Eye (2005) 19, 210–214

www.nature.com/eye

LABORATORY STUDY

Detection of point mutations at codon 12 of KI-*ras* in ophthalmic pterygia

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Abstract

Aims **Ophthalmic pterygium is a potentially vision-threatening lesion of unknown etiology, related to an exposure to solar light. Mutations to the *ras* genes are frequently observed in lesions related to an exposure to solar light. The present study aims at screening pterygia for mutations at codons 12 and 13 of the *ras* genes.**

Methods **In all, 50 pterygia were examined, together with respective blood samples and specimens of normal conjunctiva. A PCR reaction was performed to amplify sequences containing codons 12 and 13 of Ki-*ras*, H-*ras*, and N-*ras*. An RFLP analysis was then performed to detect point mutations at codon 12. The mutational status at codons 12 and 13 was further explored with sequencing of PCR products.**

Results **RFLP analysis revealed Ki-*ras* mutations at codon 12 in five (10%) of pterygia, whereas H-*ras* or N-*ras* mutations were not observed. Sequencing confirmed Ki-ras mutations at codon 12 and revealed absence of mutations at codon 13. The presence of Ki-*ras* mutations was significantly correlated with postoperative recurrence ($P = 0.02$) and young age ($P = 0.04$). Mutations were not observed in specimens of blood or normal conjunctiva for any of the genes examined.**

Conclusions **The absence of N-*ras* mutations is in agreement with previous reports concerning mucosal lesions. The detection of Ki-*ras* mutations and the association with postoperative recurrence implies a possible role of Ki-*ras* in the clinical profile of pterygium. The mechanism of Ki-*ras* mutations is unclear and could be independent of the action of UV light.**

Eye (2005) **19,** 210–214. doi:10.1038/sj.eye.6701452
Published online 2 July 2004

Keywords: pterygium ras gene mutation

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Received: 5 September 2003
Accepted: 12 January 2004
Published online: 2 July 2004

Introduction

Ophthalmic pterygium is a lesion of the corneoscleral limbus that, apart from local inflammation and disfigurement, can also expand towards the central cornea and threaten vision.[1] Although the pathogenesis remains obscure, the importance of heredity, possibly in an autosomal dominant form, as well as environmental factors, such as microtrauma by dust particles or exposure to solar radiation, especially scattered light (albedo), has long been recognised.[2] The ultraviolet radiation (UVR), especially UVR-A and UVR-B (290–400 nm), is considered the most dangerous.[3] There is growing evidence that pterygium is a proliferative, rather than degenerative, condition.[4] Experimental findings, such as detection of loss of heterozygosity (LOH),[4,5] association with oncogenic viruses,[6] and mutations in p53,[7] as well as clinical evidence, such as progressive growth and frequent postoperative recurrences,[1,5] support the concept that pterygium may be considered a benign neoplastic condition. Based on immunohistochemistry with monoclonal antibodies against p53 and vimentin, it has been proposed that pterygia may arise from a vimentin-expressing, altered limbal epithelial basal cell, the so-called 'pterygium cell' [8]

However, the exact molecular events leading to pterygium development are not clear. The frequent detection of LOH, which may result from exposure to UVR,[4] supports the involvement of tumour suppressor genes (TSGs) in the pathogenesis of pterygium.[5] Proto-oncogene sequences may also be affected by UVR.[9,10] Mutations in genes of the *ras* family, including Harvey-Ras (*H-ras*), Kirsten-Ras (*Ki-ras*), and Neuroblastoma-ras (*N-ras*), have been detected in cultured cells of mouse skin tumours following exposure to near-UVR[9] and are also frequently detected in human neoplastic conditions of sun-exposed body

areas, such as cutaneous melanomas.[10] Such mutations may convert these genes into active oncogenes.[11] Therefore, it is reasonable to assume that mutations in these genes may also be present in ophthalmic pterygia. The purpose of the present study is to evaluate the presence of such mutations and to explore the role they could possibly play in the clinical profile of ophthalmic pterygium.

