

Possible Prognostic Markers in Conjunctival Dysplasia and Squamous Cell Carcinoma

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Abstract: Conjunctival squamous cell carcinoma (SCC) can develop from carcinoma in situ or severe dysplasia known as conjunctival intraepithelial neoplasia (CIN). Conjunctival intraepithelial neoplasia and SCC are histopathologically well-defined conditions. However, it is difficult to determine the grading of dysplasia by clinical morphologic findings. Recently, proliferating cell nuclear antigen (PCNA) immunostaining, p53 immunostaining, and argyrophilic nucleolar organizer regions (AgNORs) staining have been established as valuable means of studying the biologic behavior of malignant cells. In the present study, these three staining techniques were used to examine histologic preparations of three conjunctival dysplasia and one SCC lesion. Five conjunctival tumor samples were obtained from four patients between July 1993 and October 1995. Following formalin fixation and embedding in paraffin, PCNA, and p53 immunostaining and AgNORs staining was performed with all tissue specimens. The PCNA-positive rate was the highest in SCC, followed by severe dysplasia and mild dysplasia. The p53-positive rate was the highest in severe dysplasia, followed by mild dysplasia, and negative in SCC. The AgNORs-count increased as malignancy advanced. These staining methods, which are markers for proliferative potency and cell differentiation, will be useful for early detection of changes in malignancy and will aid in decisions on treatment and prognosis. **Jpn J Ophthalmol 1998;42:256-261** © 1998 Japanese Ophthalmological Society

Key Words: Argyrophilic nucleolar organizer regions stain, conjunctival dysplasia, p53 stain, proliferating cell nuclear antigen stain, squamous cell carcinoma.

Introduction

In 1942, McGavie proposed that intraepithelial tumor of the conjunctiva be called Bowen's disease of the conjunctiva based on the histologic similarity to carcinoma in situ of the skin (Bowen's disease).¹ Since then, many cases of disease of the conjunctiva have been reported as Bowen's disease of the conjunctiva.²⁻⁴ However, intraepithelial tumors of the conjunctiva and Bowen's disease of the skin do not seem to be sufficiently correlated to warrant the same name; primary malignant tumors of the viscera do not occur as often with intraepithelial tumor of the conjunctiva as with Bowen's disease, and tumor cells of the two diseases differ morphologically. In 1978, Pizzarello and Jakobiec⁵ proposed designating

intraepithelial tumor as conjunctival intraepithelial neoplasia (CIN) and classified the tumor into two types; mild dysplasia and severe dysplasia. In mild dysplasia, dysplastic cells occupy less than half the conjunctival epithelium and in severe dysplasia, more than half or the entire layer.⁵ The World Health Organization (WHO) classifies carcinoma in situ with dysplasias and actinic keratosis as a CIN.⁶

When intraepithelial dysplastic cells, even partially, invade the basement membrane and infiltrate the subepithelium of the conjunctiva, the condition is called squamous cell carcinoma (SCC). Conjunctival intraepithelial neoplasia and SCC are histopathologically well-defined conditions. However, it is difficult to determine the grading of dysplasia by clinical morphologic findings. Recently, and antibody to the nuclear proliferation marker, proliferating cell nuclear antigen (PCNA), an antibody to the putative tumor suppressor gene, p53, and the silver-binding nuclear proliferation marker, argyrophilic

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nucleolar organizer regions (AgNORs), have been used to establish staining methods that are a valuable means of studying the biologic behavior of malignant cells. Although CIN usually remains in the epithelium and rarely infiltrates beyond the basement membrane, a few cases have developed into SCC. We studied the possible development of CIN into SCC and the degree of malignancy of CIN by examining histopathologic specimens of conjunctival dysplasia and SCC using the above three staining methods.

Materials and Methods

Five conjunctival tumor samples were obtained from four patients between July 1993 and October 1995 at the Department of Ophthalmology, Fukui Medical University, Japan. The clinical data for each patient are shown in Table 1. Normal conjunctiva obtained from excised pterygium tissue was used as the control. All tumor tissue specimens were formalin-fixed and paraffin-embedded. Those stained with hematoxylin and eosin had been previously diagnosed according to the WHO classification.⁶ Serial sections (5- μ m thick) were cut, deparaffinized, and processed for PCNA immunostaining, p53 immunostaining, and AgNORs staining.

