

Evaluation of Epithelial Dysplasia at the Surgical Margins in Patients with Early Tongue Carcinoma — Immunohistochemical Study of Recurrence of Epithelial Dysplasia

Yuichiro Okazaki,¹ Kazumichi Sato,² Atsushi Takada,² Shigeki Morisaki,² Yutaka Watanabe,² Yasuhiro Ozawa,² Mitsuaki Morimoto,² Morio Tonogi,² Yoichi Tanaka,³ Gen-yuki Yamane^{1,2}

¹Oral Cancer Center and ²Department of Oral Medicine, Oral and Maxillofacial Surgery, Tokyo Dental College, and ³Division of Surgical Pathology, Clinical Laboratory, Ichikawa General Hospital, Tokyo Dental College, Chiba, Japan

Abstract

Objective: The diagnosis of epithelial dysplasia at the surgical margins in oral cancer plays an important role in its prognosis and requires careful evaluation. Epithelial dysplasia is usually asymptomatic, but often associated with recurrence and malignant transformation.

Patients and Methods: The patients included 9 men and 5 women (mean age, 54.6 years; range, 31 to 76 years) with early tongue carcinoma and tumour-cell-“free” surgical margins. Immunohistochemical analysis of p53 and Ki-67 expression was performed in order to evaluate epithelial dysplasia and pathological changes.

Results: None of the patients had cancer recurrence, but 2 showed recurrence of epithelial dysplasia. The resection margins at initial surgery and at recurrence were positive for p53 and Ki-67 in both these patients.

Conclusions: Epithelial dysplasia, with positive staining for p53 and Ki-67, has the potential for morphological changes, with possible recurrence of epithelial dysplasia or malignant transformation. Such patients will require careful follow-up for local recurrence. Examination of surgical margins for these markers is useful for the early detection of pathological changes.

Key words: Ki-67 antigen, Precancerous conditions, Recurrence, Tongue neoplasms, Tumor suppressor protein p53

Introduction

The accurate diagnosis and treatment of premalignant oral lesions and epithelial dysplasia in the periphery is an important factor in improving oral cancer cure rates and prognosis. Diagnostic criteria for epithelial dysplasia differ between medical centres, and differential diagnosis from early cancer is often difficult. Epithelial dysplasia is often asymptomatic, but recurrence and malignant transformation are common. The diagnosis of epithelial dysplasia and early carcinoma requires uniform evaluation criteria and the prognosis of these oral lesions requires careful evaluation of the surgical margins in each patient. The immunohistochemistries of p53, a tumour suppressor gene, and Ki-67, a

marker of cell proliferation, have been used to evaluate pre-malignant and malignant oral lesions,¹⁻⁴ but few studies have investigated the clinical usefulness of these tumour markers in the surgical margins of resected lesions.⁵⁻⁷

To establish prognostic factors for epithelial dysplasia and early invasive cancer, we performed immunohistochemical staining for p53 and Ki-67 in epithelial dysplasia of surgical margins from patients undergoing surgery at our hospital for early tongue cancer. Few, if any, studies have compared the histopathology findings of initially resected lesions and recurrence. In this report, we describe findings in recurrent epithelial dysplasia after tongue surgery.

Patients and Methods

Patients

This study included 14 patients with T1 and early T2 tongue cancer, diagnosed and treated at our hospital between April 1998 and March 2005, with histological findings of

Correspondence:

Yuichiro Okazaki, Oral Cancer Center, Tokyo Dental College, 5-11-13 Sugano, Ichikawa City, Chiba 272 8513, Japan.
Tel: (81 47) 322 0151; Fax: (81 47) 324 8533;
E-mail: okazaki@tdc.ac.jp

carcinoma (negative) and dysplasia (positive) at the surgical margins. There were 9 men and 5 women, with a mean age of 54.6 years (range, 31 to 76 years). Surgery consisted of tumour excision after preoperative biopsy or total excision and biopsy after preoperative cytology. Vital staining with iodine was done in all patients, and the surgical margins were about 5 mm from unstained areas. If intraoperative rapid histological examination showed severe dysplasia, further resection was performed. Serial sections were prepared by bread-loaf step sectioning.⁸ The surgical margins were examined in all patients. The tumour type in all cases was squamous cell carcinoma (N0). Tumour invasion was limited to the lamina propria mucosa, without invasion into the muscularis propria. The study was performed in accordance with the guidelines of the ethics committee at our hospital.