Materials and methods

Specimens included in this study were obtained from patients treated at the University Hospital of Heraklion, Crete, Greece. All participants gave written informed consent to a protocol approved by the Institutional Review Board in conformity with the tenets of the Declaration of Helsinki. Pterygia were excised *in toto*, under local anaesthesia, using the bare sclera technique.[1] In each case, a blood sample and a conjunctival biopsy from a site relatively protected from solar radiation (corneoscleral limbus, 12 O'clock) were obtained. Immediately after surgery, tissue specimens (either pterygia or conjunctiva) and blood samples were stored at −70 and 4°C, respectively, until DNA extraction.

In all, 50 pterygia were obtained, 24 (48%) from males. The average age (mean ± standard error mean, range) was 65 ± 1.96 (30–92) years. In 48 cases (96%), the location of the pterygium was nasal and in two cases (4%) temporal to cornea. In 26 cases (52%), pterygium did not extend beyond 1 mm on the corneal surface, in 22 cases (44%), it was moderately advanced (2–3 mm); and in two cases (4%), it was advanced (>3 mm).[2] In 13 cases (26%) pterygium was recurrent (up to four previous operations). Pterygium was reported to exist on the average for 11.89 ± 1.37years (0.5–35 years) prior to the excision. A family history (occurrence of pterygium among first-degree relatives) was reported in 11 patients (22%). The average altitude of patients' residence was 208.8 ± 30.60 (0–800) m.

DNA extraction was performed using a standard protocol with organic detergents.[12] The yield of DNA from the excised pterygia and conjunctival specimens was on the average 10 μg. Initial DNA concentration was determined by agarose gel electrophoresis and then titrated to 200 μg/ml. Subsequently, a 50 μl PCR reaction (35 cycles) was performed. The same PCR system was used in all cases (MJ Research Inc., USA). The premix solution contained 200 ng of genomic DNA, 1 μM of each primer, 250 μM dNTPs, 5 μl of 10X buffer (670 mM Tris.HCl, pH 8.5;166 mM ammonium sulphate; 67 mM magnesium chloride; 1.7 mg/ml BSA; 100 μM β-mercaptoethanol and 1% (w/v) Triton X-100), and 1 U of *Taq* DNA polymerase (Life Technologies Ltd, UK). Primers used were selected to amplify a sequence containing codons 12 and 13 in N-*ras*, Ki-*ras*, and H-*ras*, as described elsewhere.[13,14] The primer sequences and the length of PCR products are shown in Table 1.

Reactions were denatured at 94°C for 3 min initially. In all, 35 cycles were used in all cases. In each cycle, denaturation was performed at 94°C for 60 s (in all cases), annealing was performed at 58°C for 40 s (Ki-*ras* and N-*ras*) and 64°C for 40 s (H-*ras*), and extension was performed at 72°C for 50 s (in all cases). A final extension was also used in all cases (72°C for 7 min).

An RFLP analysis was then performed to detect mutations in codon 12, as previously described.[13–15] The PCR products were processed with the Wizard PCR Preps DNA purification Clean kit (Promega, Southampton, UK) to remove unincorporated primers and dNTP. In the case of Ki-*ras* and N-*ras*, 10 μl of the PCR product was digested overnight with 30 U of *Bst*NI endonuclease (New England BioLabs Inc., USA) at 60°C, in a buffer solution containing 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μg/ml BSA (pH = 7.9). In the case of H-*ras*, overnight digestion of 10 μl of PCR product at 37°C in a buffer solution (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH = 7.9) containing 30 U of *Msp*I endonuclease (New England BioLabs Inc., USA) was employed. The final volume of the restriction digests was 15 μl (including 1 μl of buffer solution, 3 μl of distilled water, 1 μl of enzyme and 10 μl of PCR product). Subsequently, 10 μl of the incubation solution was electrophoresed in a 10% polyacrylamide gel, together

