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LABORATORY STUDY

K-ras oncogene mutation in pterygium

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Abstract

Purpose **Pterygium is claimed to be a benign proliferation triggered by prolonged exposure to ultraviolet radiation. The frequency of *K-ras* oncogene mutation, which is among the initial mutations in tumorigenesis, is evaluated in this study.**

Patients and methods **In this prospective randomized clinical, trial pterygium tissues and normal conjunctiva tissue specimens are obtained from the superotemporal quadrant of patients who underwent primary pterygium excision with autograft transplantation. DNA extraction from tissues was performed using the QIAamp DNA FFPE tissue kit. A PCR reaction was performed to amplify sequences containing codons 12, 13, and 61 of the *K-ras* gene in DNA. These PCR products then underwent the 'pyrosequencing' procedure for mutations at these codons.**

Results **Pterygium and normal conjunctival tissue samples of 25 patients (10 females, 15 males) were evaluated in the study. The mean age of the patients was 54.54 ± 13.13 years. Genetic analysis revealed no *K-ras* mutations in normal conjunctival tissues, whereas pterygium tissues of the same cases demonstrated mutation at codon 12 in one case and mutations at codon 61 in seven cases, which was statistically significant ($P < 0.05$). The point missense mutations at codon 61 were glutamine to arginine (Glu61Arg CAA > CGA) in four cases and glutamine to leucine (Glu61Leu CAA > CTA) in three cases.**

Conclusion **The significantly higher frequency of codon 61 mutation of the *ras* oncogene in primary and bilateral pterygium specimens compared with normal conjunctiva supports the tumoral origin of pterygium, and thus set the stage for research into a targeted therapy for pterygium with better outcomes than surgical excision.**

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Introduction

The original description of pterygium dates back to the time of Hippocrates; however, the pathogenesis of this benign fibrovascular proliferation advancing on the corneal surface is still not fully understood.[1,2] The lesion has a characteristic wing-like appearance and occurs more frequently on the nasal limbus than the temporal. It may cause irritation and redness due to tear film irregularity and visual impairment related to irregular astigmatism. Treatment is limited to surgical excision and there is a high recurrence rate, which has led researchers to seek potential prognostic and predictive factors to pave the way for novel, targeted treatment alternatives other than surgery.[2,3]

Although several risk factors have been proposed, the higher incidence in tropical and subtropical regions supports the role of ultraviolet (UV) radiation in the formation of pterygium.[4,5] However, exactly how UV radiation triggers pterygium formation is a matter of debate. Some authors hypothesized that scattered light follows an alternative transcameral optical path and hits limbal stem cells from their inner surface. Other studies have provided evidence implicating antiapoptotic mechanisms, immunological mechanisms, cytokines, growth factors, extracellular matrix modulators following UVR injury. There is also compelling evidence toward uncontrolled cell proliferation as a mechanism, as pterygium requires cell migration, proliferation, and local angiogenesis.[2,6–8]

Ultraviolet radiation is known to lead to tumor formation in other tissues in the human body. For example, in skin tumors UV light has been shown to affect tumor suppressor genes such as p53 and oncogenes such as *K-ras*.[9,10] The role of the p53 gene in pterygium pathogenesis had been evaluated in several studies.[11] However, there is only one study by Detorakis *et al*[12] investigating the frequency of *K-ras* mutation.

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K-ras is the most frequently mutated oncogene in cancer.[13,14] It encodes low molecular weight G-proteins that are located on the internal surface of cell membranes and have GTPase activity.[15–17] They function as molecular switches, regulating critical cellular processes including mitosis, cell differentiation, apoptosis, and gene expression.[16–18] Mutational activation of *K-Ras* has been found in 22% of tumors. It has been shown to play an important role in colorectal cancer progression and in 90% of pancreatic adenocarcinomas because it causes the downstream cascade proteins to be permanently switched on.[18] Approximately 40% of colorectal cancers present point mutations in the *K-ras* gene.[19] These mutations are generally located at codons 12 and 13 in the majority of cases and less frequently at other codons, like codon 61 or 64.[20] These point substitutions in codons 12 and 13 were confirmed to be negative predictors of response to targeted therapies with anti-epidermal growth factor receptor antibodies.[21] Therefore, determining the *K-ras* mutational status of tumor samples prior to treatment has become an essential step toward getting optimal benefit from treatment.

