Original Article

# Expression of p53 and Ki-67 proteins in patients with increasing severity and duration of pterygium

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**Purpose:** Pterygium is a triangular fibrovascular subepithelial ingrowth of degenerative bulbar conjunctival tissue over the cornea. It is now considered to be a result of uncontrolled cellular proliferation as overexpression of p53 protein and Ki-67 nuclear protein was found in the epithelium. This study was done to find the expression of p53 and Ki-67 with the severity and duration of the pterygium to explain the etiopathogenesis. **Methods:** Data were analyzed from 43 Indian participants of all age groups. All patients were divided according to the severity of pterygium (mild, moderate, and severe groups) and according to the duration of pterygium (<4 years and >4 years). The samples were studied by immunohistochemistry by using antibodies against p53 and Ki-67 proteins considering >5% expression as significant. **Results:** Of 43 cases, p53 and Ki-67 expression were positive in 33 cases. In mild, moderate, and severe cases p53 positivity was 33.3%, 78.4%, 100%, respectively. P53 expression increased with duration, 79.3% positive in <4 years, and 92.9% positive in >4 years. With increasing severity of pterygium, mild, moderate, and severe cases, Ki-67 positivity was 66.7%, 78.37%, 66.7%, respectively. Ki-67 expression with duration, 79.3% positive in <4 years, and 85.7% positive in >4 years of the duration of pterygium with no statistical significance. **Conclusion:** Our study revealed that with increasing duration and severity of pterygium, p53 expression was observed to be increasing. Ki-67 expression increased with the duration of pterygium but not with the severity.

**Access this article online**

**Website:** www.ijo.in

**DOI:** 10.4103/ijo.IJO_1034_20

**Quick Response Code:**

**Key words:** Immunohistochemistry, Ki-67, P53, paraffin embedding, pterygium

Pterygium is an abnormal, proliferative overgrowth of fibrovascular tissue that develops from the bulbar conjunctiva over the cornea. It is well known that pterygium is one of the most common eye diseases. In advanced cases, it can induce significant astigmatism and decrease visual function caused by loss of corneal transparency.[1,2] The prevalence rates of pterygium mentioned in different population-based studies vary greatly from 2.8% to 33.0%.[3,4] The exact cause of pterygium remains unclear; however, some risk factors like residence, age, race, sex, sunlight exposure were associated with risk for pterygium.[5-10] Among these long-term ultraviolet radiation exposure was the most important.[1,2] With respect to the mechanisms, many genetic factors have been suggested in many studies. Some genes associated with DNA repair play an important role in pterygium development.

Pterygia share many similar traits with tumors, such as cell proliferation, invasion of the cornea, and recurrence after resection.[11] however, after abnormal expression of p53 protein was found in the epithelium, pterygium is now considered to be a result of uncontrolled cellular proliferation, like a tumor.[12-18] p53 is a well-known tumor suppressor gene that regulates the cell cycle and is involved in DNA repair, synthesis, cell differentiation, and apoptosis.[19] The p53 gene product is a nuclear phosphoprotein that binds to the DNA and can be identified by immunohistochemical (IHC) staining, using a specific monoclonal antibody.[20] The proliferative cellular activity can be evaluated by the detection of Ki-67 nuclear protein that is essential for the maintenance of the cell cycle. The expression of both p53 and Ki 67 both have been found in the pterygium. This study was done to find the correlation between p53 and Ki 67 expression with the severity and duration of the pterygium. This will be helpful to explain the etiopathogenesis, treatment planning, and prevention. No such study has been done previously in our part of the country.

## Methods

### Study design and population

It was a hospital-based, cross-sectional epidemiological study, which was conducted in the Department of Ophthalmology for 16 months. In this study patients with primary pterygium of all age groups were included. Patient having illnesses which could affect the parameters been tested such as any conjunctival and systemic malignancy, any previous ocular surgery (squint, cataract), previously treated pterygium with or without mitomycin, any case of recurrent pterygium, and patients with moderate to the severe dry eye were excluded from the study.

### Examination methods and the definition of pterygium

The study was undertaken after obtaining ethical clearance from the institute ethics committee. Written and informed consent was taken. Ocular examination was done for all patients which included best-corrected visual acuity, intraocular pressure measurement by non-contact tonometer (NT 510, Nidek Corporation, USA), ocular movements, slit-lamp

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Received: 19-Apr-2020 Revision: 18-Sep-2020
Accepted: 28-Sep-2020 Published: 16-Mar-2021



**Cite this article as:** Mahesh M, Mittal SK, Kishore S, Singh A, Gupta N, Rana R. Expression of p53 and Ki-67 proteins in patients with increasing severity and duration of pterygium. Indian J Ophthalmol 2021;69:847-50.

biomicroscopy (SL120, Carl Zeiss GmBH, Germany), Schirmer's test without anesthesia, tear-film-break up time (TFBUT) and fundus examination. Vitals were recorded and routine laboratory investigations, including complete blood counts, random blood sugar, bleeding time, clotting time, and activated partial thromboplastin time were done for all patients. At diagnosis, the staging of pterygium was done, based upon the position of pterygium tissue in relation to the limbus.[21]

Grade 0: Pingeculum, posterior to the limbus
Grade I: Tissue involvement to the limbus
Grade II: Tissue just on to the limbus
Grade III: Tissue between the limbus and the pupillary margin
Grade IV: Tissue central to the pupillary margin.

