

# Evaluation of loss of heterozygosity and microsatellite instability in human pterygium: clinical correlations

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## Abstract

**Aims**—To evaluate the incidence of loss of heterozygosity (LOH) and microsatellite instability (MI) in pterygia and their possible correlation with clinical variables.

**Methods**—50 pterygia, blood, and conjunctival specimens were obtained. A personal and family history was recorded for each patient. Amplification of 15 microsatellite markers at regions 17p, 17q, 13q, 9p, and 9q was performed using the polymerase chain reaction. The electrophoretic pattern of DNA from pterygia was compared with the respective pattern from blood and conjunctiva.

**Results**—LOH incidence was the highest at 9p (48%), followed by 17q (42%). Only three cases displayed MI. LOH incidence at individual markers was positively correlated with recurrence (D9S59,  $p=0.11$  and D9S270,  $p=0.16$ ), family history of neoplasia (D13S175,  $p=0.09$ ), altitude of present residence (D9S112,  $p=0.1$ ), duration of the existence of pterygium (D9S144,  $p=0.06$ ), and inversely correlated with age (D9S59,  $p=0.09$ ). Concerning chromosome arms, LOH was positively correlated with the altitude of present residence (13q and 17p,  $p=0.03$ ) and duration of the existence of pterygium (13q and 17p,  $p=0.09$ ).

**Conclusions**—LOH is a common event whereas MI is a very uncommon one at the examined markers in pterygium, indicating the presence of putative tumour suppressor genes implicated in the aetio-pathogenesis of the disease. The fact that LOH at 9q31-33 was more frequent in recurrent pterygia and also correlated with known risk factors such as young age and high altitude of residence, implies a possible predictive value of this finding for postoperative recurrence.

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Pterygium is a conjunctivalisation of the limbal cornea leading to loss of transparency of the adjacent corneal region. It is often bilateral, and grows at the interpalpebral limbal area, in general, nasally.<sup>1</sup> The pathogenesis of the condition is not yet fully understood despite the diversity of theories that have been proposed.<sup>1</sup> A strong correlation with environmental exposure such as sunlight, dust, and wind has been accepted.<sup>2</sup> Pterygium can threaten vision in many ways and is the subject of thousands of surgical procedures annually.<sup>2</sup> Major indica-

tions for surgery include progressive growth towards the visual axis, irregular astigmatism, and restriction of ocular motility while other indications are contact lens intolerance, chronic irritation, and disfigurement.<sup>2</sup> Apart from the tendency to grow in size, pterygia often recur after excision. Recurrent pterygium usually grows more aggressively and requires more sophisticated surgery.<sup>2</sup> Fibroblasts from pterygia have been shown to behave as neoplastic cells in vitro<sup>3</sup> and random histological examination has revealed neoplastic features.<sup>4</sup> Polymerase chain reaction (PCR) studies have revealed viral presence (herpes simplex virus, cytomegalovirus, human papillomavirus) in pterygia.<sup>5,6</sup> Human papillomavirus in particular, has been correlated with neoplastic lesions of conjunctiva.<sup>7</sup> Thus, it has been suggested that pterygium possesses similarities to neoplasia and could be considered a benign neoplastic lesion.<sup>8</sup>

Among the genes that play a key role in multistage carcinogenesis are the tumour suppressor genes.<sup>9</sup> Tumour suppressor genes are often inactivated by mutation or loss of genetic material in one allele. If the remaining normal allele is lost (loss of heterozygosity, LOH), a tumour can potentially develop.<sup>9</sup> Additionally, neoplastic cells show instability of the microsatellite DNA (MI), reflecting an elevated mutational rate.<sup>10</sup> The highly polymorphic microsatellite DNA, composed of repetitive sequences of dinucleotides (CA)<sub>n</sub> and rarely trinucleotides,<sup>11</sup> has been used for genetic linkage analysis, in various conditions including neoplasias. The finding of LOH at a microsatellite marker locus implies that a nearby gene could be affected by the genetic lesion.<sup>12,13</sup> A previous study on pterygium revealed a significant incidence of LOH for 17q11.2-q21 (47%) and a moderate incidence of MI (13%).<sup>8</sup> Apart from evaluating the occurrence and incidence of LOH and MI in pterygial tissues, the present study examined the possible association of these phenomena with clinical and epidemiological variables. The results imply the involvement of tumour suppressor genes located in certain chromosomal sites and that LOH at 9q31-33 correlates with known risk factors.