The preliminary study of Detorakis *et al*,[12] investigating the possible role of *K-ras* mutation in pterygium pathogenesis, evaluated *K-ras* mutation at codons 12 and 13 in pterygium samples and found a mutation rate of 10% at codon 12, especially evident in recurrent and young cases. In this study we endeavored to elucidate the mutation rate of *K-ras* oncogene at codon 61 in addition to codons 12 and 13. As a difference from the study of Detorakis *et al*[12] which included both primary and recurrent cases, our study enrolled only primary pterygium tissues and compared it with the patients' own normal conjunctival tissue. Of the several mutation analysis methods available, the highly sensitive method of pyrosequencing is preferred because it allows direct sequencing by the synthesis of short fragments of DNA via a novel enzymatic cascade system.[22]

Materials and methods

Patients with pterygium who were scheduled for an excisional pterygium surgery with conjunctival autograft transplantation were enrolled in the study. The nature of the study was explained to all participants before obtaining written informed consent for the use of collected samples or any accompanying images in this research project. The research presented in this manuscript was performed in compliance with the Helsinki Declaration according to the study ethics proposal approved by the Ethics Board of Selçuk University.

Patients completed an information form including occupation, duration of outside activity, sunglass use, family history, duration of pterygium, and treatment history. Patients who had received any ocular medication or chemical agent or underwent previous surgery for any ocular disease including pterygium were excluded from the study.

Bilateral best-corrected visual acuity and biomicroscopic findings were recorded for each patient. Pterygium was classified as type 1 if it did not extend beyond the limbus, type 2 if it extended 2–4 mm over limbus but did not encroach onto the optic zone, and type 3 if it extended into the optic zone. In subjects with bilateral pterygium the eye with more advanced type has been preferred for excision.

Pterygium surgery with the conjunctival autograft transplantation technique was performed under local anesthesia, and 1×1 mm samples were taken from both the pterygium specimens and from the superotemporal bulbar conjunctiva after taking a conjunctival autograft. All fresh tissues were put in separate Eppendorf tubes filled with 0.9% NaCl and stored at -80 °C until DNA isolation.

Laboratory procedures

Frozen pterygium and normal conjunctival tissues were allowed to return to room temperature, then incubated overnight at 56 °C in proteinase K (Qiagen, Hilden, Germany) for lysis. Afterwards, DNA from the lysate was precipitated using AL buffer and ethanol. DNA purification was performed using the spin column procedure (QIAamp DNA FFPE Tissue Kit; Qiagen) and AW1, AW2, and ATL buffers were added sequentially according to the manufacturer's protocol. Finally, the DNA content was measured with a Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at 260 and 280 nm. Samples with a DNA quantity less than 100 ng/ml were excluded and reanalyzed from the stored tissue samples.

Mutation in the *K-ras* gene at codons 12, 13, and 61 in the pterygium and normal tissues were detected with the pyrosequencing method using the PyroMark *K-ras* Kit (Qiagen, Catalog Nr: 971450). Prior to pyrosequencing, the corresponding mutation sequences at codons 12, 13, and 61 underwent PCR amplification using 10 ng/μl of genomic DNA with the HotStarTaq Plus Master Mix Kit and PyroMark *K-ras* Kit (Qiagen) including sequencing primers for codons 12, 13, and 61. The primary PCRs were generated in 20 μl reaction volumes, which contained 1 μl DNA, 1 μl PCR primers for codon 12/13 or codon 61, 12.5 μl HotStarTaq Plus Master Mix, 2.5 μl Coral Load, 3 μl Q solution, and 1 μl $MgCl_2$. The primary PCR cycling conditions were 95 °C for 5 min for activation of HotStarTaq Plus DNA polymerase; denaturation at 92 °C for 20 s, annealing at 53 °C for 30 s, elongation at 72 °C for

20 s, and a final hold at 72 °C for 5 min. In addition to the patients' samples, PCR was also conducted on positive and negative controls.