Patients were divided according to the severity of pterygium into mild (grade1,2), moderate (grade 3), and severe (grade 4). They were also divided according to the duration of pterygium as recalled by them, into two arms to compare the gene expression in pterygia of prolonged duration as compared to those relatively recent in onset, respectively. The cut-off duration between the two groups was also reported.

The pterygium tissue excised during surgery was marked and harvested from 43 patients who underwent pterygium surgery. All specimens of pterygium tissue were formalin-fixed and paraffin-embedded. Sections (3 μm of thick) were cut, mounted on glass, and dried overnight at 37°C for IHC. All sections were deparaffinized in xylene, rehydrated through a graded series of alcohols, and washed in phosphate-buffered saline. This buffer was used for all subsequent washes. IHC using the streptavidin-biotin peroxidase method was performed on paraffin-embedded tissues using an anti-p53 monoclonal antibody, DO7 (diluted 1:100; DakoCytomation, Glostrup, Denmark), which recognizes the N-terminus of the human p53 protein (amino acids 19 to 26). Besides, the antibody reacts with both types of wild and many mutant p53 proteins. The IHC results were scored for the percentage of cells with positive reactivity. P53 and Ki-67 levels were considered positive if they were found to be overexpressed in more than 5% of cells [Figs. 1 and 2, respectively]. Internal validation of the IHC technique was done by comparison of staining in samples with 1 mm × 1 mm normal supero-temporal conjunctiva taken from the same patient while harvesting the conjunctival-limbal autograft for transplantation. To annul the difference in p53 and Ki-67 expression within the samples of pterygium, all sections were drawn from the head of the pterygium tissue.

### Data analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS version 21, IBM Inc). Descriptive data were reported for each variable. Shapiro-Wilk test was used to check if the variables were following a normal distribution. Subsequently, bivariate analyses were performed using the parametric independent *t* test. Chi-square test was used for qualitative analysis, taking *P* value <0.05 statistically significant.

## Results

Of 43 cases included in the study, 31 were males (Mean ± SD age 44.71 ± 12.5 years) and 12 were females (Mean ± SD age 51.25 ± 9.81 years). Of 43 cases, 3 (7%) were mild, 37 (86%) were moderate and 3 (7%) were severe.

Internal validation of the staining technique showed 100% accuracy in staining for p53 as well as Ki-67, wherein the expression of both these gene products was detected to be < 5% in normal conjunctiva.

### Severity

In cases of mild pterygium ($n$ = 3), p53 was positive in one case. In moderate pterygium ($n$ = 37), p53 was positive in 29 cases. In severe pterygium ($n$ = 3), p53 was positive in all 3 cases. No statistically significant relation was noted in the p53 expression with the duration of pterygium [$P$ = 0.451, Table 1].

In cases of mild pterygium ($n$ = 3), Ki-67 was positive in 2 cases. In moderate pterygium ($n$ = 37), Ki-67 was positive in 29 cases. In severe pterygium ($n$ = 3), Ki-67 was positive in 2 cases. No statistically significant relation was noted in the Ki-67 expression with the severity of pterygium [$P$ = 0.607, Table 1].

### Duration

Our patients recalled that their pterygia were present for 3.4 ± 1.8 years (Mean ± SD). As per our methodology, the 43 patients were divided into three categories viz. 14 cases with prolonged history and 29 cases with relatively recent onset. The cut off between the two groups was kept as four years.

With <4 years of duration ($n$ = 29), p53 expression was positive in 23 cases. With >4 years of duration ($n$ = 14), p53 expression was positive in 13 cases. No statistically significant relation was found in the p53 expression with the duration of pterygium [$P$ = 0.25, Table 2]

With <4 years of duration ($n$ = 29), Ki-67 expression was positive in 23 cases. With >4 years of duration ($n$ = 14), p53 expression was positive in 12 cases. No statistically significant relation was found in the Ki-67 expression with the duration of pterygium [$P$ = 0.478, Table 2].