Table 1 Primer sequences used and respective PCR product in PCR amplification of Ki-*ras*, *H*-ras and N-*ras* sequences

Gene		*Primer sequences*	*Product*
Ki-ras	Forward	5′-ACTGAATATAAACTTGTGGTAGTTGGACCT-3′	157 bp
	Reverse	5′-TCAAAGAATGGTCCTGGACC-3′	
H-ras	Forward	5′-GAGACCCTGTAGGAGGACCC-3′	312 bp
	Reverse	5′-GGGTGCTGAGACGAGGGACT-3′	
N-ras	Forward	5′-GGGTGCTGAGACGAGGGACT-3′	83 bp
	Reverse	5′-ATATTCATCTACAAAGTG GTCCTGGA-3′	

Table 2 Postdigestion sequences for N-*ras*, H-*ras*, and Ki-*ras*

Gene	*Initial PCR sequence (bp)*	*Enzyme*	*Post-digestion sequences*	
			Wild type	*Mutated type (codon 12)*
N-ras	83	*Bst*NI endonuclease	19 bp + 23 bp + 41 bp	23 bp + 60 bp
H-ras	312	*Msp*I endonuclease	21 bp + 55 bp + 236 bp	21 bp + 291 bp
Ki-ras	157	*Bst*NI endonuclease	15 bp + 113 bp + 29 bp	15 bp + 142 bp

with undigested PCR product, and silver stained. Postdigestion sequences[15] for N-*ras*, H-*ras*, and Ki-*ras* are presented in Table 2.

Further exploration of the mutational status of specimens was performed with sequencing of PCR products. Sequencing enabled the evaluation of codons 12 and 13, located in the same amplified *ras* region (exon 1). The PCR products were resolved through 2% agarose gels, excised, and processed with the Wizard PCR Preps DNA purification Clean kit (Promega, Southampton, UK) to remove unincorporated primers and dNTP. The sequencing reaction contained: 4 μl Big Dye Terminator ready-reaction mix (PE ABI, Warrington, UK), 2 μl of cleaned PCR product, and 1.6 pmol of sequencing primer in a total reaction volume of 10 μl. Reaction conditions were: 96°C for 10 s, 50°C for 10 s and 60°C for 4 min, 25 cycles. Sequencing products were precipitated with isopropanol to remove unincorporated dye terminators and resuspended in 4 μl of loading buffer (formamide:dextran sulphate/ethylenediamine tetraacetic acid, 5:1). Products were run on a 377 ABI PRISM automatic sequencer and analysed with the Sequencing Analysis™ software (PE ABI, Warrington, UK). Both strands were sequenced for each PCR product.

A statistical analysis of results was performed using SPSS 8.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at 0.05.

Results

The RFLP analysis revealed heterozygous Ki-*ras* mutations at codon 12 in five cases (10%) of the examined pterygia. No H-*ras* or N-*ras* mutations were detected in any of the examined pterygia specimens. Mutations were also not detected in blood or conjunctiva specimens for any of the genes examined. Examples of specimens displaying heterozygous Ki-*ras* mutations (RFLP analysis) are shown in Figure 1.

Sequencing confirmed Ki-*ras* mutations at codon 12. Specimens mutated at codon 12 of Ki-*ras* displayed a transition from GGT (glycine) to GTT (valine) in all cases. The lack of mutations at codon 12 of Ki-*ras* for conjunctival and blood specimens was also confirmed with sequencing. Furthermore, no Ki-*ras* mutations at codon 13 were detected for any of the examined pterygia, conjunctiva, or blood specimens. With respect to H-*ras* and N-*ras*, sequencing revealed a lack of mutations at codons 12 and 13 for all examined specimens, including pterygia, normal conjunctiva, and peripheral blood. Sequencing profiles for a pterygium specimen mutated at codon 12 of Ki-ras as well as a nonmutated pterygium specimen are presented in Figure 2.