The amplicons were immobilized on Streptavidine High Performance beads. Two microliters of Streptavidine Sepharose High Performance Beads were added to 40 μl binding buffer and mixed with 10 μl PCR product and 28 μl distilled water for 10 min at room temperature. The beads were captured on the filter probes using the Pyrosequencing Vacuum Prep Tool after the vacuum was applied and then washed with 70% ethanol for 5 s, denaturation solution (0.2 M NaOH) for 10 s, and washing buffer for 5 s. The vacuum was then stopped and the beads were released into a PyroMarkQ24 Plate containing 24.2 μl annealing buffer and 0.8 μl sequencing primer. Pyrosequencing reactions were then performed using the PyroMark Gold Q24 Reagents, PyroMark Q24 instrument (Qiagen), and PyroMark Q24 version 1.0.6.3 software.

Data were analyzed using the Statistical Package for the Social Sciences (SPSS version 13.0) for Windows. Differences between pterygium and control tissues were analyzed using χ^2 and Fisher exact tests. All tests were performed at an error level of 5%.

Results

Primary pterygium tissue and normal conjunctival tissues as the control group were harvested from 25 subjects whose mean age was 54.54 ± 13.13 years. Ten (40%) of the patients were women and 15 (60%) were men. The ratio of sunglasses use was 16% (4/25) and the mean duration of outdoors work was 4.08 (2–8) h/day. Family history for pterygium was not reported.

Pterygia were located nasally in all patients and exceeded the cornea by 2–4 mm (type 2) in 18 (72%) cases and by more than 4 mm (type 3) in 7 (28%) cases (Figure 1). Fifteen cases presented with bilateral pterygium. Pterygium grading in the fellow eyes were type 1 in eight subjects, type 2 in three subjects, and type 3 in four subjects.

No *K-ras* mutations were found at codons 12, 13, and 61 in normal conjunctival tissues (Figure 2). In pterygium tissues of the same subjects, only one patient exhibited a missense mutation from GGT (glycine) to GTT (valine) at codon 12. No mutations were detected at codon 13, as in normal conjunctiva. Statistical analysis using Fisher's exact test demonstrated no significant difference between pterygium and normal tissue in respect to codons 12 and 13 ($P = 0.32$, $P = 1$) (Table 1).

Pyrosequencing analysis of codon 61 revealed mutations at codon 61 in seven subjects (Table 2). All were missense mutations; the CAA (glutamine) was replaced with CGA (arginine) in four cases and CTA

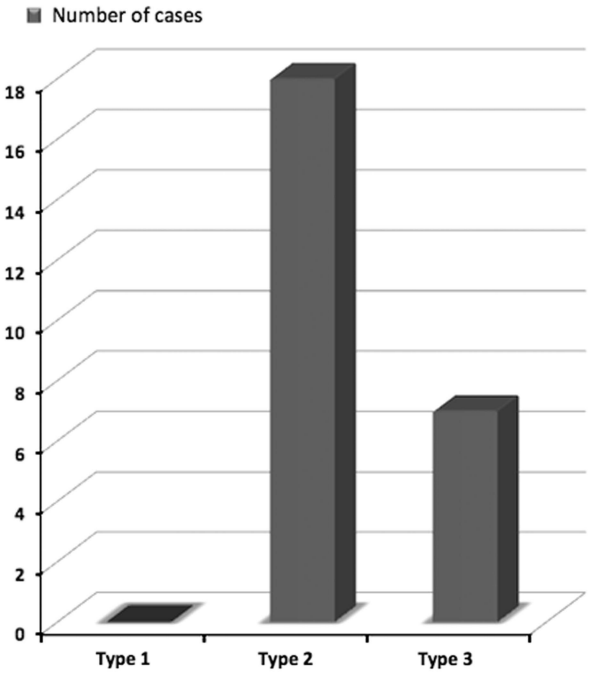


Figure 1 Distribution of cases according to pterygium types.

(leucine) in three cases (Figures 3a–c). The frequency of codon 61 mutation in pterygium tissue compared with normal tissue was found to be statistically significant according to Fisher's exact test ($P = 0.01$).