The Z-ratio was calculated to determine the significance of the difference between the two independent proportions of p53 and Ki-67 positivity. The difference between the proportions was -0.0374 the Z-statistic was -0.418. The corresponding $P$ = 1 (2-tailed probability) implied that the difference between two proportions was not significant [Table 3].

## Discussion

Pterygium has been long considered to be a chronic inflammation occurring in the limbal conjunctival vessels or conjunctival epithelium. Various opinions regarding the mechanism of pterygium development have been concluded. But the exact pathogenesis is still a dilemma. Pathophysiology of pterygium had reported exposure to UV rays, is perhaps the most accepted risk factor for the occurrence of pterygium.[22] In many of the latest research, the expression of biomarkers of proliferation and apoptosis has been studied. The p53 gene is located on the short arm of chromosome 17 and its main function is as a tumor suppressor gene. The p53 gene controls the cell cycle and is involved in DNA repair and synthesis, cell differentiation, and apoptosis. Mutation in the p53 gene is believed to lead to increased stability of the protein, allowing its more pronounced immunohistochemical detection. UV radiation can cause mutation in genes such as p53, which when inactivated through mutation and loss of heterozygosity can lead to cell proliferation and genomic instability.[20]

Weinstein *et al*. concluded that pterygium is a growth disorder rather than a degeneration in their study. The abnormal p53 expression in the pterygium samples might suggest that they contained transformed cells and that there was a failure in the regulation and control of the cell cycle. Previous studies found no significant difference in p53 expression between primary pterygia samples and those of recurrent pterygia.[17] Anthwal *et al*. revealed that of 29 pterygia specimens that stained positive

**Table 1: Comparison of P53 and Ki 67 protein expression among different grades of Pterygium**

| | | | P53 | | | Ki 67 | | |
|---|---|---|---|---|---|---|---|---|
| | | | Positive | Negative | Total | Positive | Negative | Total |
| Grade | Mild | Count | 1 | 2 | 3 | 2 | 1 | 3 |
| | | %within grade | 33.33% | 66.66% | 100% | 66.7% | 33.3% | 100.0% |
| | Moderate | Count | 29 | 8 | 37 | 29 | 8 | 37 |
| | | %within grade | 78.4% | 21.6% | 100% | 78.37% | 21.62% | 100% |
| | Severe | Count | 3 | 0 | 3 | 2 | 1 | 3 |
| | | %within grade | 100.0% | 0.0% | 100.0 | 66.7% | 33.3% | 100.0% |
| Total | | Count | 33 | 10 | 43 | 33 | 10 | 43 |
| | | %within grade | 76.74% | 23.25% | 100% | 76.74% | 23.25% | 100.0% |
| *P* | | | | 0.451 | | | 0.607 | |

**Table 2: Distribution of P53 and Ki 67 protein expression according to different duration**

| Duration | | | P53 | | | Ki 67 | | |
|---|---|---|---|---|---|---|---|---|
| | | | Positive | Negative | Total | Positive | Negative | Total |
| | <4 years | Count | 23 | 6 | 29 | 23 | 6 | 29 |
| | | %within duration | 79.3% | 20.7% | 100.0% | 79.3% | 20.7% | 100.0% |
| | >4 years | Count | 13 | 1 | 14 | 12 | 2 | 14 |
| | | %within duration | 92.9% | 7.1% | 100.0% | 85.7% | 14.3% | 100.0% |
| Total | | Count | 36 | 7 | 43 | 35 | 8 | 43 |
| | | %within duration | 83.7% | 16.3% | 100.0% | 81.4% | 18.6% | 100.0% |
| *P* | | | | 0.255 | | | 0.478 | |

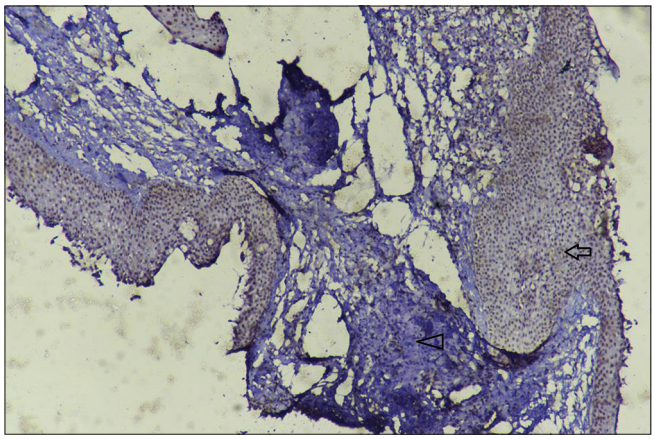

**Figure 1:** Photomicrograph showing immunohistochemical staining with DO-7 of a section of p53 positive pterygium specimen at 40×. Cells staining darkly are p53 positive (arrow head) compared to those which are p53 negative (arrow)

