



Oxidative stress in pterygium: relationship between p53 and 8-hydroxydeoxyguanosine

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Purpose: Ultraviolet (UV) radiation is known to cause oxidative DNA damage and is thought to be a major factor implicated in the pathogenesis of pterygium, a benign invasive lesion of the bulbar conjunctiva. Among all the photooxidative DNA products, 8-hydroxydeoxyguanosine (8-OHdG) is regarded as a sensitive and stable biomarker for evaluating the degree of DNA damage. The protein p53 is a major cell stress regulator that acts to integrate signals from a wide range of cellular stresses. UV radiation can cause mutations in the p53 tumor suppressor gene that, when inactivated through mutation and loss of heterozygosity, can lead to cell proliferation and genomic instability. In many types of UV-radiation damaged cells, p53 is overexpressed and immunohistochemically detectable. Recent data on tissues exposed to factors inducing oxidative stress have provided evidence of the concomitant presence of increased levels of 8-OHdG and protein p53. To verify a possible significant association between p53 and 8-OHdG, we examined a series of 31 Ecuadorian pterygia for the expression of the two markers. Moreover, we evaluated if clinical variables such as patient's age, gender, geographic location, and disease stage, might play a role affecting the 8-OHdG and p53 immunohistochemical staining results.

Methods: Primary pterygium samples were treated for immunohistochemical evaluations of 8-OHdG and p53 protein. Mouse monoclonal antibodies to 8-OHdG and p53 were used. Statistical analyses were performed using the SPSS 12 statistical software package.

Results: In our study, 21 (67.74%) pterygial samples were positive for 8-OHdG staining, 11 (35.48%) specimens were positive for p53 expression, and all negative control samples showed no staining. The staining for 8-OHdG was limited to the nuclei of the epithelial layer. No substantial staining was visible in the subepithelial fibrovascular layers. No differences in the pattern of staining between 8-OHdG and p53 were observed. All samples positive for p53 (11/31, 35.48%) were also positive for 8-OHdG immunostaining, and all specimens negative for 8-OHdG (10/31, 32.26%) were also negative for p53. When analyzed by Fisher's exact test, 8-OHdG expression was significantly associated with p53 positivity ($p=0.0049$). Student's t-test demonstrated statistically significant association between the expression of p53 and age ($p=0.02$). The correlation between the two markers and the other clinical variables revealed no statistically significant association.

Conclusions: Although pterygium is a lesion with limited local invasion and an inability to metastasize, the concomitant presence of altered p53 in 8-OHdG-immunoreactive cells could provide evidence of apparent genetic instability, which is in contrast to its benign clinical course.

Pterygium is a common ocular surface disease, apparently only observed in humans [1,2]. It is a chronic condition characterized by the encroachment of a fleshy, wing-shaped or triangular overgrowth of the bulbar conjunctiva into the cornea, more often on the nasal than the temporal side [2-5]. It has been proposed that the typical location of pterygia is due to corneal focusing of incident sunlight on the medial limbus [6-8]. Progression of the lesion with central migration into the visual axis results in vision loss [9,10]. It is generally considered an inflammatory and degenerative lesion, consisting

of a superficial growth of a highly vascularized loose connective tissue covered by an epithelium, which shows alternately thickening or thinning, with elastoid and basophilic degeneration of the underlying connective tissues [11,12].

Recent studies have provided evidence that a genetic component might be involved in the pathology of pterygium [13-15], while antiapoptotic mechanisms [16,17], cytokines [18-20], growth factors [21,22], angiogenic factors [23], extracellular matrix remodeling [24], immunologic mechanisms [12,25], and viral infections [15,26-28] have all been implicated in its pathogenesis. Studies by Clear et al. [29] suggest that pterygia may represent a precancerous condition of the mucosal epithelium, analogous to cutaneous actinic keratosis. Since pterygium can recur aggressively after removal, it has been suggested to be a neoplastic-like growth disorder and

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not merely a simple degenerative condition of the conjunctiva [21,30,31].