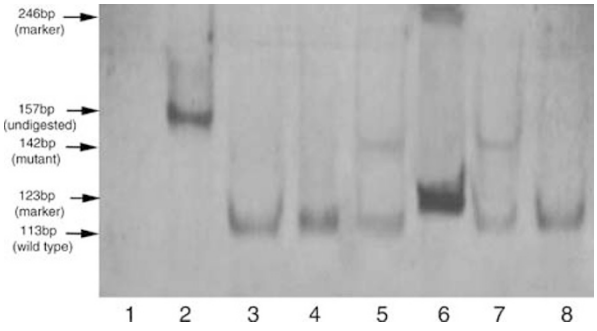

Figure 1 Silver-stained 10% polyacrylamide gel showing two heterozygous K-*ras* mutations in specimens of ophthalmic pterygia. Specimens in lanes are as follows: Lane 1 = blank, lane 2 = undigested PCR product, Lanes 3, 4, 8 = wild-type specimens, lanes 5 and 7 = mutated specimens, lane 6 = 123bp marker.

Mutations in Ki-*ras* were more common in recurrent pterygia (Fisher's exact test, one-tail P value = 0.02, two-tail P value = 0.02). Furthermore, patients displaying Ki-*ras* point mutations at codon 12 were of significantly younger age compared with patients not displaying such mutations (54.50 ± 8.31 and 66.31 ± 1.97 years, respectively, independent-samples t-test value = 1.96, $P = 0.04$). The correlation between Ki-*ras* mutations and gender, altitude of present residence, family history of pterygium, size and location of the lesion, and time interval that pterygium was reported to exist was statistically insignificant.

Discussion

With the present study, we aimed at screening specimens of ophthalmic pterygium for mutations at codons 12 (RFLP and sequencing analysis) and 13 (sequencing analysis) of the members of the *ras* gene family. *Ras* genes

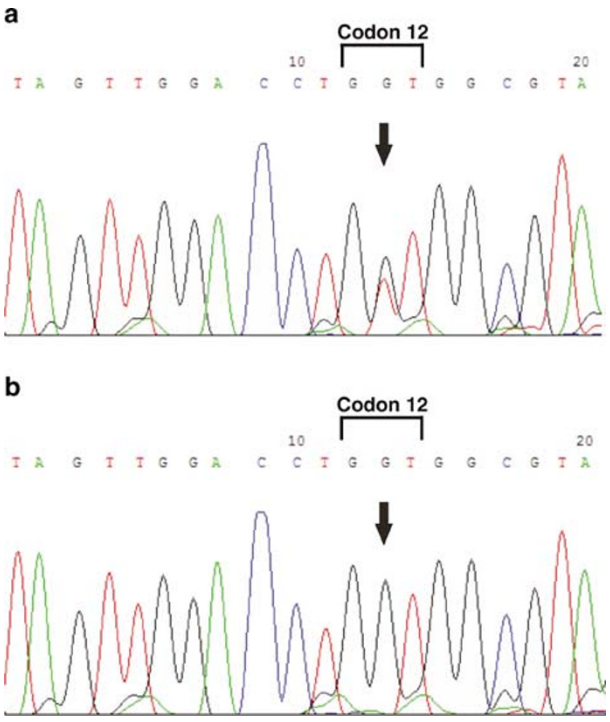

Figure 2 Sequencing profiles of mutated (a) and nonmutated (b) pterygia specimens. A heterozygous point mutation at the second base of codon 12 (G to T) is detected, signifying a change from glycine to valine at that codon for the p21 protein.

are implicated in several different malignancies and have been shown to be mutated in 10–15% of human solid tumors.[16,17] Point mutations at critical domains, such as codons 12, 13, and 61[18] is a common mechanism of *ras* gene activation.[17]

Mutations in members of the *ras* family genes, especially N-*ras*, are often found in cutaneous conditions, such as cutaneous melanoma, associated with exposure to UVR.[10,11] However, it has been reported that N-*ras* mutations are considerably more rare in mucosal lesions than in cutaneous lesions.[10] In a previous study assessing the role of N-*ras* mutations in conjunctival melanomas, no such mutations were detected.[11] Our findings are in agreement with these reports and indicate that N-*ras* and H-*ras* are possibly not mutated at codons 12 and 13 in the case of ophthalmic pterygium.