## Materials and methods

The samples were obtained from patients treated at the ophthalmological clinic of the University Hospital of Heraklion, Crete. A personal and family medical history was recorded for each patient including infor-

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mation on age, sex, profession, place of birth and residence, previous pterygium surgery and recurrences, duration of the existence of pterygium, familial occurrence of pterygium, neoplastic diseases, ophthalmic and skin infections, as well as medications used. A slit lamp examination was also performed and pterygia were photographed before surgery. The lesions were excised in toto, under local anaesthesia, using the bare sclera technique.<sup>2</sup> In each case, a blood sample and a conjunctival biopsy from a site located far from the pterygium and relatively protected from solar radiation (12 o'clock) was obtained. Immediately after surgery, tissue specimens (either pterygia or conjunctiva) and blood samples were stored at  $-70^{\circ}\text{C}$  and  $4^{\circ}\text{C}$  respectively, until DNA extraction and PCR amplification.

Fifty pterygia were obtained, 24 (48%) from male and 26 (52%) from female patients. The average age (mean (SD)) was 65 (1.96) (range 30–92) years, 69 (2.95) (30–92) years in men and 61 (2.43) (39–84) years in women. The excision was performed on the right eye in 22 (44%) and on the left eye in 28 (56%) cases. 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).<sup>2</sup> In 37 cases (74%) pterygium was operated for the first time, in six (12%) for the second time, in six (12%) for the third time, and in one case (2%) for the fourth time. Pterygium was reported to exist on average for 11.89 (1.37) years (0.5–35 years) before the operation. Further clinical examination and history recording revealed cataract in 15 (30%), conjunctivitis in seven (14%), glaucoma in three (6%), ophthalmic allergic reactions in one (2%), and no specific finding other than pterygium in 24 (48%) of the

Table 1 Frequency and percentage of loss of heterozygosity (LOH) and microsatellite instability (MI) for the selected markers

Marker	Location	LOH (%)	MI (%)
THRA1	17q11.2-q12	8 (16)	1 (2)
D17S579	17q21	5 (10)	0
D17S855	17q21	13 (26)	0
TP53	17pter-p11.22	5 (10)	1 (2)
D17S515	17p13	3 (6)	0
D17S678	17p13	1 (2)	0
D13S155	13q14.3-q21.2	0	0
D13S168	13q14.3	9 (18)	0
D13S175	13q11	9 (18)	0
D9S59	9q31-q33	8 (16)	0
D9S112	9q31-q34	4 (8)	0
D9S109	9q31	11 (22)	0
D9S50	9p21	11 (22)	1 (2)
D9S270	9p23-p22	8 (16)	0
D9S144	9pter-p22	11 (22)	0

Table 2 Frequency and percentage of loss of heterozygosity (LOH) and microsatellite instability (MI) for each chromosome arm examined

Chromosome arm	LOH (%)	MI (%)
17q	21 (42)	1 (2)
17p	14 (28)	1 (2)
13q	14 (28)	0
9q	19 (38)	0
9p	24 (48)	1 (2)