PCNA and p53 Immunostaining

Tumor tissue sections were deparaffinized and dehydrated through a graded series of alcohol. Sections were placed in 0.3% hydrogen peroxide in methanol solution in order to block endogenous peroxidase activity. After washing in phosphate buffer solution (PBS, 0.1 mol/L, pH 7.4), the specimens were incubated for 10 minutes with 10% normal rabbit serum diluted in PBS. Specimens were washed in PBS and then incubated with antiproliferating cell nuclear antigen (PCNA) monoclonal antiserum (PC10, a monoclonal mouse antihuman antibody; Novocastra Laboratories, UK) diluted 1:400 in PBS for the PCNA analysis; with anti-p53 polyclonal antibody

(Ab-6, a polyclonal rabbit antihuman antibody; Oncogene Science, NY, USA) diluted 1:50 in PBS for the p53 analysis. Following overnight incubation at 4°C, specimens were washed in PBS and incubated with secondary antiserum (biotinylated rabbit anti-mouse immunoglobulin; Nichirei, Tokyo) at room temperature for 30 minutes and then rewashed in PBS. Treatment with avidin-biotin-peroxidase complex (avidin-peroxidase-conjugated streptavidin; Nichirei, Tokyo) followed at room temperature for 30 minutes. Immunoreaction products were visualized using 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Nakarai, Kyoto) containing 0.05% hydrogen peroxide. All samples were counterstained with hematoxylin. Negative control samples were incubated with nonimmunized normal rabbit IgG overnight at 4°C. The PCNA-positive rate was calculated as the average percentage of PCNA-positive cells counted per 100 cells in three sites throughout the conjunctiva. The p53-positive rate was calculated in the same way.

Silver-Binding AgNORs Staining

Sections were incubated in a mixture of 50 mL of solution A (1% gelatin, 2% formic acid in double distilled water) and 100 mL of solution B (50% silver nitrate in double distilled water) for 27 minutes in the dark at room temperature. Nucleolar organizer regions (NOR) were visualized as stained black dots in the nuclei. The AgNORs-count was calculated as the number of NOR per nucleus averaged over 100 tumor cell nuclei throughout the conjunctiva.

Results

Age, sex, treatment, and histologic diagnosis of each case of conjunctival tumor are shown in Table 1. The patients ranged in age from 77 to 83 years. Conjunctival tumors were diagnosed histopathologically as conjunctival intraepithelial neoplasia (CIN) in three cases and squamous cell carcinoma (SCC) in one case. The clinical findings of Case 1 observed

Table 1. Clinical Profile for Each Patient

Case	Age (years)	Sex	Treatment	Histopathologic Diagnosis
1a	79	F	Tumor excision	CIN with mild dysplasia
1b (recurrence)			Extended excision	CIN with severe dysplasia
2	77	F	Extended excision	CIN with mild dysplasia
3	83	F	Extended excision	CIN with severe dysplasia
4	82	M	Extended excision	SCC

F: Female. M: Male. CIN: Conjunctival intraepithelial neoplasia. SCC: Squamous cell carcinoma.

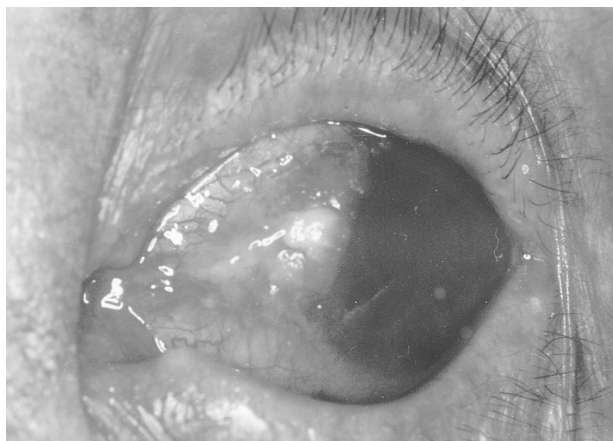


Figure 1. Anterior portion of affected eye in Case 3. Elevated leukoplakic limbal tumor extends one third of entire length of limbal conjunctiva. Lesion partially extends toward cornea.

during initial surgery (Case 1a), and a photograph of the anterior portion of the eye in Case 3, show opaque conjunctival epithelium and a levee-shaped protruding lesion that occupied one third of the entire limbal conjunctiva (Case 3, Figure 1). The lesion extended partially toward the cornea (Case 3, Figure 1). A localized protruding lesion was observed in the limbus at the nasal side in Case 2 and at the temporal side in Case 4 (Case 4, Figure 2). Histopathologically, Case 1 (at initial surgery, Case 1a) and Case 2 were diagnosed as CIN; mild dysplasia in the conjunctival epithelium occupied less than half the epithelium (Case 2, Figure 3). Case 1 (at recurrence, Case 1b) and Case 3 (Figure 4) had dysplastic cells in all the