Epidemiological studies indicate that chronic exposure to the sun, and most probably ultraviolet B-light (UV-B), is an important factor in the development of pterygia [6,32-34]. This hypothesis is supported by epidemiological data, ray-tracing models, and histopathological changes that share common features with UV-damaged skin. Several aspects of cutaneous malignant melanoma resemble processes that characterize pterygium. Although this is a potentially fatal condition, the most important environmental risk factor associated with this tumor is UV exposure, and, like pterygium, there appears to be a familial component [2]. Pterygium is prevalent in populations living in peri-equatorial and tropical regions and in laborers who work outdoors or in specific factory environments [35]. The detrimental effects of UV irradiation are either directly due to a UV phototoxic effect or indirectly to formation of radical oxygen species (ROS). ROS, causing so-called oxidative stress, are harmful to cells, because they injure cellular DNA, proteins, and lipids. Among numerous types of oxidative DNA damage, the formation of 8-hydroxydeoxyguanosine (8-OHdG) is a ubiquitous marker of oxidative stress [36,37]. Based upon the premise that pterygium is a UV-related lesion, Tsai et al. [38] recently demonstrated the presence of 8-OHdG in pterygium, providing evidence that oxidative stress plays an important role in the development of pterygium.

The protein p53 is a major cell stress regulator that acts to integrate signals from a wide range of cellular stresses. Detection of DNA damage or replication errors may lead to cell cycle arrest and apoptosis if the damage cannot be repaired. This mechanism prevents the accumulation of potentially oncogenic DNA mutations. UV radiation can cause mutations in genes such as the p53 tumor suppressor gene that, when inactivated through mutation and loss of heterozygosity, can lead to cell proliferation and genomic instability [39]. Studies on p53 in pterygia have provided inconsistent results. Some investigators have reported overexpression of p53 in pterygium [16,30,31,40-43], although lower levels of p53, using ELISA [44] or other immunohistochemical techniques [45] have also been reported. Reisman et al. [39] demonstrated that the p53 gene has undergone monoallelic deletions.

In a previous study on rat fibroblasts exposed to substances inducing oxidative stress, increased levels of 8-OHdG were found to be accompanied by changes in p53-related pathways involved in cell proliferation and apoptosis [46]. Kim et al. [47] reported the immunohistochemical staining of p53, Fas, and 8-OHdG in UVB-exposed living skin equivalent. To verify a possible significant association between p53 and 8-OHdG, we used immunohistochemistry to examine a series of 31 Ecuadorian pterygia for the expression of the two markers. Moreover, we statistically evaluated clinical variables such as age, patient's gender, geographic location, and disease stage, to see if they might play a role affecting the 8-OHdG and p53 immunohistochemical staining results.

TABLE 1.

Variables	Number of patients
8-OHdG expression	
Positive	21
Negative	10
p53 expression	
Positive	11
Negative	20
Gender	
Male	11
Female	20
Age	
<42*	17
≥42*	14
Disease stage	
Quiescent	8
Inflamed	23
Residency	
Urban	12
Rural	19

This table presents clinical variables of the study's participants and the immunohistochemical analysis. The asterisk indicates the median value.

METHODS

Primary pterygia were harvested from 31 patients (11 males and 20 females), whose ages ranged from 21 to 68 years (mean age, 43.13; standard deviation, 2.42). Nineteen patients lived in the countryside and 12 resided in an urban setting; they were all outdoors workers. All patients underwent excision by bare sclera technique at the Department of Pathology, Cancer Center of Solca, Cuenca, Ecuador. Most of the lesions were located on the nasal side and only the head of primary pterygium was used as pterygium sample. The study included 23 inflamed and 8 quiescent lesions. Normal conjunctiva samples as controls were collected from medial conjunctiva of five patients without pterygium and pinguecula while undergoing cataract surgery. Patients did not receive any medication prior to surgery, except for a topical anesthetic, and no drugs or chemical agents were used during intervention. The study protocol was approved by the local research ethics committee, and informed consent was obtained from all volunteers in this study, after explanation of the nature of the research; complete information on patients was available in all cases.

