Hypermethylation of the p16 gene promoter in pterygia and its association with the expression of DNA methyltransferase 3b

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A pterygium has long been considered a degenerative disease. However, after p53 protein was found to be abnormally expressed in the epithelium, a pterygium is now considered to be uncontrolled cell proliferation related to exposure to UV light, similar to a tumor [1-7].

Our previous studies revealed that mutations in the p53 gene (TP53) occur in pterygia [8,9]. However, only 15.7% of pterygia had p53 mutations [8]. Several mutations are required for a normal cell to be converted to a tumoral cell, and several oncogenes or tumor suppressor genes are reported to be involved in tumoral formation. Therefore, it is logical to assume that tumor-related genes other than p53 are involved in the formation of pterygia. The oncogene Ki-ras was recently found to be mutant in 10% of pterygia [10]. Hence, we suggest that tumor suppressor genes or oncogenes besides p53 and Ki-ras genetic mutations are involved in pterygial formation.

The p16 gene (CDKN2A) is a tumor suppressor gene, and its product, p16 protein, controls the cell cycle and prevents tumoral formation. This gene is inactivated in many cancers [11-14]. Hence, like p53, p16 is another important gene involved in tumorigenesis. However, unlike p53, which mutations frequently inactivate, p16 is frequently inactivated by hypermethylation of its promoters [15].

Hypermethylation of regulatory elements, a well-know epigenetic change, is an important alternative to genetic alteration for inactivating genes, and it plays an important role in the pathogenesis of human cancers [16]. At least three independently encoded DNA methyltransferases (DNMTs) are known. They are DNMT1, DNMT3a, and DNMT3b, which are involved in hypermethylation [17,18]. Overexpression of DNMT1 and DNMT3b is common in human tumors, but DNMT1 is constitutively expressed in proliferating cells. Therefore, DNMT3b is commonly evaluated in tumors [17,18].

We hypothesized that the p16 gene loses its function in pterygia and that, besides genetic mutations, epigenetic changes (e.g., hypermethylation of genetic regulatory elements) occur. To test these hypotheses, we analyzed hypermethylation of the p16 gene promoter in pterygia and investigated the relationship between this hypermethylation and the expression of p16 and DNMT3b protein.

Purpose: A pterygium has long been considered as a degenerative condition. After p53 protein was found to be abnormally expressed in the epithelium, researchers suggested that a pterygium may be a tumor, but additional evidence is required to support this hypothesis. Aberrant methylation of the p16 gene (CDKN2A) promoter and resultant gene silencing play important roles in the pathogenesis of many types of human cancers. The purpose of this study was to investigate hypermethylation of the p16 promoter in pterygia and the relationship between this hypermethylation and the expression of p16 and DNA methyltransferase 3b (DNMT3b) proteins.

Methods: We studied the methylation status of p16 and the expression of p16 and DNMT3b proteins by performing methylation-specific polymerase chain reaction and immunohistochemistry, respectively, in specimens of 129 pterygia and 16 normal conjunctiva. The results were statistically analyzed.

Results: Hypermethylation of the p16 gene promoter was detected in 21 (16.3%) of 129 pterygial specimens. Among them, 46 (35.7%) were positive for p16 protein expression, and 83 (64.3%) were negative. Staining for p16 was limited to the nuclei of the epithelial layer. We observed a significant reverse correlation between hypermethylation of the p16 promoter and the expression of p16 protein (p=0.006). Thirty-eight (29.5%) pterygial specimens were positive for DNMT3b protein expression, and 91 (70.5%) were negative. DNMT3b staining was limited to the nuclei of the epithelial layer. A significant correlation was found between hypermethylation of the p16 promoter and the expression of DNMT3b protein (p<0.001).

Conclusions: The p16 gene promoter was hypermethylated in pterygia, and this hypermethylation was strongly linked to expression of the positive expression of DNMT3b protein and to the suppression of p16 protein. These data provided molecular evidence that methylation occurs in pterygia and that it may play a role in the their development.
METHODS
Patients and control subjects: Pterygial samples were harvested from 129 patients undergoing surgery to treat pterygia with apexes that invaded the cornea by more than 1 mm. For controls, normal samples were collected from the superior conjunctiva of 10 patients and from the medial conjunctiva of six patients without pterygia and pingueculae who undergoing cataract or vitreoretinal surgery.

Immunohistochemical analysis of p16 and DNMT3b protein expression: All specimens were fixed in formalin and embedded in paraffin. Sections of 3 µm thickness were cut, mounted on glass, and dried overnight at 37 °C for DNA extraction and immunohistochemical analysis. The sections were then deparaffinized in xylene, sequentially rehydrated in alcohol, and washed in phosphate-buffered saline. This buffer was used for all subsequent washes.

