Human Immunodeficiency Virus Infection, Bcl-2, p53 Protein, and Ki-67 Analysis in Ocular Surface Squamous Neoplasia

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Objectives: To determine the age and human immunodeficiency virus (HIV) status of patients with ocular surface squamous neoplasia (OSSN), and to analyze tumor proliferation, Bcl-2, and p53 oncoprotein expression in OSSN.

Methods: Only patients with histologically proved neoplasia were included in this study. The HIV status was obtained only with informed consent. Monoclonal antibodies to p53 and Bcl-2 protein were used after microwave antigen retrieval to enhance immunohistochemical staining of the sections. Proliferation was assessed by means of Ki-67 antigen expression. Positive staining in each specimen was expressed as a percentage and graded accordingly.

Results: Forty-one eyes in 40 black patients with a mean age of 37 years were found to have OSSN. Of the 41 lesions, 35 represented in situ or invasive carcinoma. The remaining 6 had mild or moderately dysplastic lesions. Seventeen patients agreed to an HIV test and, of these, 12 (70.6%) were HIV positive. All 12 were younger than 50 years, and 11 had either carcinoma in situ or invasive lesions. Twenty-two of 40 lesions expressed significant (greater than 50% of neoplastic cells) p53 positivity, while Bcl-2 expression was detected in 10. Ki-67 expression was low, even in the HIV-positive lesions.

Conclusions: At our institution, OSSN occurs in young patients, many of whom are HIV positive. Expression of p53 is a common finding, whereas Bcl-2 immunoreactivity occurs in the minority of cases. Ki-67 analysis showed that OSSN is a slow-growing tumor, even in the presence of HIV infection.

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Ocular surface squamous neoplasia (OSSN) is a rare condition, but interest in this disease has been revived in the literature through association with the human immunodeficiency virus (HIV) epidemic. The diagnosis of OSSN includes a spectrum of conditions ranging from mild squamous dysplasia of the conjunctiva through moderate dysplasia, carcinoma in situ (CIS), and, in the advanced stage, invasive squamous carcinoma. To the best of our knowledge, the relationship between OSSN and HIV infection has not yet been explored in Kwa-Zulu Natal, South Africa. Furthermore, whereas Dushku et al reported p53 expression to be confined to the limbal basal stem cells of pterygia and limbal tumors, other genetic and protein abnormalities in OSSN have not yet been reported.

Genes can be roughly categorized into 2 types: oncogenes and tumor suppressor genes. The latter exist as proto-oncogenes that then become converted into tumor-promoting genes as a result of a variety of genotoxic events that lead to translocations, mutations, deletions, etc. The tumor suppressor genes have a protective function in the normal state, and any genetic abnormality in these genes results in loss of this protective role. All types of genes produce proteins that are amenable to detection by immunohistochemistry. This is a simple, relatively inexpensive, and reproducible technique that uses specific monoclonal and polyclonal antibodies directed against the protein of interest. A chromagen is used for visualization purposes.

Tumor cell progression results from the accumulation of multiple mutations in both tumor suppressor genes and oncogenes. Tumor suppressor genes and their proteins play a major role in preventing tumorigenesis by negatively regulating cell growth. The p53 is a prototype tumor suppressor gene. It consists of 11 exons and is located on chromosome 17p13. Mutations and deletions of p53 are said to be the most common genetic abnormality in hu-
PATIENTS AND METHODS

Patients with localized conjunctival lesions who were first seen at the King Edward VIII Hospital eye clinic, Natal, between January 1, 1995, and December 31, 1997, were operated on under local anesthesia by means of a “no-touch” modified Mohs micrographic technique, which aims for total excision by means of histologic surveillance of tumor margins.15,16 Large infiltrating lesions were first examined by biopsy, and exenteration was offered thereafter. Specimens were submitted to the Pathology Department at the Nelson R. Mandela School of Medicine, University of Natal, Natal, for histopathologic analysis. Only histologically proved neoplastic lesions were included in this study. Forty patients were found to have OSSN. After informed consent was obtained, an enzyme-linked immunosorbent assay test for HIV antibodies was performed.

