P53 gene mutation spectrum and the relationship between gene mutation and protein levels in pterygium

Yi-Yu Tsai,1,2 Ya-Wen Cheng,3 Huei Lee,3 Fuu-Jen Tsai,4,5 Sung-Huei Tseng,6 Kong-Chao Chang7

Purpose: To investigate the spectrum of p53 gene mutations and the relationship between gene mutation and p53 protein levels in pterygium.

Methods: Pterygial samples were harvested from 51 patients undergoing pterygium surgery. DNA samples for p53 mutation analyses were extracted from epithelial cells and subjected to DNA sequencing for examination of mutations in exons 4, 5, 6, 7, and 8 of the p53 gene. In situ levels of p53 protein were studied by immunohistochemistry (IHC) and the percentage of positively stained cells quantified. Ten normal conjunctiva samples were included in this study as controls.

Results: Mutations within the p53 gene were detected in 8 pterygial samples (15.7%) with only one mutation found in each sample. All the mutations observed were point mutations, with 6 being substitutions and 2 deletions. Three mutations were identified in exon 6, two in exon 7, and a single mutation found in each of exons 4, 5, and 8. P53 protein levels were scored as 0 (negative) in 31 pterygial specimens (60.8%), +1 in 9 samples (17.6%), +2 in 5 samples (9.8%), and +3 in 6 samples (11.8%) by IHC. The 8 samples found to have p53 gene mutations were equally distributed among the different levels of p53 protein observed using IHC, with 2 samples in each group. The two deletion mutations, which caused a frame shift to occur, were found in samples negative for p53 immunostaining (score 0), while substitution mutations were found in samples positively stained (score +1, +2, and +3).

Conclusions: Mutations within p53 gene exons 4-8 were detected in pterygial epithelium and the mutations showed no correlation with p53 protein levels as seen by IHC.

The p53 gene is a tumor suppressor gene with important functions in cell cycle control and the prevention of tumor formation. Mutations in the p53 gene could lead to tumor formation and have been found in various types of tumor cells [1]. Previous studies of the p53 gene have been conducted using either DNA sequencing or via immunohistochemical (IHC) detection of the p53 protein.

Pterygium is a chronic condition characterized by the encroachment of a fleshy triangle of conjunctival tissue into the cornea. Pterygium has long been considered a degenerative condition, however, after abnormal levels of the p53 protein being found in epithelium, some researchers feel that pterygium is a UV related, uncontrolled cell proliferation consistent with that of a tumor [2-7].

Nearly all previous reports of the p53 gene in pterygium have been conducted using IHC staining. The reported prevalence of p53 positive staining, or presumed p53 gene mutation, varies widely, from 7.9% to 100% [2-9]. In normal, unstressed cells, p53 is a short lived protein, which is maintained at low, often undetectable, levels in the cell. Mutations in the p53 gene are believed to lead to an increased stability of its protein in the cell, allowing its detection by antibodies recognizing several p53 epitopes. In previous studies, over 85% of mutations detected in the p53 gene were missense mutations that encode for altered forms of the protein that still gave positive p53 immunostaining. However, other mutations, including non-in-frame deletions, splice mutations, and nonsense mutations, were negative following IHC [10-13]. Therefore, DNA sequencing is a far more accurate means to evaluate nature of p53 gene mutations [12].

To our knowledge, there have been only two previous studies using DNA sequencing to identify p53 gene mutations in pterygium. In the study of Reisman et al. [13], they found that the p53 gene had undergone a mono-allelic deletion, but the remaining allele remained wild type. In the study of Shimmura et al. [14], they reported that no mutation was found in exons 5 through 8 of the p53 gene. However, only 9 and 6 cases, respectively, were examined in these two studies.

In this study, we used DNA sequencing to detect mutations of the p53 gene in exons 4 to 8 in 51 pterygium specimens. P53 protein is reportedly found only in the thin layer of epithelium [2-9], therefore, laser capture micro-dissection (LCM) was used to remove the subepithelial fibrovascular layer to then permit isolation of only the epithelial cells. P53 protein levels in these cells were also evaluated by IHC. Finally, the results of IHC and DNA sequencing were compared.

METHODS

Pterygial samples were harvested from 51 patients undergoing pterygium surgery. All specimens were formalin fixed and
paraffin embedded. Sections (3 μm thick) were cut, mounted on glass, and dried overnight at 37 °C for IHC and DNA sequence analysis.