Sections used for DNMT3b detection were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0). Mouse anti-p16 monoclonal antibody at a dilution of 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-DNMT3b monoclonal antibody at a dilution of 1:50 (Gene Therapy Systems, San Diego, CA) were the primary antibodies. The sections were incubated with the primary antibodies for 60 min at room temperature, and the signals were detected by using a conventional streptavidin peroxidase method (LSAB kit K675; Dako, Copenhagen, Denmark). Signals were developed with 3,3'-diaminobenzidine for 5 min and counterstained with hematoxylin. Negative controls that did not include the primary antibodies were also analyzed.

Three observers independently evaluated the results and scored the percentage of positive nuclei, where 0 was no positive staining, + was 1-10%, ++ was 11-50%, +++ was more than 50%. Samples with scores of ++ or +++ were considered to have positive immunostaining, and those with scores of 0 or + were considered to have negative immunostaining.

Methylation-specific polymerase chain reaction and direct sequencing: To analyze hypermethylation of the p16 promoter, DNA was extracted from paraffin-embedded pterygial tissues by means of laser capture microdissection [8]. Extracted DNA was treated with sodium bisulfite (Sigma-Aldrich, St. Louis, MO) and purified by using the Wizard DNA Clean-Up System (Promega Corporation, Madison, WI) according to the manufacturer’s instructions. Purified DNA was mixed with 3.3'-diaminobenzidine for 5 min and counterstained with hematoxylin. Negative controls that did not include the primary antibodies were also analyzed.

Three observers independently evaluated the results and scored the percentage of positive nuclei, where 0 was no positive staining, + was 1-10%, ++ was 11-50%, +++ was more than 50%. Samples with scores of ++ or +++ were considered to have positive immunostaining, and those with scores of 0 or + were considered to have negative immunostaining.

RESULTS
Samples in the pterygial group were obtained from 76 men and 53 women aged 50-83 years (mean age 64.7 years), and control samples came from eight men and eight women aged 55-81 years (mean age 68.2 years).

Hypermethylation of the p16 gene promoter: Hypermethylation of p16 gene promoter was detected in 21 (16.3%) of 129 pterygial specimens. In the normal conjunctiva group, all specimens were negative for hypermethylation. Figure 1A shows representative data from methylation-specific PCR analysis. All cytosines in the CpG dinucleotides in this region were completely methylated in pterygia lacking p16 genetic expression (Figure 1B,C).

Relationship between hypermethylation of the p16 gene promoter and p16 protein expression: Table 1 summarizes the immunohistochemical results for p16 in the pterygial samples. Among them, 46 (35.7%) were positive for p16 protein expression and 83 (64.3%) were negative. Staining for p16 was limited to the nuclei of the epithelial layer (Figure 2). No substantial staining was visible in the subepithelial fibrovascular layers.

Table 2 shows the relationship between hypermethylation of the p16 gene promoter and the expression of p16 protein. Of 21 pterygial samples with this hypermethylation, 19 (90.5%) were negative for p16 protein expression. This rate was higher than the 59.3% found among pterygia without such hypermethylation. We observed a significant reverse correlation between hypermethylation of the p16 gene promoter and the expression of p16 protein (p=0.006).

Relationship between hypermethylation of the p16 gene promoter and DNMT3b protein expression: Table 1 lists the immunohistochemical results for DNMT3b protein. Thirty-eight (29.5%) specimens were positive for DNMT3b protein.
expression, and 91 (70.5%) were negative. DNMT3b staining was limited to the nuclei of the epithelial layer (Figure 2). No substantial staining was visible in the subepithelial fibrovascular layers.

Table 3 shows the relationship between hypermethylation of the p16 gene promoter and DNMT3b protein expression. Of 21 pterygia with this hypermethylation, all were positive for DNMT3b protein expression, and 91 pterygia negative for DNMT3b protein expression, did not have this hypermethylation. The correlation between hypermethylation of the p16 gene promoter and DNMT3b protein expression was significant (p<0.001).

**DISCUSSION**

Hypermethylation of the promoter for the p16 gene is found in several types of cancer. To understand the role of this hypermethylation in pterygial progression, we used methylation-specific PCR and DNA sequencing to analyze the methylation status of CpG islands of the p16 gene. The site we tested was on that included a 5’ CpG island whose hypermethylation was associated with complete loss of genetic expression, as observed in many cancers [20-23].

Although some researchers believe that a pterygium is actually a tumor, others believe it is a degenerative condition [1-5]. In our previous study, 15.7% of pterygia had p53 genetic mutations [8], and, in this study, 16.3% of pterygia had hypermethylation of the p16 gene promoter. These data suggest that genetic mutations and epigenetic changes occur in pterygia and these alterations are similar to those observed in several types of cancer [24,25]. Hence, the finding of inactivated p53 and p16 genes in pterygia supports the hypothesis that a pterygium may be a tumor.