Tissue segments were fixed by immersion in cold 10% formalin in 0.2 M phosphate buffer, pH 7.3, for 4-6 h., and processed for paraffin embedding. Microtome sections (6-7 µm) were treated for the immunohistochemical demonstration of p53 using the streptavidin-biotin alkaline phosphatase method. Briefly, they were rehydrated in PBS, and antigen retrieval was performed by microwave heating for 20 min (4x5 min) in 10 mM citrate buffer solution (pH 6.0). Sections were

treated for 45 min with 10% normal horse serum (NHS) in PBS. Mouse monoclonal antibody to human p53 protein (clone DO-7, 1:50; Dako, Glostrup, Denmark) was used as primary antiserum and incubated for 60 min at room temperature, while biotinylated anti-mouse IgG was used as secondary antiserum (1:200; Vector Laboratories, Burlingame, CA) by incubation for 30 min at room temperature. The sections were further incubated in alkaline phosphatase streptavidin (1:1000; Vector Laboratories, Burlingame, CA) for 30 min at room temperature, reacted with Fast Red Substrate System (Dako) and then counterstained with Mayer hematoxylin and mounted in glycerol gelatin (Sigma, St. Louis, MO).

Adjacent microtome sections were treated for the immunohistochemical demonstration of 8-OHdG, using the Dako EnVision™ +System peroxidase. Antigen retrieval was performed by microwave heating for 20 min (4x5 min) in 10 mM citrate buffer solution, pH 6.0. After gradual cooling for 20 min, the slides were treated with 10 µg/ml proteinase K (Roche, Nutley, NJ) in PBS, pH 7.4 for 40 min at 37 °C and then incu-

bated in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.4 mM NaCl, 100 µg/ml RNase for 15 min at 37 °C. To denature DNA, slides were soaked in 4 N hydrochloric acid for 7 min and then neutralized by incubation in 50 mM Tris-base for 5 min at room temperature. To block nonspecific sites, specimens were incubated in PBS containing 10% fetal bovine serum for 1 h at 37 °C; they were then treated with mouse monoclonal anti-8-OHdG (1:300; Trevigen, Gaithersburg, MD) in PBS, pH 7.5 containing 10% fetal bovine serum overnight at 4 °C. Samples were further incubated with goat antimouse immunoglobulins (Ig) conjugated to peroxidase labeled dextran polymer (Dako EnVision™ +System, Carpinteria, CA) for 30 min at room temperature, reacted with 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, MO) for 3 min, dehydrated in graded alcohols, clarified in xylene and mounted in Entellan (Merck, Frankfurter, Germany). In both experiments, the sections were thoroughly rinsed in PBS between each step.

Sections of human melanoma were used as positive control tissues for p53 staining. A suitable positive control sec-

