

Expression of survivin protein in pterygium and relationship with oxidative DNA damage

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Abstract

Ultraviolet radiation is known to cause oxidative DNA damage and is thought to be a major factor implicated in the pathogenesis of pterygium. Among all the photo-oxidative DNA products, the 8-hydroxydeoxyguanosine (8-OHdG) is regarded 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 has a carcinogenic effect resulting in DNA damaged cells with loss of normal growth control. This assumption is supported by the association between UV-B exposure and activation of survivin, a member of the inhibitor of apoptosis protein family (IAP), highly up-regulated in almost all types of human malignancy. In this study we demonstrate, for the first time in pterygium, the immunohistochemical presence of survivin, and investigate the correlation between survivin, p53 and 8-OHdG. Our results demonstrate that oxidative stress could lead to a significant activation of survivin expression, suggesting that this might be an important event in the development of pterygium, inducing and supporting a hyperproliferative condition. Survivin expression in pterygium would counteract UV-B-induced apoptosis and would cooperate with loss of p53. The co-operation between survivin and functional loss of p53 might provide a general mechanism for aberrant inhibition of apoptosis that could be responsible for the development of pterygium and its possible progression to neoplasia.

Keywords: survivin • oxidative stress • pterygium • immunohistochemistry

Introduction

Pterygium is a surface ocular lesion that begins growing from limbal epithelium and invades the cornea centripetally followed by conjunctival epithelium, exhibiting degenerative and hyperplastic changes as well as proliferative, inflammatory features and a rich vasculature.

Recent studies have provided evidence that a genetic component might be involved in the pathology of pterygium [1–3], while anti-apoptotic mechanisms [4, 5], cytokines [6], growth factors

[7–10], angiogenic factors [11, 12], extracellular matrix remodeling [13], immunologic mechanisms [14, 15] and viral infections [3, 16–18] have all been implicated in its pathogenesis. Studies by Clear *et al.* [19] 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 not merely a simple degenerative condition of the conjunctiva [9, 20, 21]. To support this hypothesis, in a previous study [22] we reported in pterygium the presence of conjunctival melanocytic pigmented lesions, and, among these, two primary acquired melanosis with atypia.

Epidemiological studies indicate that chronic exposure to the sunlight, and most probably ultraviolet B (UV-B) irradiation, is an important factor in the development of pterygia [23–26]. It is

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known that UV irradiation has a carcinogenic effect resulting in DNA-damaged cells with loss of normal growth control [27, 28]. Recent studies by Aziz *et al.* [29] demonstrated that UV-B exposure leads to a significant activation of survivin, a protein only expressed in embryonic or proliferating normal adult tissues, and highly up-regulated in almost all types of human malignancy [30–32]. As its name infers, survivin is cytoprotective, belonging to the inhibitor of apoptosis (IAP) gene family, but it has also a role as an essential cell division protein [33, 34]. Overexpression of survivin seems to inhibit apoptosis initiated *via* the extrinsic or intrinsic pathways, relative to its ability to inhibit terminal caspase-3 and -7 [35, 36]. *In vitro*, UV irradiation has been shown to induce typical caspase-dependent cell death in normal conjunctival cells [37].

The primary goal of the present study was to demonstrate, for the first time in pterygium, the immunohistochemical presence of survivin. Because of its strong expression in several preneoplastic lesions [38, 39], including hypertrophic actinic keratosis [40] and in the vast majority of cancers [30, 41], the presence of survivin would emphasize the idea of the origin of pterygium from an anti-apoptotic mechanism, and the trend of this lesion towards a neoplastic-like growth disorder *versus* a simple degenerative condition of the conjunctiva. Moreover, since in our previous study [42] we demonstrated the concomitant presence of altered p53 in 8-hydroxydeoxyguanosine (8-OHdG)-immunoreactive cells, the other purpose of the study was to verify a possible correlation between survivin, p53 and 8-OHdG, in order to provide a further evidence of an apparent genetic instability which is in contrast to the pterygium's benign clinical course.