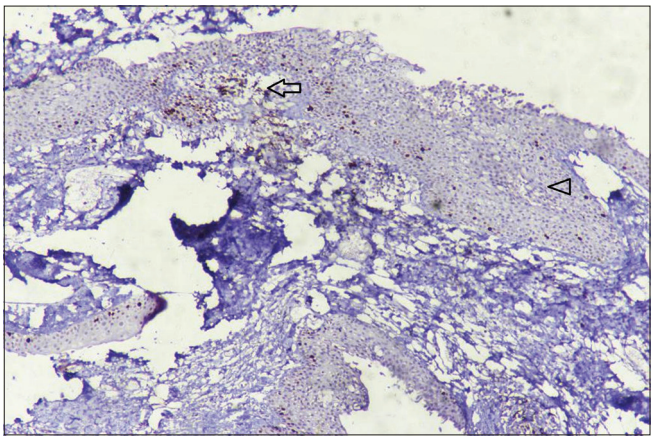

**Figure 2:** Photomicrograph showing immunohistochemical staining with Ki-67 labelling antibody of a section of Ki-67 positive pterygium at 10×. Cells staining darkly are Ki-67 positive (arrow) compared to those which are Ki-67 negative (arrowhead)

for p53 gene expression, the altitudinal variation in p53 gene expression in pterygium was found insignificant in their study.[23]

The proliferative cellular activity can be evaluated by the detection of Ki-67 nuclear protein that is essential for the maintenance of the cell cycle. The expressions of p53 and Ki-67 proteins are useful markers of early premalignant lesions.[24,25] Liang K *et al*. studied that number of immunopositive cells for Ki-67 was found to be significantly higher in the epithelial layer of pterygium than normal conjunctiva.[26]

In our study of 43 cases, p53 and Ki-67 expression was positive in 33 cases and was negative in 10 cases. In mild (grade 1, 2), moderate (grade 3) and severe (grade 4) cases p53 positivity was 33.3%, 78.4%, and 100%, respectively. It implies that with increasing severity, p53 expression seems to increase but without statistical significance (p value = 0.451). With <4 years of duration (no of cases = 29), p53 expression was positive in 23 cases (79.3%) and was negative in 6 (20.7%) cases. With >4 years of duration (total no of cases = 14), p53 expression was positive in 13 (92.9%) cases and was negative in 1 (7.1%) case. Our study revealed that with increasing duration of pterygium p53 expression was observed to increase but without statistical significance (p value = 0.255).

**Table 3: Correlation between expression of p53 and Ki-67 proteins**

| Biomarkers | Count(%) | | | p-value |
|---|---|---|---|---|
| | Negative | Positive | Total | |
| P53 | 10 (23.3%) | 33(76.7%) | 33(76.7%) | 1 |
| Ki-67 | 10 (23.3%) | 33(76.7%) | 33(76.7%) | |

Overall p53 expression was increased in pterygium tissue with both severity and duration of pterygium and it supports that mutations in genes such as the p53, result in the abnormal pterygial epithelium. So, pterygium can also be considered a neoplastic-like growth disorder.

The severity of pterygium was also positively associated with Ki-67 expression with positive in 33 cases and negative in 10 cases. With increasing severity of pterygium mild (grade 1, 2), moderate (grade 3), and severe (grade 4) cases ki-67 positivity was 66.7%, 78.37%, and 66.7%, respectively. The maximum percentage of expression of Ki-67 was observed in the moderate type of pterygium. Unlike p53, Ki-67 expression did not increase significantly with the severity of pterygium ($P$ = 0.607). Ki-67 expression was positive in 23 (79.3%) cases and negative in 6 cases (20.7%) with <4 years of duration ($n$ = 29). It was positive in 12 cases (85.7%) and negative in 2 cases (14.3%) with >4 years of duration of pterygium ($n$ = 14). It implies that with increasing duration of pterygium Ki-67 expression also increases but not significantly ($P$ = 0.478). The present study revealed that more positive expression of Ki-67 supports the hypothesis that pterygium is a proliferative rather than a degenerative condition as Ki-67 is a marker of proliferation.

The limitations of this study are unequal number of individuals in the groups of severity and duration of pterygium, and a small sample size.

## Conclusion

To conclude, the present study tried to find the correlation of apoptosis-related biomarkers (p53) and proliferation marker (Ki-67) with the duration and severity. The results indicate that the expression of cell proliferation (Ki-67) increases with duration of pterygium; and apoptosis (p53) increases with duration and severity of pterygium. As there has been a marked difference in the number of cases in mild, moderate, severe cases categories; and the sample size being small, therefore statistical significance concerning severity and duration could not be drawn and further studies are needed to identify and substantiate potential genetic factors that may be useful to predict the same.

### Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

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