**DNA sequence analysis:** Mutations in exons 4, 5, 6, 7, and 8 of the p53 gene were determined by direct sequencing. DNA was extracted from the paraffin embedded pterygium tissues. As pterygial epithelial cells are difficult to capture by LCM directly, we removed the subepithelial fibrovascular layer first by LCM and left the epithelium intact as shown in Figure 1. DNA lysis buffer was applied to lyse the epithelial cells on a glass slide and then the DNA solution transferred into an eppendorf tube for traditional proteinase K digestion and phenol-chloroform extraction. Finally, the DNA was precipitated by ethanol with the addition of linear polyacrylamide to increase the amount of DNA available [15]. Target sequences were amplified in a 50 µl reaction mixture containing 20 pmol of each specific primer, 2.5 units of Taq polymerase (TAKARA Shuzo, Shiga), 0.5 mM dNTPs, 5 µl PCR reaction buffer, and 1 µl genomic DNA as the template. Genomic DNAs extracted from the paraffin sections were not adequate for amplification of long DNA fragments and, therefore the PCR products amplified ranged from only 200 to 400 bp. β-Actin was used as an internal control. The primers used for p53 gene sequencing are listed in Table 1. An initial cycle was performed for 5 min at 94 °C, followed by 35 cycles each for 40 s at 94 °C, 40 s at 54 °C, and 1 min at 72 °C. The PCR products were sequenced by an auto-sequencing system (Applied Biosystems 3100 Avant Genetic Analyzer). All of the p53 mutations identified were confirmed by direct sequence analysis of both strands.

**IHC analysis of p53 protein levels:** All sections were deparaffinized in xylene, rehydrated through a graded series of alcohols, and washed in phosphate buffered saline. This buffer was used for all subsequent washes. IHC using the streptavidin-biotin-peroxidase method was performed on paraffin embedded tissues using an anti-p53 monoclonal antibody, DO7 (diluted 1:100; DakoCytomation, Glostrup, Denmark), which recognizes the N-terminus of the human p53 protein (amino acids 19 to 26). In addition, the antibody reacts with both wild type and many mutant p53 proteins. The IHC results were

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Exon</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4S</td>
<td>4</td>
<td>acctggtctctgactgctc</td>
</tr>
<tr>
<td>E4S 1</td>
<td>4</td>
<td>cagcagcctcctacaccggcg</td>
</tr>
<tr>
<td>E4AS</td>
<td>4</td>
<td>aggcattgaagtctcatgga</td>
</tr>
<tr>
<td>E5S</td>
<td>5</td>
<td>tgccctgactttcaactctg</td>
</tr>
<tr>
<td>E5AS</td>
<td>5</td>
<td>gctgtctcaccatcgctatc</td>
</tr>
<tr>
<td>E6S</td>
<td>6</td>
<td>ctgttctcatcctgattgct</td>
</tr>
<tr>
<td>E6AS</td>
<td>6</td>
<td>agttgcaaacagacctcag</td>
</tr>
<tr>
<td>E7S</td>
<td>7</td>
<td>cctgttgtatctcctaggttg</td>
</tr>
<tr>
<td>E7AS</td>
<td>7</td>
<td>gcacaggccagctgtgca</td>
</tr>
<tr>
<td>E8S</td>
<td>8</td>
<td>gacccgatctccctactgcc</td>
</tr>
<tr>
<td>E8AS</td>
<td>8</td>
<td>ttcctccccaggtttctg</td>
</tr>
</tbody>
</table>

Primer sequences used for sequencing the p53 gene. In the “Primer name” column, all names ending with an “S” are sense primers and all names ending with “AS” are antisense primers for the different exons.
scored for the percentage of cells with positive reactivity: score 0, being no positive staining in the nuclei of any cells; score +1, from 1% to 10% of cells viewed immunoreactive; score +2, from 11% to 50%; score +3, more than 50% positive cells. The normal conjunctival samples, collected from 10 patients without pterygium or pinguecula when they underwent cataract or vitreoretinal surgery, were included as normal controls.

This study was conducted with the approval of the Human Study Committee of the China Medical University Hospital and National Cheng Kung University Hospital. Informed consent was obtained from all individuals who participated in this study.

### RESULTS

There were 31 males and 20 females in the pterygium group (mean age: 64.2; range: 50 to 83), and 6 males and 4 females in the normal control group (mean age: 68.3; range: 55 to 81).

**DNA sequencing:** P53 mutations were detected in 8 (15.7%) of the 51 pterygial samples and each of these 8 had only one form of mutation. The spectrum of mutations identified in the exons examined of the p53 gene are shown in Table 2.