Materials and methods

Patients and study design

Primary pterygia were harvested from 31 patients (11 males and 20 females). All patients were of mixed race, between Indian and Hispanic. Ages ranged between 21 and 68 years (mean age, 43.13; standard deviation, 13.5). Nineteen patients lived in the countryside and 12 resided in an urban setting. All the patients were outdoors workers. The patients underwent excision by bare sclera technique at the Department of Pathology, Cancer Center of SOLCA, Cuenca, Ecuador. Twenty-four lesions were located on the nasal side and only the head of primary pterygium was used as pterygium sample. Pterygium morphology was clinically graded as atrophic (nine cases), intermediate (15 cases) or fleshy (seven cases), according to an assessment of pterygium translucency. Normal conjunctiva samples as controls were collected from medial bulbar conjunctiva of 10 patients (six males and four females) without pterygium and pinguecula while undergoing cataract surgery. Ages ranged between 25 and 70 years (mean age 52.1; standard deviation 16.02); the younger patients were surgically treated for traumatic cataract. Seven patients lived in the countryside and three resided in an urban setting. Patients did not receive any medication prior to surgery, except for a topical anaesthetic, and no drugs or chemical agents were used during surgical operation. The study protocol was approved by the local Research Ethic Committee and informed consent

was obtained from all participants, according to the World Medical Association Declaration of Helsinki; complete information on patients was available in all cases.

Immunohistochemistry

Tissue segments were fixed by immersion in cold 10% formalin in 0.2 M phosphate buffer, pH 7.3 for 4–6 hrs, and processed for paraffin embedding. Microtome sections (6–7 μ m) were treated for the immunohistochemical demonstration of survivin and p53 using the streptavidin-biotin alkaline phosphatase method. Briefly, they were re-hydrated in phosphate-buffered saline (PBS) and water-bath heating-based antigen retrieval was performed by immersion in 10 mM citrate buffer solution (pH 6.0) at 95°C for 40 min. After gradual cooling for 20 min., sections were treated for 45 min. with 10% normal goat or normal horse serum in PBS, respectively. Rabbit polyclonal antibody to recombinant human survivin protein (Novus Biologicals, Littleton, CO, 1:1000) and mouse monoclonal antibody to human p53 protein (clone DO-7, Dako Glostrup, Denmark, 1:50) were used as primary antisera and incubated for 60 min. at room temperature, while biotinylated anti-rabbit and antimouse IgG were used as secondary antisera (Vector Laboratories, Burlingame, CA, USA, 1:200) by incubation for 30 min. at room temperature. The sections were further incubated in alkaline phosphatase streptavidin (Vector Laboratories, Burlingame, CA, USA, 1:1000) for 30 min. at room temperature, and reacted with Fast Red Substrate System (Dako Glostrup).

Adjacent microtome sections were treated for the immunohistochemical demonstration of 8-OHdG, using the Dako EnVisionTM+ System/AP. Water-bath heating-based antigen retrieval was performed by immersion in 10 mM citrate buffer solution (pH 6.0) at 95°C for 30 min. The slides were incubated in 10 mM Tris-HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.4 mM NaCl, 100 μ g/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 non-specific sites, specimens were incubated in PBS containing 10% foetal bovine serum for 1 hr at 37°C; they were then treated with mouse monoclonal anti-8-OHdG (Trevigen, Gaithersburg, USA, 1:300) in PBS, pH 7.5 containing 10% foetal bovine serum overnight at 4°C. Samples were further incubated with antimouse immunoglobulins (Ig) conjugated to alkaline phosphatase labelled dextran polymer (Dako EnVisionTM+ System, Carpinteria, CA, USA) for 30 min. at room temperature, and reacted with Fast Red as chromogen.

In the experiment, all sections were thoroughly rinsed in PBS between each step, and finally counterstained with Mayer haematoxylin and mounted in glycerol gelatin (Sigma, St. Louis, MO, USA).

Sections of human cutaneous melanoma were used as positive control tissues for survivin and p53 staining, while human skin was used as control for 8-OHdG. Negative controls were obtained by omission of the primary antibody or by replacing the primary antibody with an isotype-matched antibody.