Subgroup analysis of the seven cases with codon 61 mutations (5 women, 2 men) yielded a mean age of 53.71 ± 14.16 years. The mean age of the patients without codon 61 mutations was 54.88 ± 13.12 years, but the age difference between patients with and without codon 61 mutation was insignificant ($P = 0.95$). With respect to the clinical findings of the patients with codon 61 mutations, six had type 2 and one had type 3 pterygium, and all had bilateral pterygium. Pterygia in the fellow eyes were type 1 in six cases and type 2 in one case. Only one of the seven patients with mutation at codon 61 used sunglasses. The median duration of outdoor work was 3 (range 2–5) h/day in patients with codon 61 mutation and 4 (range, 1–8) h/day in patients without; this difference was not statistically significant ($P = 0.10$).

Discussion

Although the clinical features and surgical treatment methods of pterygium have been well described, its pathogenesis has not been fully elucidated. It is characterized by hyperplasia and dysplasia of the conjunctiva and has several biological features in common with tumors, such as uncontrolled cell proliferation, corneal invasion, and recurrence after resection.[23–25] Another source of evidence that pterygium

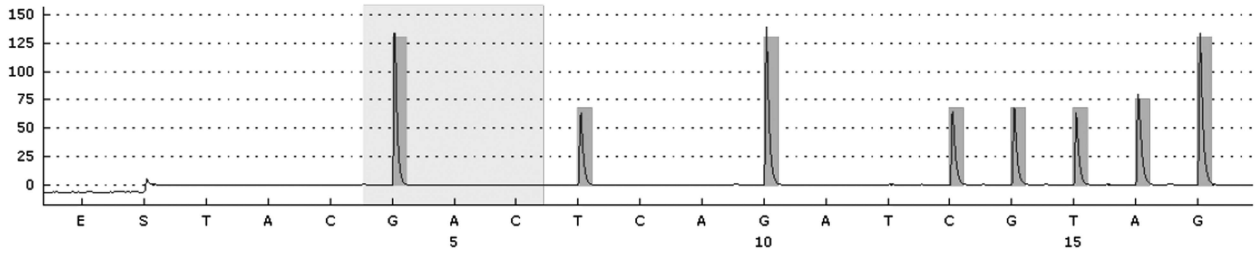


Figure 2 Sequencing graphic for codone 12 of the 25th case demonstrating normal tissue sample without any mutation (wild type).

Table 1 Frequency of mutation at codone 12, 13, and 61 in normal conjunctiva and pterygium tissues

Codone	*Normal tissue* (n = 25)	*Pterygium* (n = 25)
Codone 12	0	1
Codone 13	0	0
Codone 61	0	7

should be considered a neoplasia rather than a degenerative disease is the better visual outcome obtained with application of 5-fluorouracil or mitomycin during surgery.[26] The lack of metastasis, cell migration, local angiogenesis, and recurrence seen in cancer tissue may be ascribed to the benign nature of this tumor-like proliferation.[27]

Tan *et al*[28] concluded that pterygium resulted from disruption of the normal process of apoptosis. The increased cellular proliferation and low level of apoptosis in pterygium tissue was further confirmed by Liang *et al*.[29]

Many recent studies also consider pterygium as a neoplastic condition. The MAPK signal pathway, which is activated in nearly all cancers, has been shown to also be activated in pterygium tissue.[30–32] Loss of heterozygosity (LOH) and microsatellite instability (MI) are characteristics of transformed cells.[33–35] Detorakis *et al*[33] have found that LOH is common with pterygia most often at 9p (48%), followed by 17q (42%), but MI of DNA was uncommon. LOH was not detected in normal conjunctiva protected from UV radiation beneath the upper eyelid, proving that these genetic alterations are likely due to UV exposure.[34,36,37]