### Table 2. Mutation spectrum in exons 4 to 8 of p53 gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>n (%)</th>
<th>Mutation Spectrum</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1 (12.5%)</td>
<td>codon 117, 1 bp deletion</td>
<td>frame shift</td>
</tr>
<tr>
<td>5</td>
<td>1 (12.5%)</td>
<td>codon 179, C:G-&gt;T:A</td>
<td>CAT(His)-&gt;TAT(Tyr)</td>
</tr>
<tr>
<td>6</td>
<td>3 (37.5%)</td>
<td>codon 208, A:T-&gt;T:A</td>
<td>GAC(Asp)-&gt;GTC(Val)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>codon 213, G:C-&gt;G</td>
<td>GGA(Arg)-&gt;GCA(Pro)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>codon 213, G:C-&gt;T:A</td>
<td>CGA(Arg)-&gt;CTA(Leu)</td>
</tr>
<tr>
<td>7</td>
<td>2 (25%)</td>
<td>codon 234, T:A-&gt;A:T</td>
<td>TAC(Tyr)-&gt;AAT(Asn)</td>
</tr>
<tr>
<td>8</td>
<td>1 (12.5%)</td>
<td>codon 286, G:C-&gt;C:G</td>
<td>GAA(Glu)-&gt;CAA(Gln)</td>
</tr>
</tbody>
</table>

P53 mutations were detected in 8 pterygial samples and each sample had only one form of mutation. Three mutations occurred in exon 6, 2 in exon 7, and 1 in each of exons 4, 5, and 8. All eight mutations were point mutations, with 6 mutations being substitutions and 2 being deletions. The 6 substitutions were missense and the two deletions resulted in a frame shift.

### DNA sequencing

Mutations were detected in each IHC score group. Deletion mutations were found in the group with no p53 protein IHC reactivity (IHC score 0), and substitution mutations were found in groups with p53 IHC reactivity (IHC scores +1, +2, and +3).

<table>
<thead>
<tr>
<th>Score</th>
<th>n</th>
<th>n</th>
<th>Mutation spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>31</td>
<td>2</td>
<td>exon 7, codon 259, 1 bp deletion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>exon 4, codon 117, 1 bp deletion</td>
</tr>
<tr>
<td>+1</td>
<td>9</td>
<td>2</td>
<td>exon 8, codon 286, G:C-&gt;C:G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>exon 5, codon 179, C:G-&gt;T:A</td>
</tr>
<tr>
<td>+2</td>
<td>5</td>
<td>2</td>
<td>exon 7, codon 234, T:A-&gt;A:T</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>exon 6, codon 208, A:T-&gt;T:A</td>
</tr>
<tr>
<td>+3</td>
<td>6</td>
<td>2</td>
<td>exon 6, codon 213, G:C-&gt;C:G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>exon 6, codon 213, G:C-&gt;T:A</td>
</tr>
</tbody>
</table>

Figure 2. Representative result from p53 DNA sequencing. A: Normal DNA sequence. B: Mutant DNA sequence. The mutation at codon 208 of the patient is indicated by an arrow. The numbering of each sequence does not reflect the actual coding sequence number.
2, and DNA sequencing results of one patient was shown in Figure 2. Slightly more than one third of the mutations occurred in exon 6 (37.5%) and one fourth (25%) in exon 7. All eight mutations were point mutations, with 6 (75%) of the 8 mutations being substitutions and 2 (25%) being deletions. The 6 substitutions were missense and the two deletions resulted in a frame shift.

**IHC analysis:** Thirty-one (60.8%) pterygial specimens were scored as 0 for IHC (no immunoreactive cells visible), 9 (17.6%) were scored +1, 5 (9.8%) were +2, and 6 (11.8%) were scored +3. The p53 positive rate was 21.6%, if score 0 and +1 were considered to be p53 negative staining, and +2 and +3 to be positive staining (setting cutoff level at 10% of cells with positive reactivity). If only score 0 was considered to be negative and +1, +2, and +3 to be positive (setting cutoff level at 1% of cells with positive reactivity), the p53 positive rate was 39.2%.

P53 staining was limited to the nuclei of cells in the epithelial layer. No substantial staining was visible in the subepithelial fibrovascular layers. In normal conjunctiva group, all specimens were negative.