Micrographs were captured by a digital camera Canon PowerShot A620, (Canon Inc. China) on a microscope Zeiss Axiophot, and processed by Adobe Photoshop (7.0 version) software.

Evaluation of immunoreactivity

Results were independently evaluated by three observers in a blinded fashion. Four to six 200 \times fields covering almost the whole of each of four

sections per sample were examined with a 144-intersection point square reticulum (0.78 mm²) inserted in the eyepiece, and scored for the percentage of epithelial immunoreactive cells. The cut-off 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 six groups: p53-positive, p53-negative, 8-OHdG-positive, 8-OHdG-negative, survivin-positive and survivin-negative.

Statistics

The results were assessed with parametric analysis of variance (Fisher's exact test); data were computed by the SPSS statistical software package, version 15.0 (SPSS Inc., Chicago, IL, USA); a *P*-value of less than 0.05 was considered statistically significant.

Results

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

The data on 8-OHdG and p53 expression are imported from our previous paper [42].

8-OHdG analysis

Trevigen's anti-8-OHdG antibody allows detection of 8-hydroxyguanosine 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 [43]. 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 (Fig. 1A). In normal conjunctiva group, all specimens were negative (Fig. 1B). Positive control section from human skin demonstrated immunoreactivity for 8-OHdG in the epithelial layer (Fig. 3A). Reactivity was absent in sections incubated without primary antibody and no reactivity developed when the isotype control antibody was used (Fig. 3A¹).

P53 analysis

Analysis of overexpression of the p53 protein was performed using the monoclonal antibody DO7, 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 (Fig. 1C). No immunostaining for p53 in normal conjunctiva

Table 1 This table presents clinical variables of the study's participants and the immunohistochemical analysis. The asterisk indicates the median value

Samples	Variables	No. of patients
Pterygium		
Gender		
	Male	11
	Female	20
Age		
	<42*	17
	≥42*	14
Residency		
	Urban	12
	Rural	19
8-OHdG expression		
	Positive	21
	Negative	10
p53 expression		
	Positive	11
	Negative	20
Survivin expression		
	Positive	14
	Negative	17
Conjunctiva		
Gender		
	Male	6
	Female	4
Age		
	<55*	5
	≥55*	5
Residency		
	Urban	3
	Rural	7