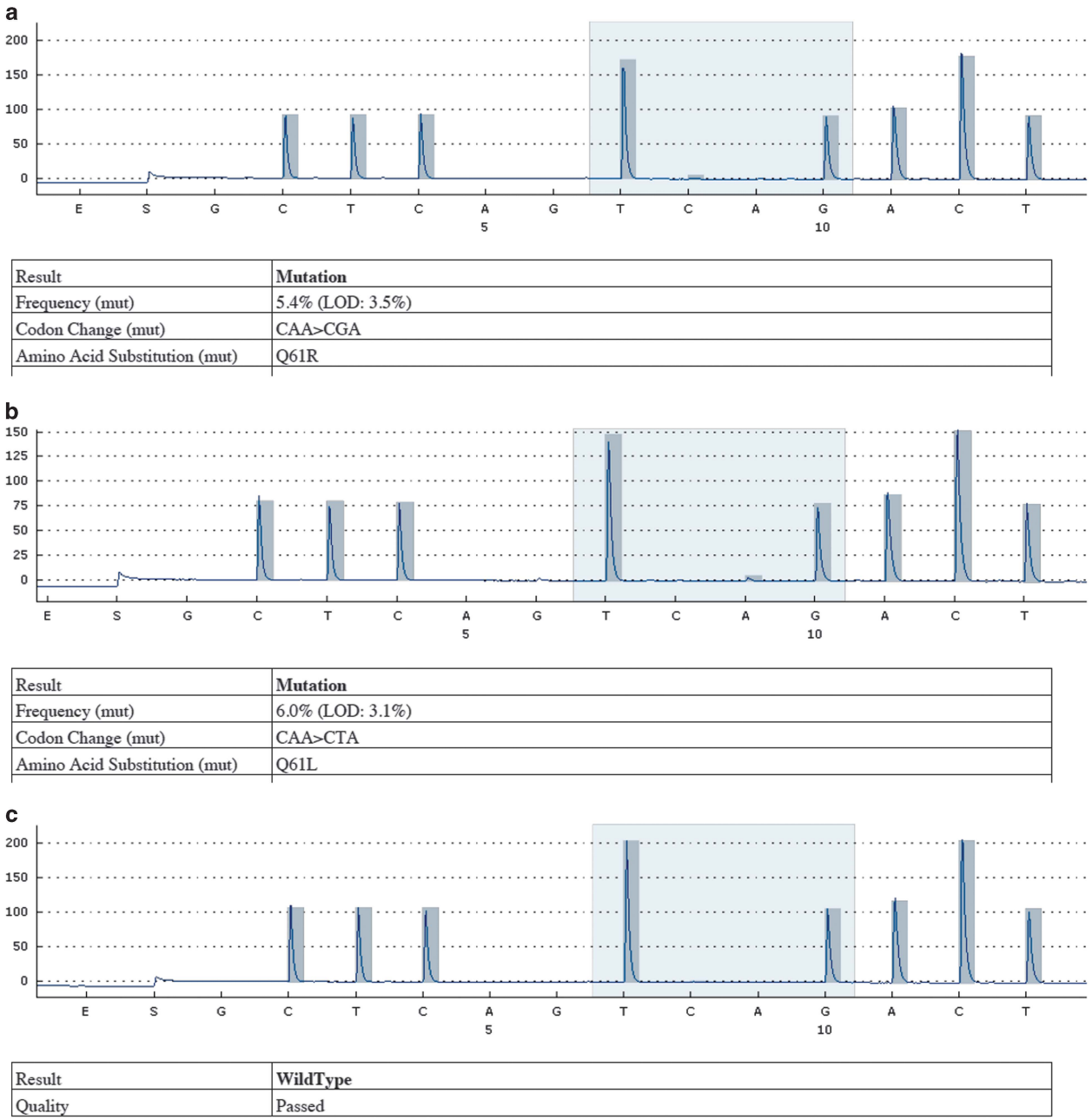
UV light exposure induces formation of pyrimidine dimers containing thymine and cytosine bases in DNA via photochemical reactions. These dimers are mutagenic if not repaired.[38] Among various types of lesions formed in DNA after UV light exposure, cyclobutane pyrimidine dimers are the most mutagenic based on their frequency, slow repair, and distinct mutagenicity.[39,40] Studies on UV light-induced skin tumors have shown that one of the