The p16 protein is a key tumor-suppressor gene product that blocks progression of the cell cycle by binding to either cyclin dependent kinase 4 or cyclin dependent kinase 6 and by inhibiting the action of cyclin D. Reduced p16 immunoreactivity is commonly due to promoter hypermethylation; however, mutations, loss of heterozygosity, and polymorphisms also play a role [26-29].

In our study, 90.5% of pterygia with p16 promoter hypermethylation were negative for p16 protein expression, a rate higher than observed in pterygial samples without methylation. We noted a significant reverse correlation between this methylation and p16 protein expression (p=0.006). Hence, reduced p16 expression in pterygia was related to hypermethylation of the p16 promoter. Further study of p16 mutations, loss of heterozygosity, and polymorphisms in samples with p16 repression and without hypermethylation

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Table 1. P16 and DNMT3b protein expression in pterygium analyzed by immunohistochemistry.

Figure 1. Representative results of methylation-specific polymerase chain reaction (PCR) and direct DNA sequencing analysis of p16INK4a CpG islands from epithelial cells in seven patients with pterygia. **A:** Lanes M and U show products obtained with primers specific for methylated and unmethylated DNA, respectively. DNA templates P1-6 were prepared from epithelial cells of various patients. Templates from Calu-1 cells served as positive controls for methylated and unmethylated reactions, whereas distilled water was used as a templates for N (negative control). **B:** CpG-methylated cytosines remained as cytosines, whereas 5-methylcytosines were unaltered in this example (star). C: p16INK4a unmethylated cytosines changed to thymidines in the PCR products.
of the p16 promoter is suggested.

Tumor suppressor genes, such as p53, p16, RB (Rb protein), VHL, E-cadherin (CDH1), and hMLH1, can be inactivated by means of gene mutation or gene silencing due to DNA methylation [15]. Aberrant promoter hypermethylation of the tumor suppressor genes inactivates the gene, and resultant gene silencing plays an important role in the pathogenesis of most, if not all, human cancers [16]. However, the mechanisms involved in hypermethylating DNA loci remain unclear.

Global cytosine-methylation patterns in mammals appear to be based on a complex interplay of at least three independently encoded DNMTs, including DNMT1, DNMT3a and DNMT3b. DNMTs are commonly classified as de novo (DNMT3a and DNMT3b) or maintenance (DNMT1) enzymes. DNMT1 is constitutively expressed in proliferating cells, and overexpression of DNMT1 and DNMT3b is common in human tumors [17,18].

In this study, 29.5% of pterygial specimens were positive for DNMT3b protein expression. Moreover, all pterygia with hypermethylation of the p16 promoter were positive for DNMT3b expression, and all pterygia negative for DNMT3b protein expression were without this hypermethylation. However, some pterygia with DNMT3b expression were positive for p16 methylation, but some were negative. Hence, we suggest that DNMT3b protein is a necessary but insufficient criterion for hypermethylation of the p16 gene promoter. Further study about DNMT1 is suggested.

A study in an animal model showed that exposure to specific carcinogens is associated with hypermethylation of genes. For example, cigarette smoking is associated with p16 hypermethylation in human lung cancers [30-32]. Therefore, we suspect that certain carcinogens may cause p16 hypermethylation in pterygia. Several factors have been related to pterygial formation, including UV exposure, immunoinflammatory processes, viral infections, and genetic factors. Of these, UV exposure is reported to be most important [33-35]. UV light is reported to induce p16 genetic mutations in cutaneous tumors, and loss of p16 function may reduce the ability of cells to repair UV-induced DNA damage [36,37]. However, other factors have also been associated with p16

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<th>Table 2.</th>
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<tr>
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Relationship between p16 gene promoter hypermethylation and p16 protein expression. p=0.006.

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<th>Table 3.</th>
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<tr>
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<td>91</td>
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Relationship between p16 gene promoter hypermethylation and DNMT3b protein expression. p<0.001.
hypermethylation [38,39]. Hence, the cause of p16 promoter hypermethylation in pterygium needs further evaluation.

In addition to the p53 genetic mutations found in our previous study, the hypermethylation of the p16 promoter observed in this study further suggests that pterygia have tumoral properties. However, the potential effect of p16 promoter methylation on the clinical features of pterygia (e.g. atrophic, intermediate, or flesh type) and on its postoperative recurrent rate is unknown. Further studies in this area are necessary.

In conclusion, we believe our study is the first to demonstrate hypermethylation of the p16 gene promoter in pterygia, as well as a strong link between such hypermethylation and DNMT3b protein expression. This hypermethylation suppresses the expression of p16 protein. These data provided molecular evidence that methylation and epigenetic changes occur in pterygia and that they may play a role in their development of pterygia. Further study is suggested.

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REFERENCES


