An association between BPDE-like DNA adduct levels and P53 gene mutation in pterygium

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Purpose: A previous report of ours noted that not only p53 protein overexpression, but also p53 gene mutation, were indeed detected in pterygium. BaP 7,8-diol 9,10-epoxide (BPDE), an ultimate metabolite of BaP, attacks deoxyguanosine to form a BPDE-N2-dG adduct resulting in p53 mutations. The relationship between BPDE-like DNA adduct levels and abnormal p53 has not been clear in pterygium. Therefore, BPDE-like DNA adduct, p53 protein expression and p53 gene mutation were examined in this study to provide more molecular evidence to understand the cause of p53 gene mutation in pterygium.

Methods: In this study, immunohistochemical staining, using a monoclonal antibody (DO7) against p53 and a polyclonal antibody against BPDE-like DNA adducts, was performed on 73 pterygial specimens. DNA samples for p53 mutation analysis were extracted from epithelial cells and then subjected to DNA sequencing for the determination of mutations in exons 4, 5, 6, 7, and 8 of the p53 gene.

Results: BPDE-like DNA adducts were detected in 36.1% (26/73) pterygium samples. No correlation between adduct levels and p53 protein expression was found in these samples. Additionally, the p53 gene mutation and p53 mutation pattern also did not correlate with BPDE-like DNA adduct levels.

Conclusions: Our data provides evidence that BPDE-like DNA adducts are indeed detected in pterygium samples, and they are only minor contributors to the abnormal p53 gene.

Pterygium is a chronic condition characterized by the enroachment of a fleshy triangle of conjunctival tissue into the cornea and has long been considered a chronic degenerative condition; however, after finding abnormal expression of the p53 protein in epithelium, researchers now consider pterygium to be an ultraviolet-related uncontrolled cell proliferation, like a tumor [1-7].

The p53 tumor suppressor gene is one of the most commonly mutated genes observed in human tumors. The mutation of the p53 gene has been noted in more than 50% of all human cancers [8-10]. In normal unstressed cells, p53 is a short-lived protein which is maintained at low, often undetectable levels in the cell, but mutations in p53 lead to increased stability of its protein in the cell, which can be detected by antibodies to several epitopes of p53. Nearly all reported studies about the p53 gene in pterygium are via immunohistochemical (IHC) staining. The reported prevalence of p53 positive staining or presumed p53 gene mutation have a wide range, from 7.9% to 100% [3-7]. A previous study of ours noted that mutations within the p53 gene were detected in 15.7% of the pterygial samples. Deletion mutations were found in the same samples with p53 negative staining, and substitution mutations were found in samples with p53 positive staining [11]. However, the cause of p53 mutation in pterygium is still unclear.

Polycyclic aromatic hydrocarbons (PAHs) might be responsible for the mutagenicity of airborne particulates in Taiwan [12,13]. The model environmental pollutants, benzo[a]pyrene (BaP), which are PAHs, have been found to cause p53 mutations, leading to lung tumorigenesis. The levels of PAHs in airborne particulates in Taiwan are higher than levels found in other countries, especially levels of BaP, benzo[b]fluoranthene, and benzo[g,h,i]perylene [12,13]. BaP 7,8-diol 9,10-epoxide (BPDE), an ultimate metabolite of BaP, attacks deoxyguanosine to form a BPDE-N2-dG adduct which results in p53 mutations. The mutation hotspots of p53 in human lung tumors (codons 154, 157, 158, 245, 248, and 273) are caused by the BPDE-N2-dG adduct [14]. Thus, an evaluation of DNA adducts induced by BaP and other PAHs is suitable as a risk marker of p53 mutation.

In this study, we examined the BPDE-like DNA adducts using immunohistochemistry in 73 pterygium specimens and compared them with p53 exon 4-8 gene mutations and p53 protein expression to understand the relationship between environmental exposure and the abnormal p53 gene in pterygium.
METHODS

Patients and methods: Pterygial samples were harvested from 73 patients (44 males and 29 females) undergoing pterygium surgery. Patient age range was 48-85 and the average was 66.9 years old. All specimens were fixed in formalin and embedded in paraffin. Sections at a thickness of 3 μm were cut, mounted on glass, and dried overnight at 37 °C for immunohistochemistry analysis.