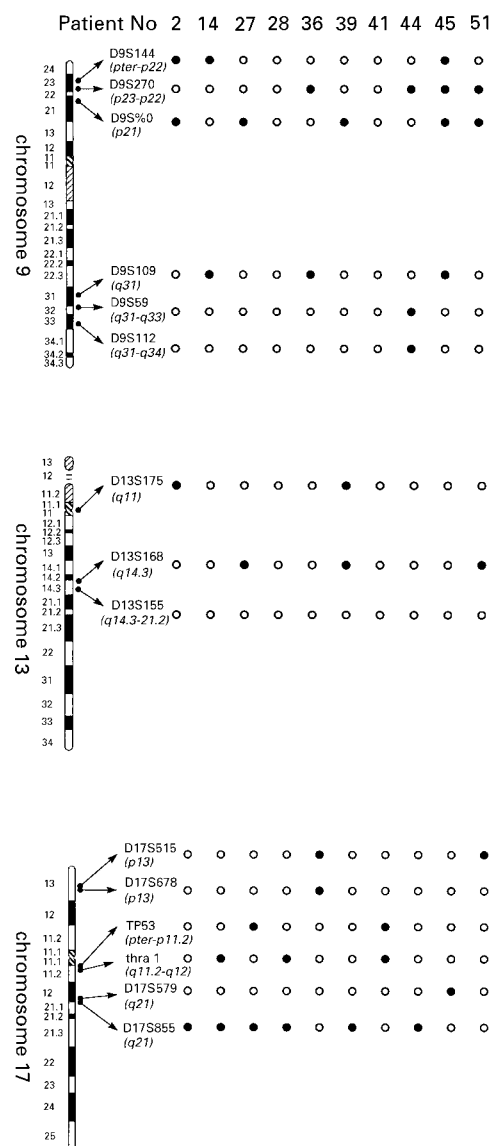


Figure 1 Location of selected markers at chromosome 9, 13, and 17 and loss of heterozygosity in 10 randomly selected cases (●, loss of heterozygosity; ○, heterozygosity).

patients. Thirty nine (78%) patients had not been using any topical medication before the operation, seven (14%) had been using topical steroids, one (2%) decongestants, and one (2%) antiallergic medication. Three patients (6%) had been using antiglaucomatous therapy in the operated eye for 1–6 years. All patients were from rural areas of Crete. The average altitude of their present residence was 208.8 (SD 30.608) (range 0–800) metres, while the average altitude of their residence during the first 20 years of their life was 239.1 (29.335) (0–630) metres.

The selection of chromosome regions to be evaluated was based on previous studies concerning either ocular (pterygium<sup>8</sup>) or skin (actinic keratoses<sup>15</sup>) tissues. Regions 17q, 17p, 13q, 9p, and 9q were evaluated. Overall, 15 markers were examined, three in each chromosome arm. DNA extraction was performed under a standard protocol using organic detergents. A 25  $\mu\text{l}$  PCR reaction was performed,

containing 200 ng of genomic DNA, 1  $\mu$ M of each primer (<http://www.gdb.org/gdb/>), 250  $\mu$ M dNTPs, 2.5  $\mu$ l of 10X buffer (670 mM TRIS.HCl, pH 8.5; 166 mM ammonium sulphate; 67 mM magnesium chloride; 1.7 mg/ml BSA; 100  $\mu$ M,  $\beta$ -mercaptoethanol and 1% (w/v) Triton X-100) and 1 U of Taq DNA polymerase. The reactions were denatured for 3 minutes at 94°C and DNA was subsequently amplified for 30 cycles at 94°C, then 50°C, 55°C, or 58°C (annealing temperature depending on the primer used each time) and 72°C, each step. Ten  $\mu$ l of the PCR product were electrophoresed in a 10% polyacrylamide gel and silver stained. Gels were scanned and the intensity of the bands corresponding to the microsatellite alleles was quantitated by an image analysis system. The analyses were performed twice and the results were highly reproducible. A comparison of the electrophoretic patterns of the amplified marker segments in the normal and pterygium tissues was done. MI was diagnosed in case of an addition or deletion of one or more repeat units resulting in novel alleles. LOH was evaluated as a significant decrease (more than 50%) in intensity of one allele relative to the other as determined from comparison of pathological and normal DNA. In a few cases, although LOH takes place, the PCR amplification of polymorphic microsatellite alleles results in an allelic imbalance owing to the presence, in decreased levels, of a contaminating band. This band corresponds to the deleted allele which is present in the normal tissue. Generally, this "contamination" is attributed to the presence of normal DNA derived by either peripheral blood or adjacent normal tissue. However, it has been suggested that it is possible to obtain information regarding the heterogeneity of the tumour by quantitation of the allelic imbalance