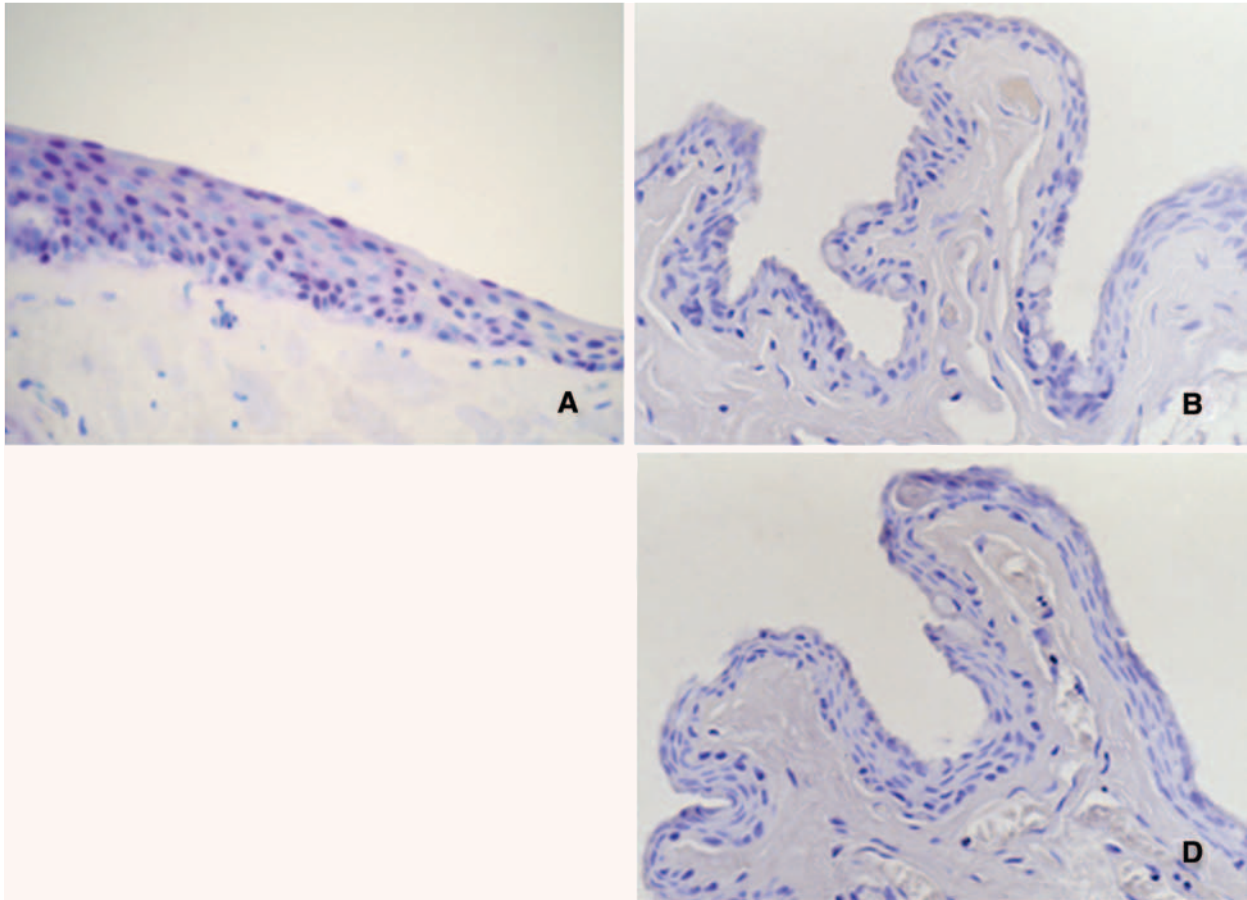


Fig. 1 Immunohistochemical staining for 8-OHdG (**A, B**) and p53 (**C, D**). In pterygium 8-OHdG (**A**) and p53 expression (**C**) was localized to the nuclei of the epithelial layer. No differences in the pattern of staining between 8-OHdG and p53 were observed. No immunostaining for 8-OHdG (**B**) or p53 (**D**) in normal conjunctiva samples was observed. Each section was counterstained with haematoxylin. Original magnification: **A, B, D**, 400X; **C**, 630X.

samples was observed (Fig. 1D). Positive control section from human cutaneous melanoma demonstrated immunoreactivity for p53 in nuclei of tumoural cells (Fig. 3B). Reactivity was absent in sections incubated without primary antibody (Fig. 3B¹) and no reactivity developed when the isotype control antibody was used.

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 staining ($P = 0.0049$).

Survivin analysis

Novus Biologicals' anti-survivin antibody recognizes the full-length recombinant human survivin. In our study, 14 (14/31, 45.16%) primary pterygia were positive for survivin staining, with immunoreactivity localized to the nuclei and cytoplasm of the epithelial cells (Fig. 2A and B). In each sample, some cells showed positivity only in nucleus, other, only in cytoplasm, while further cells showed immunoreactivity in both nucleus and cytoplasm. Cytoplasmic immunoreactivity was weaker than the nuclear. The positive cells were scattered throughout the epithelium; no substantial staining was visible in the subepithelial fibrovascular layers. No immunostaining for survivin in normal conjunctiva was present (Fig. 2C). Positive control section from human cutaneous melanoma demonstrated immunoreactivity for survivin in nuclei and cytoplasm of tumoural cells (Fig. 3C). Reactivity was absent in sections incubated without primary antibody