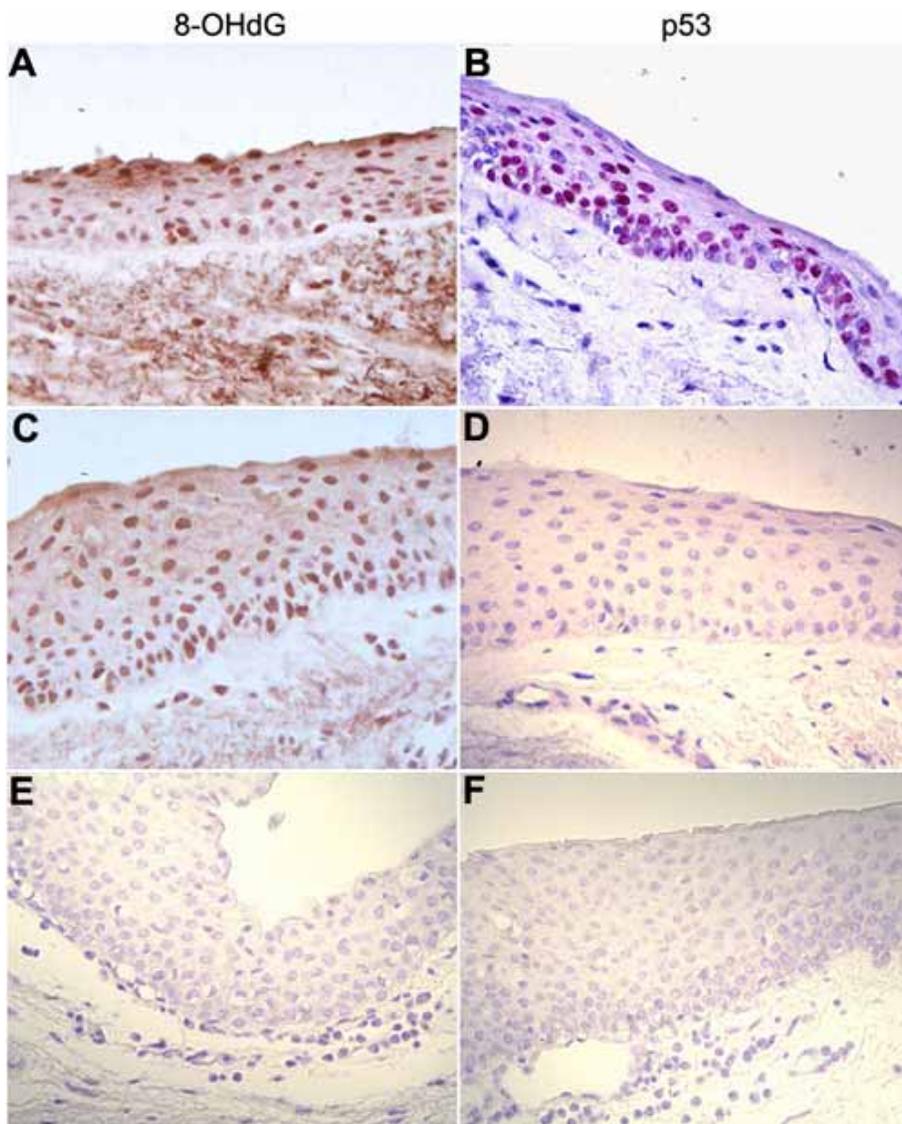


Figure 1. Immunohistochemical study of adjacent sections of pterygium from different patients. **A** and **B**: 8-OHdG+/p53+ in patient number 19; **C** and **D**: 8-OHdG+/p53- in patient number 25; **E** and **F**: 8-OHdG-/p53- in patient number 5. 8-OHdG and p53 expression was limited to the nuclei of the epithelial layer. No differences in the pattern of staining between 8-OHdG and p53 were observed. No substantial staining was visible in the subepithelial connective tissue. Original magnification was 400x.

tion for 8-OHdG was obtained by treatment of unfixed sections of rat liver with 10 μm H_2O_2 in complete medium for 15 min at 37 °C. Negative controls were obtained by omission of primary antibodies. Digital micrographs were taken and processed with Adobe Photoshop.

Results were evaluated independently by three observers and scored for the percentage of positive nuclei. The cutoff level for immunohistochemical analysis was set at 10%, meaning that those samples with more than 10% of cells stained were considered to be positive. Patients were divided into 4 groups, p53-positive, p53-negative, 8-OHdG-positive, and 8-OHdG-negative. The results were analyzed using the Fisher's exact test by the SPSS statistical software package, version 12.0 (SPSS Inc., Chicago, IL). A p-value of less than 0.05 was considered statistically significant.