resulting from the PCR amplification of the microsatellite markers.<sup>14</sup>

Statistical analysis of the results was performed with the package SPSS 6.0 (for windows). Statistical significance was set at  $p < 0.05$ .

## Results

Fifteen specimens (30%) displayed LOH at one locus, 12 (24%) at two loci, six (12%), at three loci, 11 (22%) at four loci, and one (2%) at five of the examined loci. LOH incidence was highest at 9p (48%) followed by 17q (42%). Only three cases of MI were noted. The number and percentage of specimens displaying LOH and MI for each of the markers tested are shown in Table 1 and the respective percentages for chromosome arms are shown in Table 2. The location of selected markers at specific chromosome regions as well as LOH in 10 randomly selected cases are shown in Figure 1. Representative examples of specimens displaying LOH are shown in Figure 2.

The cumulative evaluation of LOH and MI for all markers used did not reveal any statistically significant ( $p < 0.05$ ) correlation with the clinical and epidemiological variables examined. The same was true for the incidence of MI or LOH concerning whole chromosome arms. There were however individual markers or chromosome arms for which LOH incidence displayed a significant or nearly significant correlation with specific clinical variables. Recurrence, as shown by the number of reoperations for the same pterygium, was positively correlated with LOH incidence at marker D9S270 (one tail Fisher's exact test,  $p = 0.09$  and two tail Fisher's exact test,  $p = 0.16$ ) and D9S59 ( $\chi^2 = 2.55$ ,  $p = 0.11$ , Mantel-Haenszel test = 2.49,  $p = 0.11$ ). The time interval the pterygium was reported to