One of the 41 histologic specimens could not be retrieved and, therefore, immunostaining was not performed on this lesion, which was an invasive squamous carcinoma in a 66-year-old woman whose HIV status was not known. Sections 3 µm thick, embedded in paraffin wax, were picked up onto poly-l-lysine–coated slides and incubated for 10 minutes at 70°C. Sections were cleared through xylene and rehydrated through descending grades of alcohol. Microwave antigen retrieval was accomplished by placing the specimens in 0.01M buffered sodium citrate at pH 6.0 at 85°C for 10 minutes in a microwave oven processor (H2500; Energy Beam Sciences, Inc, Agawam, Mass). After cooling in water, the specimens were placed in a jar of phosphate-buffered saline (PBS) at pH 7.4. The slides were then incubated in 3% hydrogen peroxide in a microwave oven (Sharp carousel R7280, 650 W; Sharp Electronics Corp, Mahwah, NJ) at low heat for 2 minutes 30 seconds. Each section was rinsed with PBS; the specific antibody was placed over the tissue and thereafter heated in a microwave oven for 4 minutes 30 seconds. The antibodies used were MIB-1 (Biomed Corp, Foster City, Calif; dilution, 1:40); monoclonal mouse anti–human Bcl-2 oncoprotein (clone 124; DAKO, Glostrup, Denmark; dilution, 1:40), and the monoclonal mouse anti–human p53 protein (D07; DAKO; dilution, 1:100). The latter reacts with wild-type and mutant p53 protein.

Appropriate positive controls were run simultaneously: high-grade lymphoma for p53, a reactive lymph node for Bcl-2, and a reactive tonsil for MIB-1. Each specimen was rinsed thoroughly with PBS and then incubated with biotinylated link antibody in the microwave oven for 3 minutes 30 seconds. After washing in PBS, sections were incubated with peroxidase-labeled streptavidin in the microwave oven for 3 minutes 30 seconds. The slides were again washed in PBS and a substrate chromogen (DAB; DAKO, Carpinteria, Calif) was placed over each specimen. After incubating with the substrate chromogen for 5 minutes at room temperature, the sections were rinsed in distilled water, then counterstained with Mayer hematoxylin for 1 minute and rinsed in running tap water. Finally, sections were rinsed in ammoniated water and tap water, then dehydrated in alcohol and xylene and coverslipped with dibutylphthalate-xylene mountant. A positive reaction was indicated by brown staining of the nucleus (p53 and MIB-1) or perinuclear region and cytoplasm (Bcl-2).

Immunostaining for MIB-1, p33, and Bcl-2 was assessed on each slide by counting the number of positive cells and the total number of cells in at least 10 fields at ¥40 magnification. Positivity was expressed as a percentage in each field, and the average percentage for each slide was scored as follows: zero, less than or equal to 5%; 1+, 6% to 25%; 2+, 26% to 50%; 3+, 51% to 75%; and 4+, more than 75%.

RESULTS

Forty-one eyes in 40 patients were found to have OSSN during the period of this study. There were 20 men and 20 women. The ages of the patients ranged from 24 to 76 years, with a mean age at presentation of 37 years. Of the 40 patients, 27 (67.5%) were younger than 50 years. During the period of this study. There were 20 men and 20 women. The ages of the patients ranged from 24 to 76 years, with a mean age at presentation of 37 years. Of the 40 patients, 27 (67.5%) were younger than 50 years.Clinical illustrations are seen in Figure 1. Of the 41 lesions, 35 were either CIS or invasive squamous carcinomas. Table 1 indicates the ages of patients and the histologic grade of their tumors.

Results of the HIV test confirmed that 12 of the 17 patients were infected with the virus. All 12 were younger
than 50 years. Three of the 5 HIV-negative patients were
younger than 50 years. A 52-year-old woman with bilat-
eral OSSN was HIV negative. The histologic findings of
18 lesions excised from these 17 patients are shown in
Table 1. Eleven of the 12 HIV-positive patients had CIS
or invasive squamous cancer at the initial examination.

Details of p53, Bcl-2, and MIB-1 staining in the spec-
trum of OSSN are given in Table 2. MIB-1–positive stain-
ing limited to the basal cells of the conjunctival epithe-
ilium was not considered part of the lesional pattern of
staining, as these are normally dividing cells and are ex-
pected to be MIB-1 positive. Thirteen specimens dis-
played 1+ MIB-1 positivity and 5 specimens were 2+. MIB-1 positivity of 3+ was seen in 1 case of CIS. Twenty-
one cases of OSSN showed less than 5% staining.

Positive staining for p53 was present within the nu-
clet of neoplastic cells (not confined to the basal cells)
in 28 lesions, representing 70% of cases. Positivity for
p53 of 3+ and 4+ was present in 10 cases each of CIS and
invasive squamous carcinoma (Figure 2).

Expression of Bcl-2 was found to be minimal or ab-
sent in 30 specimens. Eight cases showed 1+ staining.

One specimen representing CIS expressed focal 2+
staining, and another case of CIS showed areas of 3+
positivity.