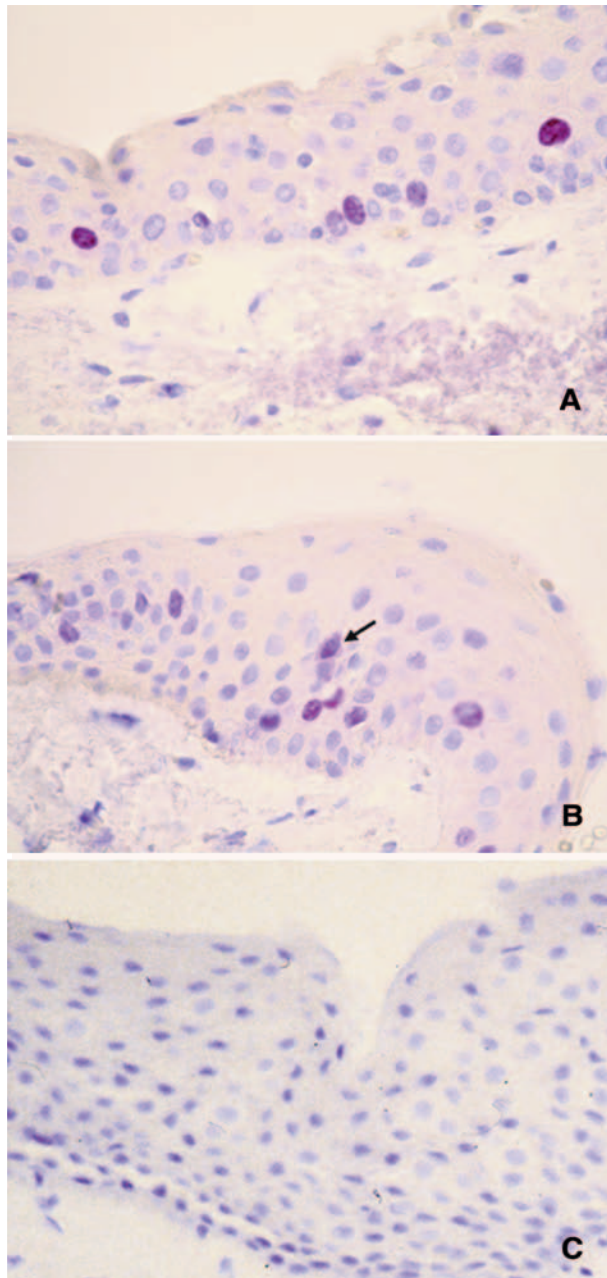


Fig. 2 Immunohistochemical staining for survivin. The immunoreactivity was localized to the nuclei and cytoplasm of the epithelial cells. Note in **B** a cell with immunoreactivity in both nucleus and cytoplasm (arrow). No immunostaining in normal conjunctiva was detected (**C**). Each section was counterstained with haematoxylin. Original magnification: **A–C**, 400 \times .