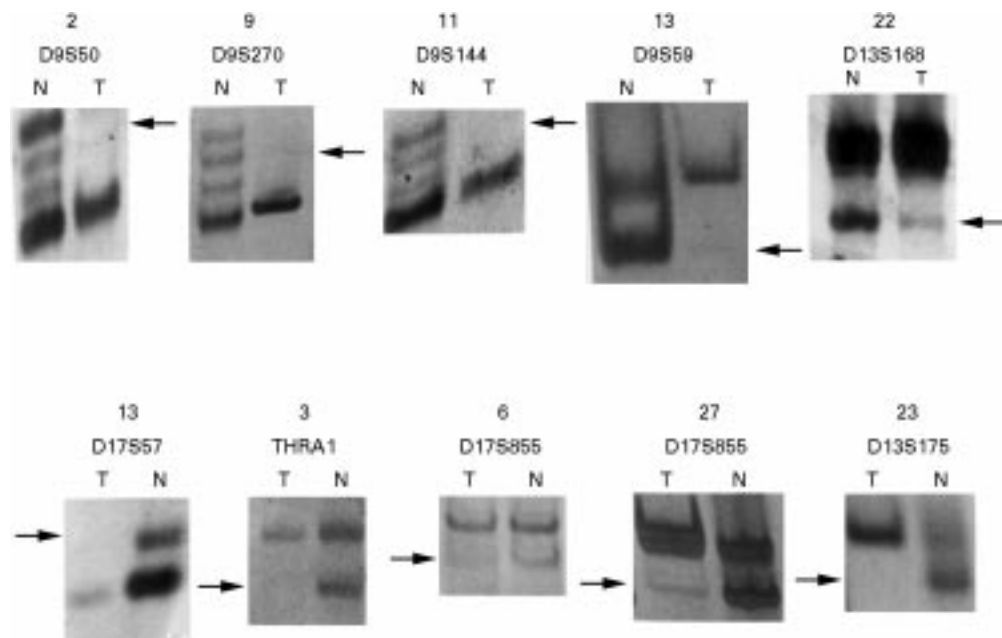


Figure 2 Representative samples of loss of heterozygosity detected in pterygium. N=normal DNA, T=pathological DNA. Arrows indicate the position of a deleted allele. Faint bands in the position of a deleted allele are interpreted as contamination by adjacent normal DNA.

have existed was positively correlated with LOH incidence at marker D9S144 (one tail Fisher's exact test,  $p=0.04$ , two tail Fisher's exact test,  $p=0.06$ ). A correlation with time was also found for D17S855 (one tail Fisher's exact test,  $p=0.08$ , two tail Fisher's exact test,  $p=0.14$ ). Family history of neoplasia displayed a positive correlation with LOH incidence at marker D13S175 (one tail Fisher's exact test,  $p=0.09$ , two tail Fisher's exact test,  $p=0.09$ ). The altitude of patients' present residence was positively correlated with LOH incidence at marker D9S112 (one tail Fisher's exact test,  $p=0.059$ , two tail Fisher's exact test,  $p=0.1$ ). The correlation of LOH incidence at the same marker with the altitude of patients' residence during the first 20 years of their life is much less significant (one tail Fisher's exact test,  $p=0.13$ , two tail Fisher's exact test,  $p=0.28$ ). Age was inversely correlated with LOH incidence at marker D9S59 (one tail Fisher's exact test,  $p=0.06$ , two tail Fisher's exact test,  $p=0.09$ ).

The positive family history of pterygium (occurrence of the condition among first degree relatives), was not significantly correlated with LOH incidence for any of the markers examined. However, patients with a positive family history displayed earlier onset of pterygium ( $r=-0.43, p=0.002$ ) and a higher rate of postoperative recurrence ( $r=0.39, p=0.056$ ).

In the case of chromosome arms, the time interval during which pterygium was reported to exist displayed a positive correlation with LOH incidence at 13q and 17p ( $\chi^2=2.81$ , Mantel-Haenszel test=2.74,  $p=0.09$ ). The altitude of patients' present residence was significantly correlated with LOH at 13q and 17p ( $\chi^2=4.27$ , Mantel-Haenszel test=4.19,  $p=0.03$ ).

### Discussion

The fact that pterygia quite often display the phenomenon of LOH, suggests that tumour suppressor genes could be involved in the development of such lesions. LOH is a key pointer to the existence of tumour suppressor genes, according to "Knudson's" two hit hypothesis, and by screening paired blood and tumour samples with markers spaced across the genome, candidate locations for tumour suppressor genes can be discovered.<sup>14</sup> Genetic instability in the form of LOH has also been found in benign conditions, such as actinic keratosis of the skin, a focal area of dysplasia in the skin with low risk of progression to squamous cell cancer, which is associated with exposure to sunlight,<sup>15</sup> in neurodegenerative diseases,<sup>16</sup> atherosclerotic plaques,<sup>17</sup> and spontaneously aborted embryonic tissues.<sup>18</sup>

LOH in pterygium has been reported to be more prominent at 17q.<sup>8</sup> In the present study, the region displaying the highest incidence of LOH was 9p (48%), but the high incidence of LOH at 17q (42%) is confirmed. The fact that areas 9p and 17q show similar findings in a variety of neoplastic lesions at various organs such as breast,<sup>19</sup> lung,<sup>20</sup> oesophagus,<sup>21</sup> kidneys,<sup>22</sup> urinary bladder,<sup>23</sup> skin,<sup>24</sup> and blood<sup>25</sup>

as well as in pterygium, suggests that candidate tumour suppressor genes located at this area may play an important universal role in the development of neoplasia.