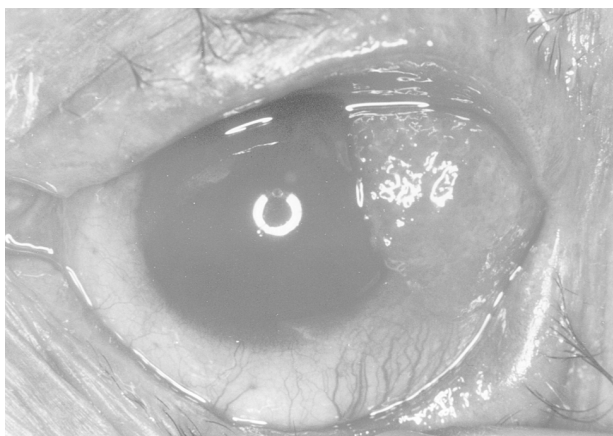


Figure 2. Anterior portion of affected eye in Case 4. Localized opaque protruding lesion on temporal side with invasion of vessels covers part of cornea.

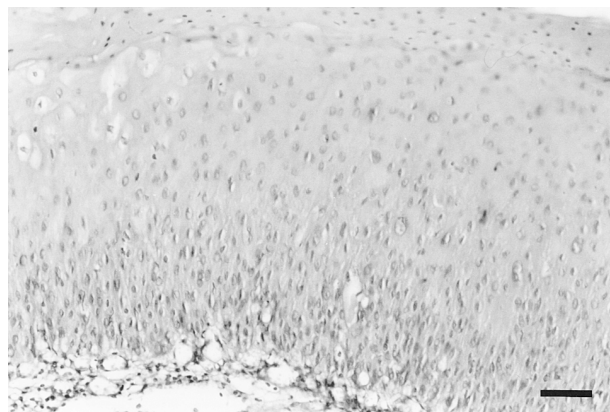


Figure 3. Hematoxylin-eosin (H-E) staining. In Case 2, diagnosed as conjunctival intraepithelial neoplasia, mild dysplasia in conjunctival epithelium occupies less than half of epithelium. Bar = 55 μ m.

layers of the epithelium. Although cells lost polarity, the basement membrane was intact. Case 4, in which a group of the advanced type of severely dysplastic cells extended beyond the basement membrane, was diagnosed as SCC (Case 4, Figure 5).

Results of Staining

The PCNA-positive rate, P53-positive rate and AgNORs-count for each case are shown in Table 2.

PCNA Staining. Although Case 2 was found to have only a few positive cells in the lower half of the epithelial layers, Case 3 demonstrated a high frequency of positive cells in these layers (Figure 6).

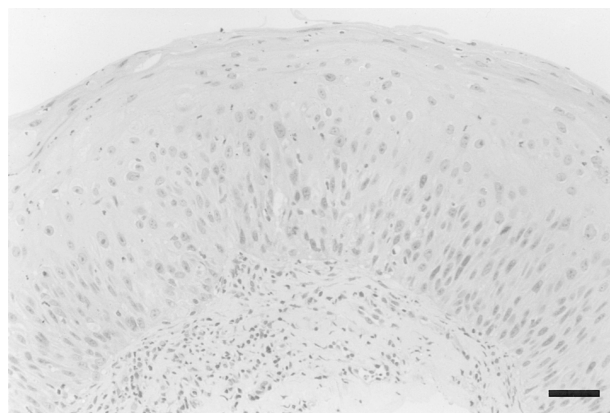


Figure 4. Hematoxylin-eosin staining. Case 3, diagnosed as conjunctival intraepithelial neoplasia, shows severe dysplasia. Dysplastic cells are present in all epithelial layers and have lost polarity. Basement membrane is still intact. Bar = 55 μ m.

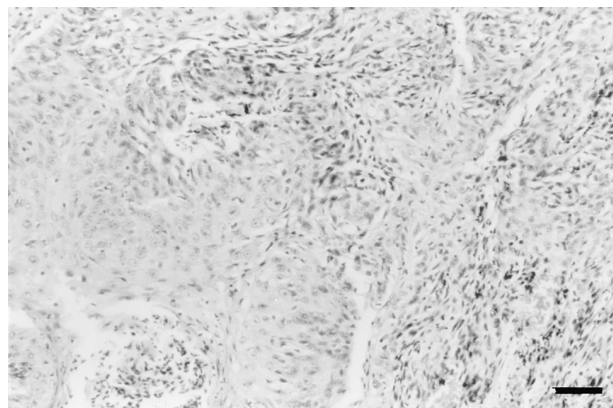


Figure 5. Hematoxylin-eosin staining. In Case 4, diagnosed as squamous cell carcinoma, severe dysplastic cells invade beyond basement membrane. Bar = 55 μ m.

