pterygium progresses. However, further studies are

needed to provide a clearer pathway regarding WWOX and p53 in pterygium. We suggest the upregulation of WWOX in the head region is in response to the invasion of pterygium.

This study had several limitations. First, we did not correlate pterygium size, volume, vascularity, and rate of progression with WWOX expression. These factors may be associated with the rate of pterygium proliferation; therefore, a comparison of primary and recurrent pterygium was not provided in this paper. Second, as previously described, WOX1 is related to the expression of p53 [25], a possible factor in pterygium formation and recurrence. Although the pathogenesis of pterygium is closely linked to the p53 gene mutation [6], the relationship between WOX1, p53, and pterygium was not examined in this study. Future studies should be performed to determine the correlation. Third, we did not have controls from patients who did not have any evidence of pterygia for comparison. In this study, we chose the conjunctiva from the normal area of the same eye to decrease the baseline individual difference in WWOX expression. With this preliminary result, we can conduct additional studies to evaluate the difference in WWOX between normal and pathological eyes.

**Conclusion:** In conclusion, this is the first study regarding WWOX expression in pterygium. Although the risk of pterygium being malignant is rare, in this paper we pointed out that with WWOX expression, which has a tumor suppression effect, pterygium may not become malignant. Increased WWOX expression, especially in the head region, is probably due to the invasion of pterygium. Our results indicate that WWOX may play a role in pterygium progression and recurrence; furthermore, pterygium could be considered as a proliferative or premalignant lesion, rather than a degenerative disease. However, further studies are needed to provide more information regarding the role of the *WWOX* gene in the pathogenesis of pterygium.

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