Normal conjunctival tissue excised from sites largely protected by the eyelids from environmental influences did not display LOH compared with constitutional blood for any of the microsatellite markers examined. This could indicate that these genetic abnormalities are tissue specific for pterygium but also implies a possible relation of LOH with sunlight exposure. Basal cell carcinomas, which are strongly related to ultraviolet light exposure, are reported to display high incidence of LOH at 9q22.<sup>26</sup> Interestingly, LOH incidence at D9S112 (9q33) was positively correlated ( $p=0.1$ ) with the altitude of patients' current residence, which in turn is known to be related to ultraviolet exposure.<sup>27</sup> Nevertheless, the correlation of LOH incidence at the same marker with the altitude of residence during the first 20 years of their life was considerably weaker ( $p=0.28$ ), despite the fact that there is a strong suggestion of a causal relation between ultraviolet light exposure and the development of pterygia during the early years of life and the cumulative exposure over the following two to three decades in occupations with a high component of reflected ultraviolet light.<sup>28</sup> The significant ( $p=0.03$ ) positive correlation of LOH incidence at chromosome arms 17p and 13q with the altitude of patients' current residence, despite the fact that individual markers located in these areas did not display a similar finding, points towards a non-specific susceptibility of genetic material at these areas to environmental influence, in particular ultraviolet light. There are reports of ultraviolet related DNA damage localised at these regions.<sup>26, 29</sup>

A genetic predisposition towards the development of pterygium has been suggested, possibly in the form of autosomal dominant heredity.<sup>30</sup> The correlation of familial occurrence of pterygium with young age at onset and postoperative recurrence found in the present study is in agreement with previous reports.<sup>31, 32</sup> The fact that in the present study, LOH did not correlate significantly with family history of pterygium or neoplasia is in accordance with the concept of LOH as a somatic event, characterising a clone of cells. The almost significant ( $p=0.06$ ) positive correlation of LOH incidence at D9S144 (9pter-p22) with the time interval pterygium was reported to exist, could suggest that during the course of the disease, new events are added which may contribute to the stabilisation or progression of the lesion. The possibility of a time proportional addition of genetic damage is further supported by the fact that D9S144 displayed a fairly high (22%) incidence of LOH, while D17S855, at which LOH also correlated weakly ( $p=0.14$ ) with time, displayed the highest one (26%). Although in a previous study on pterygia,<sup>8</sup> LOH was reported to be more common in female patients, in the present study no significant correlation of LOH incidence with sex was found for any of the markers examined. This is



in agreement with a diversity of other studies evaluating the possible relation of LOH to clinical variables.<sup>33-35</sup>

Despite the fact that pterygia often recur aggressively after excision,<sup>2</sup> attempts to establish reliable predictive factors for recurrences have failed.<sup>36</sup> In the present study, LOH incidence at the microsatellite markers examined did not generally correlate with a significant degree with recurrence, as expressed by the number of reoperations for the same lesion. There was only a weak positive correlation of LOH incidence at D9S59 (9q31-33) and D9S270 (9p23-p22) with recurrence ( $p=0.11$  and  $p=0.16$  respectively). Yet, the percentage of LOH for these markers in the specimens examined was not very high (16%) compared with other markers. This suggests that the phenomenon of LOH in pterygia could be considered generally independent of the tendency for recurrence. Nevertheless, LOH at D9S59 (9q31-q33) was more frequent among patients under 50 years of age compared with older patients at an almost significant degree ( $p=0.06$ ). Young age has been reported to be a risk factor for postoperative recurrence of pterygia.<sup>32</sup> Taking into account that region 9q33 also displayed higher incidence of LOH in patients with greater exposure to ultraviolet radiation, another recognised risk factor for pterygium, the possible predictive value of LOH at that region should be further evaluated with prospective studies and fine mapping.

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