IHC analysis of p53 protein expression and BPDE-like DNA adduct detection: All sections were then deparaffinized in xylene, rehydrated with alcohol, and washed in phosphate-buffered saline. This buffer was used for all subsequent washes. Sections for p53 and BPDE-like DNA adduct detection were heated in a microwave oven twice, for 5 min, in citrate buffer (pH 6.0). Mouse anti-p53 monoclonal antibody (at a dilution of 1:200; DAKO, Copenhagen, Denmark) and anti-BPDE-like DNA adduct polyclonal antibody (kindly provided by Dr. Huei Lee; at a dilution of 1:1000) [15] were used as the primary antibodies. The incubation time was 60 min at room temperature followed by a conventional streptavidin peroxidase method (LSAB Kit K675; DAKO). Signals were developed with 3,3'-diaminobenzidine for 5 min and counter stained with hematoxylin. Negative controls were obtained in the absence of the primary antibodies. The results were evaluated independently by three observers and scored for the percentage of positive nuclei: 0, no positive staining; +, from 1% to 10%; ++, from 11% to 50%; and ++++, more than 50% positive cells. In this study, scores +, ++, and +++ were considered to be positive immunostaining, and 0 was seen as a negative immunostaining. The normal conjunctival samples were collected from the superior conjunctiva of six patients without pterygium when they underwent cataract or vitreoretinal surgery. These samples served as controls.

DNA sequencing 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 for p53 mutation analysis [11]. DNA lysis buffer was applied to lyse the epithelial cells on the slide, and then the tissues for p53 mutation analysis [11]. DNA lysis buffer was used for p53 gene sequencing are shown in Table 1. An initial cycle was performed for 5 min at 94 °C, followed by 35 cycles at 94 °C for 40 s each, 40 s at 54 °C, and 1 min at 72 °C. PCR products were sequenced by an autosequencing system (3100 Avant Genetic Analyzer; Applied Biosystems, Foster City, CA). All of the p53 mutations were confirmed by a direct sequence of both strands.

Statistical analysis: Statistical analysis was performed using the SPSS statistical software program (SPSS Inc., Chicago, IL). The χ² and Fisher’s exact test were applied for statistical analysis. A p<0.05 was considered to be statistically significant.

RESULTS

BPDE-like DNA adduct detected in pterygium: In the pterygium group, 47 (64.3%) pterygial specimens scored as 0, 7 (9.5%) were +, 7 (9.5%) were ++, and 12 (16.7%) were ++++. The detection rate of BPDE-like DNA adduct was 26.2%, if a score of 0 and + were considered to be BPDE-like DNA adduct staining negative, and ++ and +++ to be positive (setting cutoff level at 10%). If a score of 0 was considered to be negative and +, ++, and +++ to be positive (setting cutoff level at 1%), the positive rate was 35.7%. The BPDE-like DNA adduct staining was limited to the nuclei of the epithelial layer and subepithelial fibrovascular layers (Table 2 and Figure 1).

Correlation of p53 protein expression and BPDE-like DNA adduct levels in pterygium: Our previous study noted that p53 protein expression occurs in pterygium [16], but the mo-
lecular mechanism is still unknown. To understand whether the p53 protein is induced by DNA damage in the pterygium, more study cases were included in this study. The relationship between p53 protein expression and BPDE-like DNA adduct levels in pterygium is shown in Table 3. Although there is no statistical significance between p53 protein expression and BPDE-like DNA adduct levels, the samples with high BPDE-like DNA adduct levels seem to have a higher frequency of p53 protein expression (36.8% versus 22.2%, p=0.235).

The influence of BPDE-like DNA adduct in p53 gene mutation and mutation pattern: To understand whether the p53 gene mutation is indeed caused by BPDE-like DNA adducts, the correlation between p53 gene mutation, mutation pattern, and BPDE-like DNA adduct levels were compared. As shown in Table 4, not only G->T but other types of p53 mutations were found in BPDE-like DNA adduct positive pterygium. However, there was no statistical significance between p53 gene mutation and BPDE-like DNA adduct levels as shown in Table 5 (p=0.137).

**DISCUSSION**

Theories of the pathogenesis of pterygium have implicated UV light exposure as a major causative factor. Evidence for sunlight exposure as one of the prime etiological agents de-

<table>
<thead>
<tr>
<th>Patient number</th>
<th>BPDE-like DNA adduct levels</th>
<th>p53 gene</th>
<th>mutations</th>
<th>Exon</th>
<th>Spectrum</th>
<th>Amino acid change</th>
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<tr>
<td>1</td>
<td>0</td>
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<td>G:C-C:G</td>
<td>Glu-Gln</td>
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<tr>
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<td>G:C-C:G</td>
<td>Arg-Pro</td>
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<td></td>
</tr>
<tr>
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<td>0</td>
<td>7</td>
<td>T:A-A:T</td>
<td>Tyr-Asn</td>
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<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>5</td>
<td>C:G-T:A</td>
<td>His-Tyr</td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>High</td>
<td>7</td>
<td>1-bp del</td>
<td>Frame shift</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>4</td>
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<td>Frame shift</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>6</td>
<td>A:T-T:A</td>
<td>Asp-Val</td>
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<td></td>
</tr>
<tr>
<td>8</td>
<td>High</td>
<td>6</td>
<td>G:C-T:A</td>
<td>Arg-Leu</td>
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</table>

Mutations analyzed in this study were based on mutations found in prior pterygium samples (Tsai et al. [16]).