**Concordance between IHC and DNA sequencing:** The relationship between p53 IHC protein level and gene mutation spectrum is shown in Table 3. Mutations were detected in each IHC score group. Deletion mutations were found in the group with no p53 protein IHC reactivity (IHC score 0), and substitution mutations found in groups with p53 IHC reactivity (IHC scores +1, +2, and +3).

**DISCUSSION**

To the best of our knowledge, there have only been 10 studies to date looking at p53 in pterygium [2-9,13,14]. Eight of these studies used IHC to examine p53 protein levels to detect p53 gene mutations [2-9]; the other two studies used western transfer analysis and enzyme linked immunosorbent assays [13,14]. In the 8 IHC studies, the reported p53 positive rate, or the presumed prevalence of p53 gene mutations, varied from 7.9-100% [2-9]. Although the biological causes of such variation in the prevalence of p53 positive staining in these reports are unclear, we believe that the different cutoff levels are largely to blame. In our series, 21.6% of all the specimens showed p53 staining at a cutoff level of 10% and the positive rate would double to 39.2% at a cutoff level of 1%. It has been reported that p53 immunoreactivity in less than 10% of cells was commonly associated with a high proliferative activity, not with p53 gene mutation [16]. In our series, setting the cutoff level at 10% (21.6%) is near the prevalence of p53 gene mutation by the DNA sequencing (15.7%) than setting at 1% (39.2%).

DNA sequencing was reported to be more accurate than IHC for detection of p53 gene mutations [12]. IHC simply reflects the levels of protein available for immunoreactivity in cells and mutations in a gene may not affect the immunoreactivity of the protein. P53 missense mutations usually increased the stability of the resultant p53 protein produced and showed strong positive immunostaining. Samples with deletion mutations, which resulted in stop codons or truncated proteins, did not react with the antibody and therefore, little, if any, staining was observed. The wild type p53 protein was usually not immunostained due to its short half life (6-20 min); however, sometimes the wild type p53 protein was immunostained [12,17-19]. Hence, drawing conclusions about the gene simply from examination of protein levels viewed by IHC is perilous. In our results, missense mutations resulted in positive staining by IHC, whereas deletion mutations resulted in negative staining. Because there were specimens with mutations in the p53 negative staining group and specimens without mutations in the p53 positive staining group, our results suggest IHC is not the most appropriate method to assess the prevalence of p53 gene mutation in pterygium.

Mutations in the p53 gene were found in 15.7% of our cases, determined via DNA sequencing. The result was very different from the previous studies of Reisman et al. [13] and Shimmura et al. [14]. Reisman et al. [13] found that the p53 gene had undergone a mono-allelic deletion, and the remaining allele remained wild type in 9 American patients. Shimmura et al. [14] reported that no mutation was found in exons 5 to 8 in 6 Japanese. Since there were mutations in 8 of our 51 Taiwanese patients, such differences could be due to ethnic variation, their small case number or our use of LCM, which presumably minimized the chances of the epithelium containing many fibroblast or other types of cells that may have contaminated the samples in the previous studies.

**LCM under direct microscopic visualization enables rapid one step procurement of selected human cell populations from a histological section.** This method has made micro-dissection of selected areas much easier, so that accurate study of specific target lesions is possible. As p53 staining was previously reported to be found only in the epithelial layer of pterygium and not in the subepithelial fibrovascular layer, the p53 gene mutation was presumed to be in the epithelial cells [2-7]. Hence, if DNA or RNA was extracted from whole excised pterygium, including epithelial cells and many other normal cells, it would be difficult to get specific and accurate results for epithelial cells alone. However, using the LCM method, we selectively micro-dissected epithelial cells from pterygial samples, which permitted confident examination of p53 gene mutations in the epithelial cells alone.

All the mutations in our cases were point mutations. These included single base substitutions (including missense mutations, nonsense mutations, silent mutations and single base changes at intronic sites), deletions, and insertions [20,21]. Most of the mutations (6/8, 75%) in our series were substitutions that led to missense changes and positive staining by IHC. The other 2 cases were deletion mutations, which resulted in frame shift mutations and negative staining by IHC. No insertion mutations were found in our series.