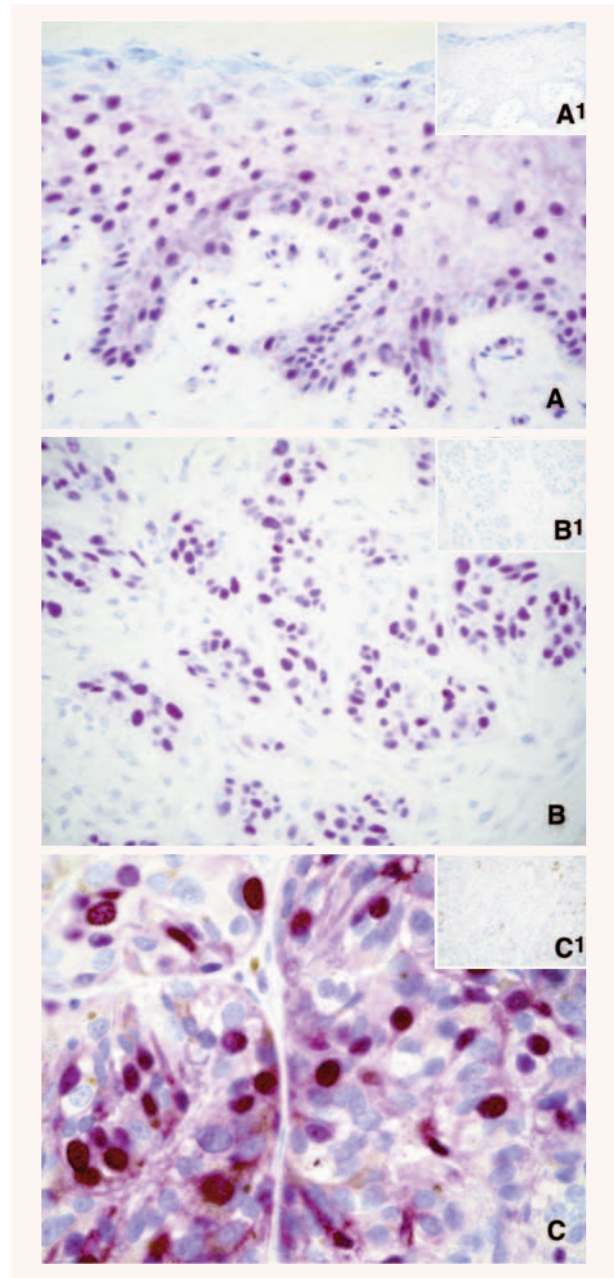


Fig. 3 Immunohistochemical staining for 8-OHdG (**A**), p53 (**B**), survivin (**C**). Sections from normal human skin (**A**) and human cutaneous malignant melanoma (**B**, **C**) were included as positive controls. Sections incubated without a primary antibody (inset **B**¹), or with an isotype control antibody (insets **A**¹ and **C**¹) displayed no immunoreactivity. Each section was counterstained with haematoxylin. Original magnification: **A**, **B**, and insets **A**¹, **B**¹, **C**¹, 400 \times ; **C**, 630 \times .

Table 2 Relationship between 8-OHdG and p53

	p53 positive	p53 negative	Total
8-OHdG positive	11	10	21
8-OHdG negative	0	10	10
Total	11	20	31
			<i>P</i> = 0.0049

Table 3 Relationship between survivin and p53

	survivin positive	survivin negative	Total
p53 positive	10	1	11
p53 negative	4	16	20
Total	14	17	31
			<i>P</i> = 0.0004

and no reactivity developed when the isotype control antibody was used (Fig. 3C¹).

Relationship between survivin, p53 and 8-OHdG

All samples positive for p53 (11/31) and for survivin (14/31) were also positive for 8-OHdG immunostaining, and all specimens negative for 8-OHdG (10/31, 32.26%) were also negative for p53 and survivin. When analysed by Fisher's exact test, the expression of total survivin showed a strong significant correlation both with p53 (*P* = 0.0004) and 8-OHdG immunostaining (*P* = 0.0005). The relationships between total survivin and p53 and 8-OHdG are shown in Tables 3 and 4.

Discussion

UV light is one of the major factors implicated in the pathogenesis of pterygium, but the mechanism by which UV radiation induces this disease remains unknown.

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 have been associated with initiation and progression in a multi-stage model of carcinogenesis [43]. 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,

Table 4 Relationship between survivin and 8-OHdG.

	survivin positive	survivin negative	Total
8-OHdG positive	14	7	21
8-OHdG negative	0	10	10
Total	14	17	31
			<i>P</i> = 0.0005

which results in G:C to T:A transversion mutations; therefore, it has been established as a sensitive marker of oxidative DNA damage [44]. 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 [45, 46]. Based upon the premise that pterygium is a UV-related lesion, the demonstration of the presence of 8-OHdG highlights the importance of the role played by oxidative stress in the development of the lesion.