To evaluate a possible correlation between the expression of 8-OHdG and p53 and some different clinical variables, such as gender, patient's age, geographic location, and disease stage, the immunohistochemical and clinical data were analyzed by Fisher's exact test or by Student's t-test; a p-value of less than 0.05 was considered statistically significant.

RESULTS

Relevant clinical features of the patients are summarized in Table 1.

8-OHdG analysis: Trevigen's anti-8-OHdG antibody permits detection of 8-OHdG in DNA, cells and tissue samples. The levels of 8-OHdG have been shown to correlate with mutagenesis and carcinogenesis in which oxidative damage is involved as causative mechanisms [48]. In our study, 21 (21/31, 67.74%) primary pterygia samples were positive for 8-OHdG staining, with immunoreactivity localized to the nuclei of the epithelium; no immunostaining was observed in the lamina propria (Figure 1A,C). In normal conjunctiva group,

all specimens were negative (Figure 2C).

p53 analysis: Analysis of overexpression of the p53 protein was performed using the monoclonal antibody DO-7, which is directed against an epitope located between amino acids 19-26 and recognizes both wild type and mutant forms of the protein. Positive staining was detected in 11 (11/31, 35.48%) pterygia. The expression of protein p53 was limited to the nuclei of the epithelial layer; no substantial staining was visible in the subepithelial fibrovascular layers (Figure 1B). No immunostaining for p53 in normal conjunctiva samples was observed (Figure 2D).

Relationship between 8-OHdG and p53: The relationship between 8-OHdG and p53 is shown in Table 2. In the group of pterygia with 8-OHdG immunostaining, there were 11 (11/21, 52.38%) samples with p53 expression. All samples positive for p53 (11/31, 35.48%) were also positive for 8-OHdG immunostaining, and all specimens negative for 8-OHdG (10/31, 32.26%) were also negative for p53. Fisher's exact test demonstrated a significant correlation between 8-OHdG expression and p53 positivity ($p=0.0049$).

Relationship between immunohistochemical results and clinical data: Student's t-test demonstrated a statistically significant association between the expression of p53 and age ($p=0.02$); no significant correlation was observed between 8-OHdG and age ($p=0.09$). The correlation between the two markers and the other clinical variables, assessed by Fisher's exact test, revealed no statistically significant association (Table 3).

DISCUSSION

Solar radiation is the primary source of human exposure to UV radiation. Overexposure without suitable protection (i.e., sunscreen and clothing) causes inflammation, gene mutation, and immunosuppression. These biological changes are respon-