Case 4 had a high frequency of positive cells in almost all layers of the epithelium. The PCNA-positive rate was the highest at 52% in the SCC case, Case 4, followed by 26.7% and 20.3% for Cases 1b and 3, respectively, which had CIN (severe dysplasia). Cases 1a and 2 (CIN; mild dysplasia) had a PCNA-positive rate of 11.0% and 11.3%, respectively. The PCNA-positive rate was the highest in SCC, followed by severe dysplasia, mild dysplasia, and the lowest in normal conjunctiva.

p53 Immunostaining. Normal conjunctiva and Case 4 (SCC) were p53-negative, while Case 2 demonstrated positive cells in the lower half of the epithelial layers (Figure 7). Case 3 had a high frequency of positive cells in almost all layers. The p53-positive rate was 21.3% and 26.7%, respectively, in Cases 1b and 3 (CIN; severe dysplasia), and 13.7% and 20.0%, respectively, in Cases 1a and 2 (CIN; mild dysplasia).

AgNOR-Count. Normal conjunctiva showed one to two small round NOR per nucleus, whereas Case 3

had a higher AgNOR-count per nucleus and these NOR were aggregated and distorted (Figure 8). The AgNOR-count was the highest, in Case 4 (3.3 per nucleus). Silver dots conglomerated in the nuclei, forming a large distorted shape. The AgNOR-count in Cases 1b and 3 (CIN; severe dysplasia) was 2.7 and 2.1, respectively, and in Cases 1a and 2 (CIN; mild dysplasia), 1.4 and 1.5, respectively. The AgNOR-count increased as the malignancy of cases advanced histologically.

Discussion

Conjunctival intraepithelial neoplasia is a common tumor of the ocular surface in elderly individuals.⁵⁻⁷ Although low malignancy and slow growth are characteristic of conjunctival intraepithelial neoplasia, the tumor often recurs following simple resection.⁸ The recurrence rate is 20–36%,^{5,7,8} and progression to SCC, although rare, has been reported.⁹ Pathomorphologic factors by which prognosis and degree of malignancy of malignant tumors are extrapolated include grade of differentiation of tumor, heteromorphic degree of tumor cells, and disease stage. In recent years, PCNA and p53 immunohistochemistry, and AgNORs have been applied to the histopathologic diagnosis of malignant tumors.^{4,10-20} We used tumor tissue specimens obtained from patients with CIN or SCC to examine the possibility that PCNA, p53, and AgNORs staining may be used as markers for malignancy and proliferative potency of CIN.

Proliferating cell nuclear antigen, a nucleoprotein with a molecular weight of 36,000, is a cofactor of DNA polymerase δ and increases in concentration from the latter half of G1-phase to the first half of S-phase in the cell cycle. The ratio of PCNA-positive to PCNA-negative cells in any given tumor tissue sample correlates well with the number of divided cells and degree of histologic malignancy.^{10,12,14,20}

Table 2. PCNA and p53 Staining and AgNOR-Count of Tumor Specimens

Case	Histology	PCNA-Positive Rate (%)	p53-Positive Rate (%)	AgNOR-Count (Per Nucleus)
1a	Mild dysplasia	11.0 (33/300)	13.7 (41/300)	1.4
1b(recurrence)	Severe dysplasia	26.7 (80/300)	21.3 (64/300)	2.7
2	Mild dysplasia	11.3 (35/300)	20.0 (60/300)	1.5
3	Severe dysplasia	20.3 (63/300)	26.7 (80/300)	2.1
4	SCC	52.0 (156/300)	Negative	3.3
Control	Dysplasia(-)	7.7 (23/300)	Negative	1.1

PCNA: proliferating cell nuclear antigen. AgNORs: argyrophilic nucleolar organizer regions. SCC: squamous cell carcinoma. Control: Normal conjunctiva obtained from excised pterygium tissue.

Carcinoma in situ with PCNA-positive nuclei in many cells tends to progress to cancer,¹⁵ and supra-basal staining is useful in predicting recurrence, change to malignancy, and response to treatment.^{4,11} The present study found that the PCNA-positive rate, which was highest in SCC, followed by severe dysplasia and mild dysplasia, correlated with the degree of histologic malignancy. Conjunctival intraepithelial neoplasia with high PCNA-positive rates have a high proliferation potency and may be at high risk of becoming malignant.