Immunohistochemistry

Serial sections of each specimen were stained by the labelled streptavidin-biotin (LSAB) method using standard procedures. Namely, 5 µm thin sections were deparaffinised with xylene and then treated with 0.3% methanolic peroxide for 15 minutes to block endogenous peroxidase. For antigen retrieval, the specimens were immersed in 0.01 M citrate buffer and irradiated with microwave energy (750 Watts) for 5 minutes. This was performed twice. Following washing with 0.01 M phosphate-buffered solution, the specimens were reacted with p53 (DO-7; DAKO, Japan; diluted 1:200) and Ki-67 (MIB-1; Immunotech, France; diluted 1:200) as primary antibodies for 60 minutes at room temperature. The slides were incubated with secondary antibody and peroxidase-labeled streptavidin using an LSAB2 Kit (Dako, Japan) and visualised with 3,3'-diaminobenzidine.

Epithelium of the surgical margins was examined under 200 times magnification, with observation of the basal, prickle, and granular cell layers for positive findings. The

findings were judged p53- and Ki-67-positive if at least 20 of 500 cells were positive.^{3,4}

Results

Of the 14 patients in this study, surgical margins were positive for p53 and Ki-67 in 5 and 7 patients, respectively. None of the patients had recurrent cancer, but 2 had recurrent epithelial dysplasia. The other patients are doing well and being followed-up on a regular basis (Table 1).

Epithelial dysplasia in the surgical margins, graded in accordance with the 1997 World Health Organization (WHO) diagnostic criteria, was mild in 4 patients, moderate in 8, and severe in 2 patients. In this study, no correlation was found between being positive for p53 or Ki-67 and the severity of dysplasia based on the WHO criteria.

Case Presentations

Patient No. 1

Initial Lesion

Histopathology findings: no tumour cells were present in the surgical margins, but drop-shaped rete ridges consistent with moderate dysplasia were observed (Figure 1a). Slightly central to the margin, immunostaining for p53 was positive in cells from the basal cell layer to just below the granular cell layer (Figure 1b). Scattered Ki-67 positive cells were found in the rete ridges (Figure 1c).

Recurrent Lesion, 3 Years 2 Months Postoperatively

1) Intraoral findings: a 6-mm area of leukoplakia was found in the mucosal transition between the tongue and floor of mouth, corresponding to the initial posterior resection margin (Figure 2). Because of a suspicion of tumour recurrence, total excision and biopsy were performed.

2) Histopathology findings: hypertrophic epithelium and hyperparakeratosis were present in the entire surgical margin,

Table 1. Results of follow-up examination of surgical margins in patients.

Case no.	Age (years)/gender	Margin dysplasia	p53 positivity	Ki-67 positivity	Follow-up period	Clinical course
1	76/female	Moderate	Positive	Positive	8 years 1 month	Recurrent dysplasia (postoperative, 3 years and 5 years)
2	63/male	Mild	Negative	Negative	7 years 9 months	
3	54/male	Moderate	Negative	Negative	7 years 1 month	
4	47/female	Moderate	Negative	Positive	6 years 3 months	
5	52/male	Mild	Negative	Negative	5 years 1 month	
6	75/female	Moderate	Positive	Negative	4 years 8 months	
7	51/female	Moderate	Positive	Negative	4 years 6 months	
8	50/male	Severe	Negative	Positive	4 years 5 months	
9	53/male	Mild	Negative	Negative	4 years 2 months	
10	37/male	Moderate	Positive	Positive	2 years 6 months	
11	57/male	Moderate	Positive	Negative	2 years 4 months	
12	46/female	Mild	Negative	Positive	2 years 3 months	
13	73/male	Severe	Negative	Positive	1 years 7 months	
14	31/male	Moderate	Negative	Positive	1 years 2 months	