<table>
<thead>
<tr>
<th>BPDE-like DNA adduct levels</th>
<th>p53 gene mutation Low</th>
<th>High</th>
<th>p-value</th>
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<tr>
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<td>6</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>5</td>
<td>3</td>
<td>0.137</td>
</tr>
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</table>

Fisher’s exact test was used for statistical analysis. The results were evaluated independently by three observers and scored for the percentage of positive nuclei: A score of - or low meant from 0% to 10%; a score of + or high indicated more than 11% positive cells.

**TABLE 3. AN ASSOCIATION BETWEEN BPDE-LIKE DNA ADDUCT LEVELS AND P53 PROTEIN EXPRESSION IN PTERYGIUM**

<table>
<thead>
<tr>
<th>p53 protein</th>
<th>BPDE-like DNA adduct levels</th>
<th>n=54</th>
<th>n=19</th>
<th>p-value</th>
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<tbody>
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<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>7</td>
<td>0.235</td>
</tr>
<tr>
<td>Positive</td>
<td>Low</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 4. RELATIONSHIP BETWEEN THE P53 GENE MUTATION SPECTRUM AND BPDE-LIKE DNA ADDUCT LEVELS**

**TABLE 5. RELATIONSHIP BETWEEN THE P53 GENE MUTATION AND BPDE-LIKE DNA ADDUCT LEVELS**

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Figure 1. Representative positive and negative immunostaining for BPDE-like DNA adducts in paraffin sections of pterygium. Representative negative immunostaining is shown in A (100X), positive immunostaining in the epithelial layer is shown in both B (400X) and C (200X). Positive immunostaining in the epithelial layer and weak positive staining in the subepithelial fibrous sarcoma are shown in D (100X).
rives both from case-control studies [17] and prevalence surveys [18-23]. Gazzard et al. [24] observed that pterygium was independently related to increasing age and outdoor activity. UV radiation is a clear fingerprint mutation in the TP53 gene due to the CC to TT double mutation in skin cancer [9,24-26]. Such mutations are typically induced by UV radiation in experimental systems [27]. C to T single mutations are also clearly associated with UV radiation [28]. In our study, no CC to TT mutation was found in our study subjects, and there was only one of eight p53 mutation positive patients with a C to T mutation. Therefore, we suspect that not only UV radiation but also other environmental exposure is related to p53 gene mutation in pterygium.

PAH-compounds are the products of incomplete combustion of organic material and thus ubiquitous in the environment (IARC, 1983;[29]). Occupational exposure to PAH-compounds increases the risk of lung, and putatively, other cancers, and is the highest in coke oven workers, other workers in the steel industry, asphalt and bitumen workers, and those exposed to exhaust and who work with gasoline. However, the absolute highest exposure comes from smoking (IARC, 1986;[30]). Nobody is ever exposed to a single PAH-compound and rarely to the group of PAH compounds only. Theoretically, it would thus be impossible to find a specific mutation spectrum in human cancers caused by a single PAH-compound, like benzo(a)pyrene (BP). The best known carcinogen in cigarette smoke, BP, induces G:CT:A transversions experimentally [31], which are the main mutation types in smoking-related lung cancer [28]. Pfeifer and colleagues [32-34] have shown that codons 157, 248, and 273 in the TP53 gene, which are the most mutated in lung cancer, are also targets for DNA-adduct formation by BP and are more prone to mutations by it. Other PAH compounds have a similar preference for adduct formation in TP53 codons 157, 158, 245, 248, and 273 [34]. To date, there have only been three reports with regard to the DNA sequence of the p53 gene in pterygium [17,35,36]. Reisman et al. [35] studied nine pterygial specimens from nine Americans and found that the p53 gene had undergone a monoallelic deletion, and the remaining allele remained wild-type. In another study of six Japanese pterygial samples, Shimmura et al. [36] al reported that there was no mutation in exons 5 through 8 of the p53 gene. No G to T mutation was found in two previous studies. In this study, the p53 gene mutation in pterygium was analyzed by a DNA sequence, and as reported in our previous study [35], only one patient showed a G to T mutation. The mutation sites of the p53 gene in pterygium are codons 117, 179, 208, 213, 234, 259, and 286 but not the mutation hotspots caused by PAHs. These data seem to show that the BPDE-like DNA adducts are only minor contributors to the p53 gene mutation of pterygium.

After abnormal expression of p53 protein was found in the epithelium, pterygium is now considered to be a result of uncontrolled cellular proliferation, like a tumor [1-7]. Nevertheless, there is no molecular evidence to support this hypothesis. In this study, we did not detect a correlation between BPDE-like DNA adducts, p53 mutation and p53 protein overexpression in pterygium paraffin sections to provide molecular evidence to support the idea, that not only UV radiation, but also environmental exposure, is involved in pterygium pathogenesis.

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REFERENCES


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