Table 2 Outcome of mutation analysis at codone 61

Case no.	*Codone 61*	
	Normal tissue	*Pterygium*
1	N	N
2	N	CAA>CGA
3	N	N
4	N	N
5	N	CAA>CGA
6	N	N
7	N	N
8	N	N
9	N	N
10	N	N
11	N	CAA>CTA
12	N	CAA>CGA
13	N	N
14	N	N
15	N	CAA>CTA
16	N	N
17	N	CAA>CGA
18	N	N
19	N	N
20	N	N
21	N	CAA>CTA
22	N	N
23	N	N
24	N	N
25	N	N

genes most sensitive to cyclobutain pyrimidine dimer formation is p53 and immunohistochemical studies further revealed overexpression of p53 in many types of UV radiation damaged cells.[41–43]

P53 is a key element in genomic stability and is involved in several aspects of cell cycle arrest, apoptosis, control of genome integrity, and DNA repair.[44] P53 mutations are known to be the most common genetic markers in human neoplasias.[45,46] Several previous studies reported increased p53 levels in pterygium tissue, while no positive staining has been observed in normal conjunctiva.[45–47] Further studies found that p53



Result	Mutation
Frequency (mut)	5.4% (LOD: 3.5%)
Codon Change (mut)	CAA>CGA
Amino Acid Substitution (mut)	Q61R

Result	Mutation
Frequency (mut)	6.0% (LOD: 3.1%)
Codon Change (mut)	CAA>CTA
Amino Acid Substitution (mut)	Q61L

Result	WildType
Quality	Passed

Figure 3 Sequencing graphic of codone 61. (a) CGA mutation of codone 61. (b) CTA mutation of codone 61. (c) Normal conjunctival tissue without mutation (wild type).

expression levels in pterygia differ between epithelial layers, being higher in basal cells compared with more superficial layers. These findings support the proposed theory of transcameral exposure of limbal basal cells to solar light.[6]

Oncogenes are also known to be affected by UV radiation. Mutations in ras family proto-oncogenes are encountered in cultured cells of mouse skin tumors and cutaneous melanoma following exposure to near-UV radiation. Such mutations may convert these proto-oncogenes into active oncogenes.[9,10] Ras genes are the most frequent proto-oncogene in tumoral proliferations of the human body. They are encoded by three genes: *H-ras, K-ras*, and *N-ras*. Of these, *K-ras* is the most frequent isoform encountered in the COSMIC data set, but there are limited data about ras mutations detected at early stages of carcinogenesis in precancerous or benign tumors.[13] It has been reported in benign skin tumors like keratoacanthoma and also in precancerous colon adenoma. Ninety-eight percent of human *K-ras* mutations

are reported at codons 12 and 13. Codon 61 mutations are rarely seen in malignancies.[13,48] Mutation of ras proteins at codon 61 impairs the coordination of water molecules necessary for phosphate binding, whereas mutations at codons 12 and 13 inhibit the formation of van der Waals linkage. The glutamine residue at position 61 (Gln61) has a vital role in catalysis as it forms a hydrogen bond with a specific residue (Arg 789) of GAP p120 to allow the nucleophilic attack of a water molecule that is crucial for GTP hydrolysis. Mutation of this residue therefore results in impaired hydrolysis of GTP, which gives rise to prolonged activation of the ras–GTP complex, subsequently overstimulating the Ras/Raf/MEK/MAPK pathway and leading to uncontrolled cell proliferation.[49]

The preliminary study about the role of K-ras mutation in pterygium performed by Detorakis *et al*[12] evaluated only codon 12 and 13 mutations and found a significant mutation rate of 10% at codon 12. They additionally noted a correlation between postoperative recurrence and young age, implying a relationship between clinical profile and locus of *K-ras* mutation. Contrastingly the mutation at codon 12 was detected in only one case in our study. This discrepancy may be attributed to the differences in the methods used for genetic analysis or to the criteria for patient selection. Detorakis *et al*[12] used the restriction fragment length polymorphism method to determine the frequency of *K-ras* mutations. As a difference we preferred pyrosequencing method as it enables regional sequencing with a higher sensitivity in addition to screening for known mutations.[50] The patient inclusion criteria demonstrated also some differences among the two studies. Detorakis *et al*[12] enrolled both primary and recurrent ptergium cases whereas only primary pterygium cases are included in our study. Regarding this fact the lower frequency of codon 12 mutations in primary pterygium cases in our study compared with the study of Detorakis *et al*[12] may be attributed to a possible correlation between pterygium recurrence and codon 12 mutation in the *K-ras* oncogene.