Pterygium formation has been reported to be related to the dose of UV irradiation [22]. UV irradiation mainly produces DNA lesions between adjacent pyrimidines, and C to T transitions on dipyrimidine sites or CC to TT tandem mutations in the p53 gene are considered as the UV related skin
In humans, different DNA damage is repaired by different DNA repair systems [24]. Single base changes are normally reversed by the base excision repair (BER) system and tandem mutations repaired by the nucleotide excision repair (NER) system [25]. Hence, the presence of a single base change of C to T transition or tandem mutation of CC to TT is reported to be related to perturbations in the different DNA repair systems [23]. CC to TT tandem mutations are usually associated with deficiency in the NER, such as in patients with xeroderma pigmentosum (XP) [23]. Thirty-six to sixty-one percent of XP patients with skin cancer were reported to be CC to TT tandem mutations, which are very rare (<10%) in non-XP patients [23]. In non-XP patients with skin cancer, 50-60% of the mutations are seen as C to T transitions [23]. In our series, all mutations were single base substitutions, including C to T transitions, but no CC to TT tandem mutations, suggesting that pterygium patients have defective BER systems. In the study of Kau et al. [26], polymorphism of HOGG1, one of the BER genes, was reported to be associated with pterygium formation. In our unpublished study, polymorphisms of XPA and XPD, two NER genes, were not associated with pterygium formation, while polymorphism of XRCC1, one of the BER genes, was reported to be associated with pterygium formation.

Mutational “hot spots” in UV related cutaneous squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), including codons 177, 196, 245, 248, and 278, have been reported [23]. None of the “hot spots” were found altered in our studies. However, a mutation at codon 213 was detected in 2 cases in our study. If we considered codon 213 as a “hot spot” of p53 gene mutations in pterygium, pterygium has the same “hot spot” as malignant melanoma, breast cancer, and colon cancer [23]. Further evaluation of correlations between potential “hot spots” of pterygium with SCC and BCC are necessary.

There were two limitations in our study. First, mutational analysis in this study was confined to exons 4 through 8, instead of sequencing the entire 11 exons of the p53 gene. We concede that other mutations may occur outside of the field examined, however, several studies have shown that tumor cells with mutations outside exons 5 through 8 are rare [27]. Exon 4 has been reported to encompass codons 32-125 and contain domains involved in the induction of apoptosis, which may be associated with tumor behavior [28]. Hence, we studied exons 4 to 8 in our series, which, we believe included nearly all possible relevant mutations in pterygium. Sondly, since we removed the fibrovascular layer by LCM and then studied the whole epithelium layer, there was the possibility of false negative due to average of all cells. Hence, the sequence analysis of all 51 pterygial samples has been rechecked by sense and antisense sequencing to avoid the false negative, and the sequences of p53 gene were repeatedly analyzed by three independent experiments. No different results were found from these analysis.

There were lower signals in Figure 2B at positions 339, 343, 344, and 355, which were not present in the normal DNA of Figure 2A. The lower signals in Figure 2B may caused by various factors, including insufficient DNA in the sequence reaction, degraded template, old or mishandled reagents, incorrect thermal cycling conditions, or electrokinetic injection failure. In this study, DNA for p53 mutation analysis was extracted from paraffin embedded pterygium tissues, base on our knowledge, it is very difficult to extract high quality DNA from paraffin stions. Therefore, we consider that the lower signals in Figure 2B at positions 339, 343, 344, and 355 were caused by insufficient DNA.

Some previous studies investigating the potential role of p53 in pterygium showed that p53 gene inactivation was predominately through p53 mutations and caused pterygium formation [3,6]. However, some reports, such as Shimmura et al. [8], Onur et al. [13], and Reisman et al. [14] do not support the above conclusion, because few p53 mutations were detected in pterygium tissues. They suggested that the inactivation of p53 was not through gene mutation, but rather through repressed expression of the gene [13]. Our results indicated that only 15.7% (8 of 51) had p53 mutations. As p53 mutation did not occur in all pterygium patients, p53 mutation was not the only cause of pterygium formation. We suggest that besides p53 gene mutations, there may be other mechanisms leading to loss of p53 function involved. Silencing of the p53 gene by alterations or mutations in its regulatory regions, methylation of important regions of the gene, or defects on other controlling factors required for p53 gene expression may play a role [13]. Moreover, there may be other tumor suppressor genes or oncogenes involved in pterygium formation.

In conclusion, p53 gene mutations were detected in some pterygium epithelium. All mutations were single base substitutions and could be found in both p53 immunoreactive and nonreactive groups. This is the first paper to investigate the spectrum of p53 gene mutations in pterygium.

ACKNOWLEDGEMENTS
The authors would like to thank Miss Ming-May Wong, Department of Ophthalmology, National Cheng Kung University Hospital.

REFERENCES

©2005 Molecular Vision