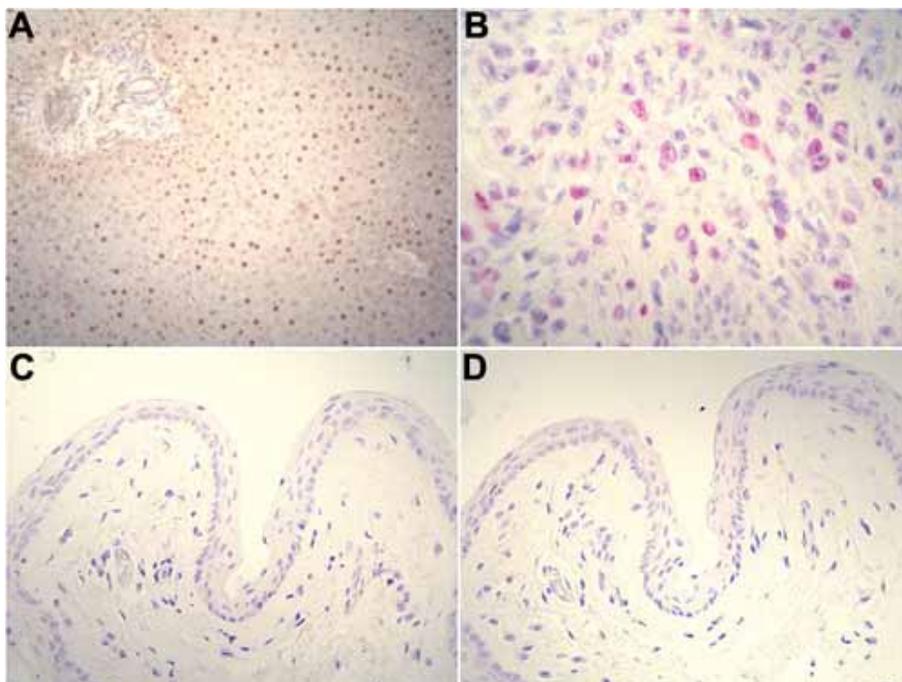


Figure 2. Immunohistochemical study of control sections. **A:** Rat liver. **B:** Human melanoma. **C** and **D:** Normal conjunctiva. **A** and **C:** 8-OHdG staining. **B** and **D:** p53 staining. Numerous 8-OHdG positive hepatocytes were observed (**A**). p53 nuclear staining was evident in scattered tumor cells (**B**). No immunostaining for 8-OHdG and p53 in normal conjunctiva was observed (**C** and **D**). The original magnification for **A**, **C**, and **D** was 200x and the original magnification for **B** was 400x.

sible for photocarcinogenesis, in which both UV-B and UV-A are causative agents. UV-B induces not only pyrimidine photoproducts but also leads to DNA lesions caused by ROS. ROS have been associated with initiation and progression in a multistage model of carcinogenesis [48]. A most typical oxidative lesion product is 8-OHdG, since guanine is the most easily oxidized naturally occurring base. 8-OHdG frequently mispairs with adenine during DNA replication, leading to G-C to T-A transversion, and thus gives rise to mutations [49]. It has also been established that it is a sensitive marker of oxidative DNA damage. G-C to T-A transversions, induced by the presence of 8-OHdG during DNA replication, have also been observed in the ras oncogene and p53 in human skin cancers of sun-exposed areas and in UV-induced mouse skin cancers [50,51]. In the present study 8-OHdG was found in 21 (21/31, 67.74%) of pterygial specimens, supporting the hypothesis that there is oxidative stress in pterygium and could provide molecular evidence of the effects of UV radiation in these lesions.

In a recent study, Tsai et al. [38] reported that 23.1% of 52 pterygia samples were positive for 8-OHdG; the discrepancy with our data might be explained by the different methods employed for immunohistochemical analysis. In our study the procedure used to immunostain for 8-OHdG was the Dako EnVision™ +System, a highly sensitive two-step immunohistochemical technique [52], in which the primary antibody is followed by a polymeric conjugate in sequential step. This polymeric conjugate consists of a large number of peroxidase and secondary antibody molecules bound directly to an activated backbone. The polymeric conjugates hold up to 100 enzyme molecules and 20 antibody molecules per backbone. The sensitivity of the method is only comparable to the catalyzed signal amplification (CSA) method [53], which has a 50 fold increased sensitivity compared to the standard ABC method, used by Tsai and colleagues.

Wild type p53 appears to monitor the genetic integrity of the cell and is required for inducing the expression of genes that are responsible for inhibiting DNA synthesis or inducing apoptosis in response to DNA damage [54,55]. In normal un-

stressed cells, the p53 protein has a short half-life and is maintained at low, often undetectable, levels. In our study, among the 31 Ecuadorian pterygia, 11 specimens (35.48%) were positive for p53 expression. The anti-p53 antibody and cutoff level (10% of cell staining) we used was the same as that used by Weinstein et al. [43] and by Tsai et al. [38]; however, the proportion of positive samples found, namely 32.28%, is different from the previously reported frequencies of 53.8% and 22.8%, respectively. This might be explained by the observation that the frequency of p53 mutations in cancer can vary among different races [56]. To support such a hypothesis, Ueda et al. [42] demonstrated that the incidence of immunoreactivity for p53 is related to differences in development of pterygium with race as well as environmental factors.