Results of the specimens belonging to the subgroup of
HIV-positive patients are summarized in Table 2. Seven cases showed either 3+ or 4+ p53 positivity. Three
HIV-negative cases showed a similar staining intensity as
well. Expression of Bcl-2 was minimal, and MIB-1 ac-
tivity was similar in HIV-positive and HIV-negative groups.

### Table 1. Patient Age, HIV Status, and Histologic Findings*

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Mild/Moderate Dysplasia, No.</th>
<th>Carcinoma In Situ</th>
<th>Invasive Carcinoma</th>
</tr>
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<tr>
<td>20-30</td>
<td>2 (1)</td>
<td>6 (5)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>31-40</td>
<td>1</td>
<td>3 (1†)</td>
<td>7 (3/2†)</td>
</tr>
<tr>
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<td>2</td>
<td>2</td>
<td>2 (1†)</td>
</tr>
<tr>
<td>&gt;70</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*Data are given as number of patients. Numbers in parentheses represent human immunodeficiency virus (HIV)–positive patients.
†Number of HIV-negative patients.

Ocular surface squamous neoplasia occurs more com-
monly in younger patients (younger than 50 years) at King
Edward VIII Hospital. This may or may not be related to
the high HIV prevalence (31%) in the 20- to 40-year age
group in Kwa-Zulu Natal. Twelve (70.6%) of 17 pa-
tients tested were HIV positive, and they were all younger
than 50 years. Of all OSSN lesions encountered in this
study, 35 (85.4%) were either CIS or frank invasive squa-
mos carcinomas. Twenty-eight (70.0%) of 40 speci-
mens showed some p53 positivity. Furthermore, in 22
of these lesions, more than 50% of the tumor cells ex-
pressed p53 protein. Significant p33 expression oc-
curred more commonly in higher-grade OSSN lesions (20
of 22). Expression of p33 was not affected by the HIV
status of the patient.

The p33 gene causes arrest of cell division in G1 and
late G2 phases by affecting transcription of key cell cycle
control genes. Overexpression of p33 protein can occur
from stress or DNA damage in the absence of p33 gene
mutations. Tumorigenesis occurs within an environ-
ment characterized by high levels of proliferation and rapid
apoptosis.

The absence of Bcl-2 expression as seen in 30 of 40
OSSN specimens analyzed in this study may be indica-

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Figure 1. A, Invasive squamous carcinoma infiltrating the orbit and destroying the globe. B, Localized invasive conjunctival squamous carcinoma.
tive of cells undergoing more rapid death, since Bcl-2 expression confers longevity to cells. The resultant synergy between the effect of increased mutant p53 and absence of Bcl-2 expression may promote rapid clonal expansion, leading to progressively increased genomic instability and ultimately to malignancy.12 Minimal Bcl-2 positivity was present in 5 cases of CIS and 4 invasive squamous carcinomas. Only 1 CIS lesion contained more than 50% of cells exhibiting Bcl-2 positivity. These findings differ slightly from Bcl-2 expression in oral squamous neoplasia reported by Singh et al,17 who concluded that expression of Bcl-2 oncoprotein was directly proportional to the degree of epithelial dysplasia and that Bcl-2 was down-regulated in differentiating carcinomas.

Overexpression of Bcl-2 was also not seen in HIV-infected cases. The Tat protein of HIV modulates the expression of various cellular genes.18 Human immunodeficiency virus causes aberrant cytokine activation, and HIV protease promotes apoptosis by depleting the cell of Bcl-2 and increasing Bax protein, which has apoptosis-inducing properties.16,19 Infection with HIV and Bcl-2 deficiency promotes neoplasia by impairing immune surveillance, thereby allowing the unchecked survival of mutation-bearing cells. However, HIV infection cannot solely be implicated as the cause of the low Bcl-2 immunoreactivity encountered in this study because both HIV-positive and HIV-negative cases displayed a similar Bcl-2 immunoprofile.

MIB-1 analysis showed a low proliferation index for OSSN, which may explain the lack of extraorbital metastases and the low mortality associated with this tumor. One case of CIS expressed MIB-1–positive staining in more than 50% of the dysplastic cells. This tumor was excised from a 76-year-old woman whose HIV status was unknown. The tumor also expressed p53 positivity in 75% of the dysplastic cells exhibiting nuclear immunolabeling for p53 protein.18

The clinical role of p53 and Bcl-2 in OSSN needs further exploration. Their use as possible markers for tumor recurrence and metastatic potential needs investigation and may influence primary management protocols. While wide margin excision remains the gold standard, the benefit of radiotherapy and chemotherapy to preserve vision or limit morbidity in large, infiltrating, and poorly localized lesions may ultimately depend on p53 status of the tumor.

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REFERENCES