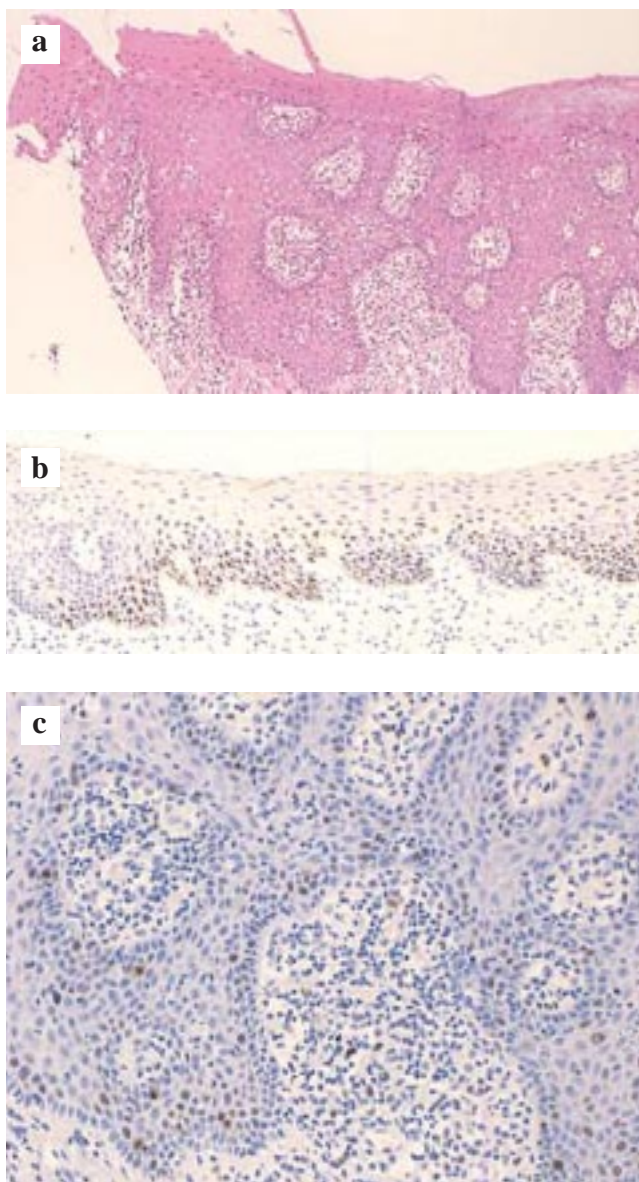


Figure 1. Patient 1, histopathology of the initial lesion. (a) Histology of surgical margin (haematoxylin and eosin, $\times 100$). (b) p53 Immunostaining (haematoxylin and eosin, $\times 100$). (c) Ki-67 immunostaining (haematoxylin and eosin, $\times 200$).



Figure 2. Patient 1 (postoperative, 3 years 2 months), intraoral findings.

with cell proliferation towards the lower basal layer. The findings were similar to those at initial resection (Figure 3a). Both at initial resection and recurrence, p53- and Ki-67-positive cells were observed from the basal cell layer to the lower prickle cell layer (Figure 3b and Figure 3c).

Recurrent Lesion, 5 Years 2 Months Postoperatively

1) Intraoral findings: two years after the previous recurrence, leukoplakia was noted again anteriorly in the mucosal