However, the detection of Ki-*ras* mutations at codon 12 and especially the significant correlation between Ki-*ras* mutations and the postoperative recurrence as well as the young age imply a possible role of such mutations in the pathogenesis and biological profile of ophthalmic pterygium. The *ras* family gene product (protein p21) belongs to the small GTP-binding proteins (G proteins) and regulates gene expression.[19] Small GTP-binding protein GDP dissociation stimulator (Smg GDS) regulates GDP/GTP exchange reaction of *ras* and the Rho and Rap1 family members and inhibits their binding to membranes.[19] In fibroblasts, Smg GDS shows mitogenic and transforming activities in cooperation with Ki-*ras*, possibly through antiapoptotic cell survival signalling.[19] It was recently reported that pterygia display different paterns of apoptosis and expression of *bcl-2* and *bax* compared to normal adjacent conjunctiva and it was suggested that deregulation of apoptosis may be an underlying mechanism leading to pterygium development.[20] The possible involvement of Ki-*ras* in the pathogenesis and biological behaviour of ophthalmic pterygium complies with these findings and further supports the concept that pterygium may be a neoplastic condition.

The transition from GGT (glycine) to GTT (valine) at codon 12 of Ki-ras has often been described in the past in various tumors, including pancreatic,[21] lung[22] and colon[23] cancers. There are conflicting reports on its clinical significance.[24,25] Some studies report that it is an innocuous mutation characteristic of indolent tumours,[24] whereas others report that it is characteristic of more aggressive forms of neoplasia, including metastatic tumours.[25] The same transition has been described in preneoplastic lesions, such as aberrant crypt foci (ACF) in the colon,[26] or even phenotypically normal mucosa located near neoplastic lesions, as in the case of non-neoplastic mucosa from bronchial carina in patients with lung adenocarcinomas.[27] In the present study, mutations were not observed in control tissues, including peripheral blood and phenotypically normal conjunctival specimens, harvested from the same patients, implying that the mutations detected may be specific for pterygium.

The mechanism of genetic damage to Ki-*ras* is unclear. Genetic lesions previously detected in pterygia, such as LOH and mutations to *P53*[4] were, in some cases, directly correlated to the level of exposure to UVR or were located in regions with known susceptibility to UVR, such as 9q or 17p.[5] In the present study, Ki-*ras* mutations were not detected in conjunctival specimens excised from areas located under the upper eyelid and thus protected from the solar light. However, mutations to N-*ras*, which have been reported to be highly susceptible to UVR,[10,11] were not detected in any case. Furthermore, the altitude of residence, which is correlated with the exposure to UVR,[5] was not significantly higher in patients displaying Ki-*ras* mutations. Therefore, although absent in areas with less exposure to solar light, mutations detected could be independent of the action of UVR.

Postoperative recurrence is an important problem in the management of pterygia and repeated operations often lead to extensive scarring and symblephara formation, thus adding to the discomfort of patients and further reducing vision.[1] Furthermore, pterygium is a

very common condition in populations with a high level of exposure to solar light.[2] Understanding the exact nature of this condition, which could lead to new, less aggressive and more effective methods of treatment, is therefore important. The role of the *ras* gene family in the pathogenesis of pterygium could be further examined by evaluating mutations to other codons, such as 61, which are also critical for the function of p21, as well as by assessing the expression of the *ras* genes in both pterygia and normal conjunctiva. Apart from helping in understanding the pathogenesis of pterygium, research towards this goal may also have strong therapeutic implications as it could enable alternative methods of treatment, such as gene therapy by introducing normal genetic material in affected cells.

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