Another information our study contributes to that of Detorakis *et al*[12] is the higher frequency of codon 61 mutations in these primary pterygium cases. Evaluation of 25 cases revealed codon 61 mutation in seven cases: Glu61Arg in four cases and Glu61Leu in three cases. Transition from glutamine to leucine as a result of transversion from A–T to T–A at codon 61 of the *H-ras* oncogene has been previously reported in the benign skin tumor keratoacanthoma, supporting a possible relationship between codon 61 mutation and benign degenerative diseases.[48] As Detorakis *et al*[12] did not evaluate codon 61 mutations, no comparison could be made with our study results.

Further evaluation of cases with *K-ras* codon 61 mutations demonstrated similar mean age and clinical stage compared with the cases without a mutation at codon 61 except for the fact that all cases with codon 61 mutation exhibited bilateral pterygium. This is a remarkable finding implying a genetic predisposition in bilateral pterygium cases. However, the higher number of bilateral cases (15 out of 25) in the study might have affected the outcome as well. The time spent outdoors was even shorter in subjects with codon 61 mutation; however, not using sunglasses might also explain the higher frequency of genetic mutation. The low reliability of patients' responses on questionnaires is another confounding factor that must be kept in mind when evaluating these data.

The significantly higher frequency of *K-ras* oncogene codon 61 mutation in primary pterygium, we detected in our preliminary analysis has not been reported previously in the literature. We found a significant difference in codon 61 mutation frequency between pterygium and normal conjunctival tissues of the same patient. For this study, normal conjunctiva tissues were obtained from the upper fornix, a location that is not exposed to UV radiation, and no mutations were found in these tissue samples. This finding suggests a relationship of pterygium with oncogenes and thus tumoral proliferation. There are also other studies in the literature supporting the relationship between *K-ras* codon 61 mutation and UV radiation. Törmänen *et al*[51] demonstrated that UV radiation targets pyrimidine dimmers, resulting in a high bias toward *ras* Q61 mutations generation. This may be considered another supporting finding for the similarity of pterygium to a benign tumor. Lack of sunglasses use in patients with codon 61 mutation raises the possibility of a cumulative effect of UV in patients with a genetic mutation, a hypothesis that is further supported by the bilaterality of their pterygia.

Evaluation of recurrent cases and more detailed documentation of the risk factors, especially quantification of UV exposure, would improve the power of this study, and their absence can be accepted as this study's limitations. The higher number of bilateral cases in the study (15/25) should be respected also as a confounding factor in the study. Evaluation of fellow eye for the same mutation in bilateral cases and comparing the outcome with the same number of unilateral patients are warranted to confirm this finding.

Until the risk factors and mechanisms leading to pterygium formation are determined definitively, surgical excision remains the sole and best treatment option for pterygium. However, excision also entails certain risks and complications, including dellen formation and permanent astigmatism. Even the high recurrence rate following surgical excision highlights the fact that more effective treatment options targeting the underlying

molecular pathways and inhibiting this tumor-like formation at the proximal stages are warranted. The significantly higher frequency of codon 61 mutation of the *ras* oncogene in primary and bilateral pterygium specimens compared with normal conjunctiva found in this study might support the tumoral origin of this conjunctival hyperplasia and be an initial step toward targeted therapies that will improve the prognosis of pterygium.

Summary

What was known before

- There is %10 mutation at K-ras gene codon 12 in pterygium tissue and no mutation in normal conjunctiva.

What this study adds

- Results of this study revealed increased mutation at codon 61 in 28% of primary pterygium cases (28%). This supports the hypothesis that pterygium is a benign tumor proliferation.

Conflict of interest

The authors declare no conflict of interest.

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