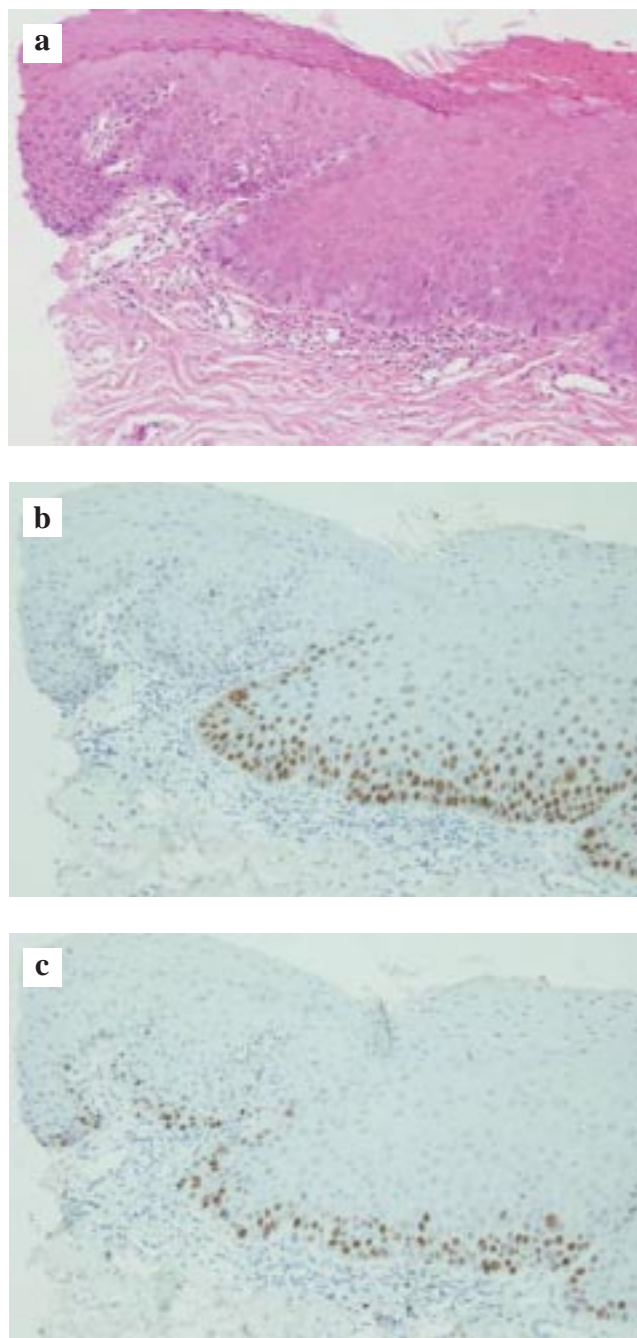


Figure 3. Patient 1 (postoperative, 3 years 2 months), histopathology of the recurrent lesion. (a) Histology of surgical margin (haematoxylin and eosin, $\times 200$). (b) p53 Immunostaining (haematoxylin and eosin, $\times 200$). (c) Ki-67 immunostaining (haematoxylin and eosin, $\times 200$).

transition of the tongue and floor of mouth (Figure 4), and total excision and biopsy was performed. This area was the initial surgical margin, and no epithelial dysplasia was found. 2) Histopathology findings: intraepithelial keratinisation, thickening of the prickle cell layer, and hyperplasia with long rete ridges were present. Scattered single cell keratinisation was also observed in the prickle cell layer (Figure 5a). As previously observed, p53- and Ki-67-positive cells were present from the basal cell layer to the prickle cell layer (Figure 5b and Figure 5c).

Patient No. 10

Initial Lesion

Histopathology findings: no tumour cells were present in the surgical margins, but epithelium with moderate dysplasia was observed (Figure 6a). Slightly central to the margin, immunostaining for p53 was positive in cells from the basal cell layer to the granular cell layer (Figure 6b). Scattered Ki-67-positive cells were found in the basal cell and parabasal layers (Figure 6c).

Recurrent Lesion, 1 Year 10 Months Postoperatively

1) Intraoral findings: a white keratotic lesion was seen in the area corresponding to lateral anterior margin in the floor of the mouth (Figure 7). Because of a suspicion of tumour recurrence, total excision and biopsy were performed. 2) Histopathology findings: findings similar to the epithelial dysplasia seen at initial resection were present (Figure 8a). Cells positive for p53 were seen primarily in the basal cell layer, with some also in the prickle cell layer (Figure 8b). Ki-67-positive cells were present in part of the basal cell and prickle cell layers (Figure 8c).

Discussion

In head and neck cancers, local recurrence rates of 10 to 30% are still seen even in patients with surgical margins free of tumour cells.⁹ In order to improve cure rates, improved methods of evaluating resected lesions are needed. Even in patients with tumour-cell-“free” surgical margins, careful follow-up evaluation is mandatory.

The presence of epithelial dysplasia in the surgical margins of oral squamous cell carcinoma is an important prognostic factor. The presence or absence of epithelial dysplasia and its degree are also important in assessing the potential for malignant transformation.¹⁰ The degree of oral epithelial dysplasia is commonly graded according to the WHO diagnostic criteria based on 13 items,¹¹ and Kurokawa et al¹² believe that these criteria are the most useful. However, in actual clinical practice, the degree of epithelial atypia does not always correlate with potential malignant transformation. Even if genetic analysis of epithelial dysplasia of the tumour periphery shows the same gene mutation as in the tumour



Figure 4. Patient 1 (postoperative, 5 years 2 months), intraoral findings.

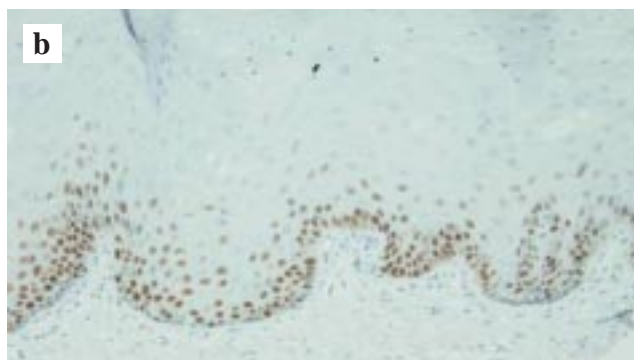
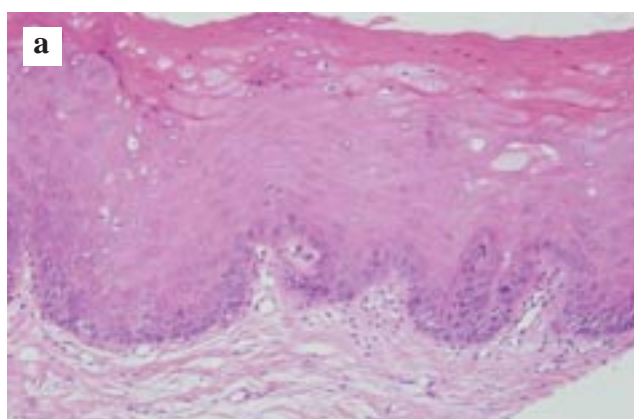