The protein p53 is a major cell stress regulator that monitors 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 [47, 48]. 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. In normal unstressed cells, the p53 protein has a short half-life and is maintained at low, often undetectable, levels. 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 mutation in genes such as p53. In our previous report [42], we observed overexpression of p53 in 8-OHdG immunoreactive pterygia, providing evidence that pterygium is a tumour-like growth disorder, related to faulty apoptosis; noteworthy is the finding that all specimens negative for 8-OHdG were also negative for p53. Moreover, other researchers demonstrated by immunohistochemistry the overexpression of p53 in pterygium, but only Tsai and co-authors [49] were able to detect mutations in all their samples, being one a G:C to T:A transversion, which is typical of oxidative lesions. In their analysis on apoptosis-related gene expression, Tan and colleagues [4] showed the presence of apoptotic cells only in restricted areas of the epithelium, consistent with aberrant expression of p53 and the anti-apoptotic effect of *bcl-2* in the remaining part of the epithelium. This datum would indicate pterygium as a result of disruption of the normal process of apoptosis. Also, Schneider *et al.* [50] showed accumulation of p53 in Caucasian pterygia, but they failed to detect mutations in p53 gene, concluding that the increased amount of p53 protein in pterygial cells does not stop cell proliferation or cause cells to die by apoptosis; they proposed that p53 protein in pterygia appears to be inactive, at least in this

function. They explained the high levels of the protein with a UV-induced defect in p53 activation.

As other tissues, homeostasis in conjunctiva is essentially regulated by a delicate balance between cellular proliferation and cellular apoptosis, thus degenerative processes are linked to either modified proliferative capacity or apoptosis. Recent studies [51] on normal apoptotic conjunctiva demonstrated the absence of Apaf-1 (cytosolic 'apoptotic protease activating factor 1'), which is shown to activate, by intrinsic pathway, caspase-3 in a cytochrome c-dependent manner. This datum would indicate that Apaf-1 does not participate in the apoptotic cascade in conjunctival cells. However, studies *in vitro* by Buron *et al.* [37] demonstrated in conjunctival cells the involvement of the extrinsic pathway and caspase-8 activation in UV-induced chromatin condensation. Investigations by Aziz *et al.* [29] on human epidermal keratinocytes demonstrated that UV-B exposure leads to a significant activation of survivin, whose overexpression seems to inhibit apoptosis initiated *via* the extrinsic or intrinsic pathways, relative to its ability to inhibit caspases [35]. In the present study we demonstrated a significant survivin overexpression in 8-OHdG- and p53-positive cells, localized in both nuclei and cytoplasm of epithelial cells. It is known, in fact, that there are different survivin proteins with unique subcellular localizations and functions [52]; among these, nuclear survivin appears to play a key role in cell division, whereas cytoplasmic survivin seems essential for the inhibition of apoptosis [53]. Thus, our immunohistochemical localization in both nuclei and cytoplasm, obtained by an antibody recognizing all survivin proteins, would represent the combined expression of all variants.

In normal cells, wild-type p53 transcriptionally represses survivin, [54–56] and overexpression of survivin is able to counteract p53-dependent apoptosis induced by ultraviolet light [54]. In our opinion, survivin, which transcription in pterygium is no more silenced by UV-inactivated p53, could exert its anti-apoptotic effect by directly inhibiting terminal caspase-3. Since there is

strong evidence that p53 also exerts its effects on the inhibition of new blood vessel development [57], an intense angiogenesis [12], typical of the histopathology of pterygium, could be another sign of the loss of p53 function.

In conclusion, this is the first report on the presence of survivin in pterygium; our results are in agreement with previous data by Aziz *et al.* [29] on mouse skin about the up-regulation of survivin by UV-B and increase in p53 expression. In this study, we demonstrate that oxidative stress may be associated with a significant activation of survivin expression, suggesting that this might be an important event in the development of a relevant number of pterygia, inducing and supporting a hyperproliferative condition. Survivin expression in pterygium would counteract UV-B-induced apoptosis and would cooperate with loss of p53. The co-operation between survivin and functional loss of p53 might provide a general mechanism for aberrant inhibition of apoptosis. Such aberrant inhibition of apoptosis may initiate pterygium growth and stimulate not only local invasiveness, but also a possible progression towards potentially malignant neoplasia, since we demonstrated in our previous paper [22] the presence in pterygium of primary acquired melanosis with atypia, with a high risk for the development of invasive melanoma. The possibility of restoring p53 function by pharmaceutical manipulation or reducing or eliminating the effect of the anti-apoptotic molecule survivin could offer hope for more effective treatment against pterygium.

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