The p53 gene belongs to the suppressor gene family. The wild type p53 protein plays a role in regulation of the cell cycle by up-regulating the expression of genes related to induction of arrest of cell proliferation and apoptosis, and down-regulating the expression of genes related to cell proliferation. The disappearance, rearrangement, or mutation of the p53 gene is thought to cause abnormal expression and transformation.^{10,11,15,21,22} Mutation in this gene has been found in over 50% of cases of human cancer.²¹ Among various antibodies to p53, Ab-6, which was used in the present study, principally stains mutant forms of the p53 protein, although the wild type is also stained slightly. At what stage of tumor formation p53 changes or mutates depends on the particular tumor in question.^{4,10,11,18,19,22} In the present study, we found that CIN was p53-positive, whereas SCC and normal conjunctiva were p53-negative. Because CIN is a precancerous lesion, this result suggests that mutation of the p53 protein occurred in these cases. SCC was negative for two possible reasons: one is that mutation of p53 varies widely, and thus, the antibody used in the present study was un-

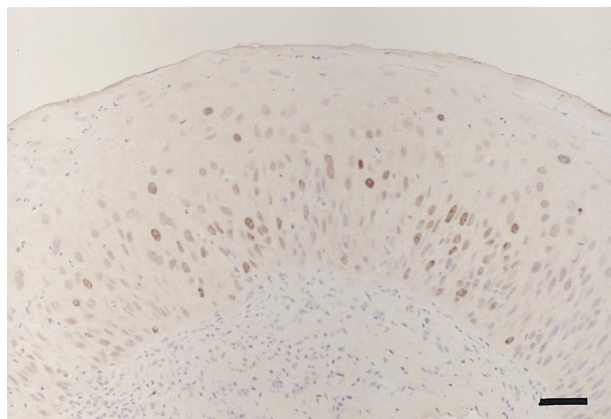


Figure 6. Proliferating cell nuclear antigen (PCNA) immunostaining. In Case 3, PCNA-positive cells are seen in lower half of epithelial layers in high frequency. Bar = 55 μ m.

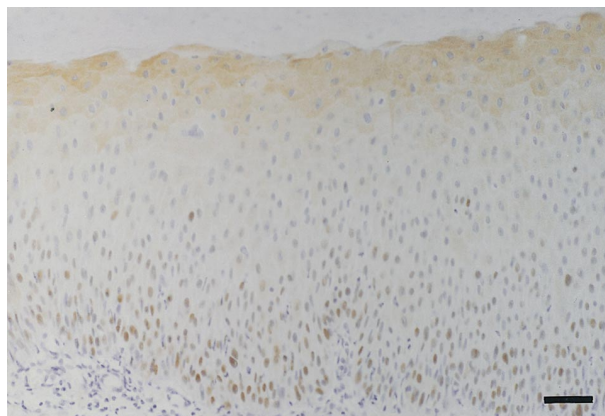


Figure 7. p53 immunostaining. In Case 2, p53-positive cells appear in lower half of epithelial layers. Bar = 55 μ m.

able to recognize the antigenic determinant and the other reason is that the tumor developed not because of mutation of p53 but because of a complete lack of p53 protein.

Nuclear organizer regions is the name of the structure that forms the ribosomal DNA loop. The numbers and size of the structures strongly affect cellular metabolism, proliferation, and differentiation and the AgNORs staining method has been used in many studies on human tumors.²³ The difference in NOR-count per nucleus between benign tumors and malignant tumors has been shown to be statistically significant.^{13,14,16} AgNORs staining of CIN and SCC specimens in the present study revealed a higher NOR-count in more malignant tissue. In addition, the morphology of the silver dots was distorted in



Figure 8. Argyrophilic nucleolar organizer regions (AgNORs) staining. In Case 3, AgNOR-count per nucleus was high and nuclear organizer regions (NORs) were aggregated and distorted. Bar = 30 μ m.

such tissue, suggesting that the NOR-count might be useful in the evaluation of malignancy in conjunctival tumors.

The three staining methods used in the present study, which are markers for proliferative potency and cell differentiation, may be useful in the early detection of the change to malignancy and may detect recurrence of CIN or progression to SCC in pathological specimens. Proliferating cell nuclear antigen and p53 staining and NOR-count may be used as aids in deciding the treatment and prognosis of such cases.

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