Mutations in the p53 gene are believed to lead to an increased stability of the protein, allowing its more pronounced immunohistochemical detection. UV radiation can cause mutations in genes such as p53, which when inactivated through mutation and loss of heterozygosity can lead to cell proliferation and genomic instability [39]. Even though Schneider et al. [57] failed to detect mutations in exons 5-8 or in the entire coding sequence in Caucasian pterygia, in their molecular analysis of p53 gene mutations in eight Chinese pterygia, Tsai and colleagues [58] detected mutations in all samples, with six substitutions and two deletions. Among the substitution, a G-C to T-A transversion was found in exon 6, codon 213, which is typical of oxidative lesions. This finding is consistent with our results showing statistically significant overexpression of p53 in 8-OHdG immunoreactive pterygia (35.48%). In our study, all specimens negative for 8-OHdG were also negative for p53.

Ueda et al. [42] evaluated the effect of age and gender on p53 expression. In their study, they reported that there was no correlation between age and p53 expression, while the prevalence of p53 positive staining was significantly higher in men than women. Tsai et al. [58] did not find significant difference between p53 expression with respect to age and gender. Our findings are not consistent with those of Ueda et al. [42] with regard to the effect of age and sex on p53 staining results, and they are not in agreement with those of Tsai and colleagues on the role of age affecting the p53 immunohistochemical staining results. Even if we agree with Tsai et al. [58] on the neces-

TABLE 2.

	p53 positive	p53 negative	Total
8-OHdG positive	11	10	21
8-OHdG negative	0	10	10
Total	11	20	31

Shown is the relationship between 8-OHdG and p53. In the group of pterygia with 8-OHdG immunostaining, there were 11 (11/21, 52.38%) samples with p53 expression. All samples positive for p53 (11/31, 35.48%) were also positive for 8-OHdG immunostaining, and all specimens negative for 8-OHdG (10/31, 32.26%) were also negative for p53. 8-OHdG expression was significantly associated with p53 positivity (p=0.0049 using Fisher's exact test).

TABLE 3.

	8-OHdG	p53
age	0.09	0.02
gender	0.055	1.00
disease stage	1.00	0.41
residency	0.24	0.056

Shown are the p-values in the relationship between the immunohistochemical results and clinical variables, as analyzed by Fisher's exact test or by Student's t-test. The statistically significant correlation is marked in red.

sity of further investigation on the role of sex in p53 expression, our study demonstrates a statistically significant correlation of p53 positive staining with increasing age. This datum might be explained by the well-known knowledge that there is decreased DNA damage repair capacity with increasing age [59]. Moreover, our detection of significant correlation between age and p53 expression is consistent with the location of our study patients, all of whom are native residents of Ecuador; due to its geographic location, Ecuador's population is continuously exposed to intense ultraviolet radiation, probably at the highest rate compared to any other population. However, our finding of no significant correlation between gender and p53 expression is probably due to the fact that in our series of 31 patients, either men or women were always engaged in outdoors activities.

Pterygium has long been considered to be a chronic degenerative condition; however, because of the finding of abnormal expression of p53 protein in the epithelium, pterygium is now considered to be a result of uncontrolled cellular proliferation, similar to that in tumors, in which there is damage to cellular regulation and control of the cell cycle [16,30,31,40-43]. To further support this hypothesis, in a previous study [60] we reported that nine conjunctival melanocytic pigmented lesions were present in a series of 80 resected Ecuadorian pterygia; among these, there were two primary acquired melanosis, which have a high probability of progressing to invasive melanoma [61].

In conclusion, although pterygium is a lesion with limited local invasion and inability to develop metastases, the concomitant presence of altered p53 in 8-OHdG-immunoreactive cells could provide evidence of apparent genetic instability which is in contrast to its benign clinical course.

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