Figure 5. Patient 1 (postoperative, 5 years 2 months), histopathology of the recurrent lesion. (a) Histology of surgical margin (haematoxylin and eosin, × 200). (b) p53 Immunostaining (haematoxylin and eosin, × 200). (c) Ki-67 immunostaining (haematoxylin and eosin, × 200).

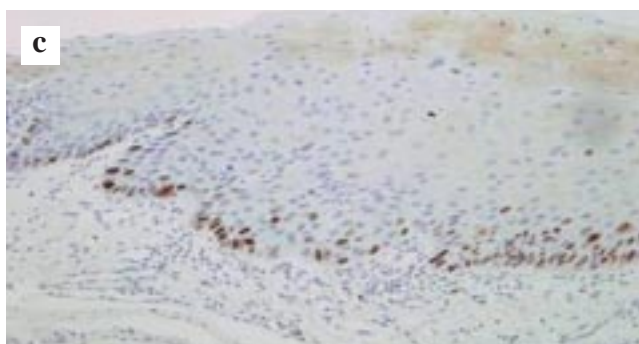
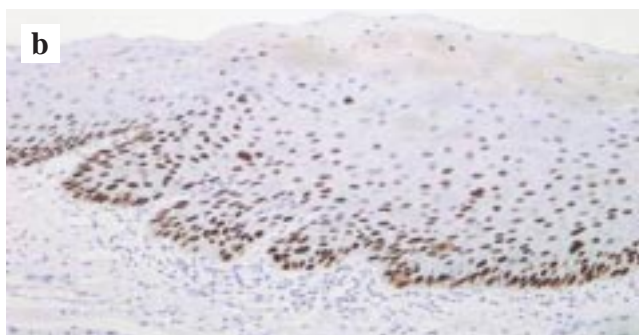
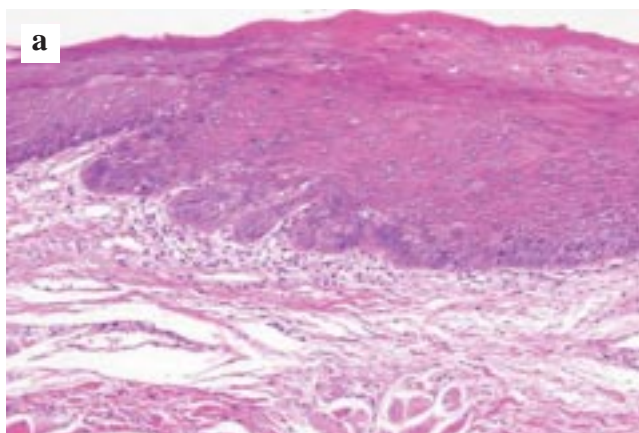


Figure 6. Patient 10, histopathology of the initial lesion. (a) Histology of surgical margin (haematoxylin and eosin, $\times 200$). (b) p53 Immunostaining (haematoxylin and eosin, $\times 200$). (c) Ki-67 immunostaining (haematoxylin and eosin, $\times 200$).



Figure 7. Patient 10 (postoperative, 1 year 10 months), intraoral findings.

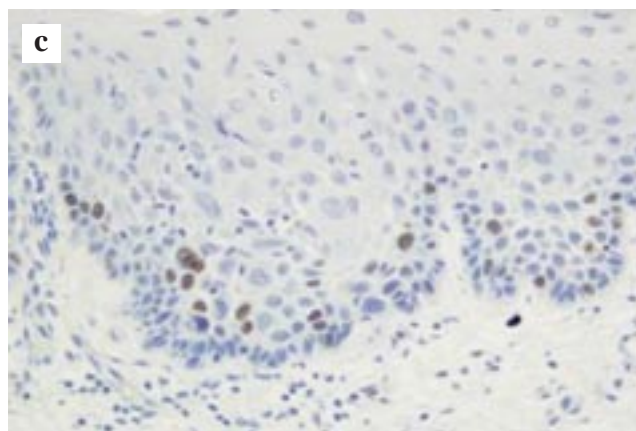
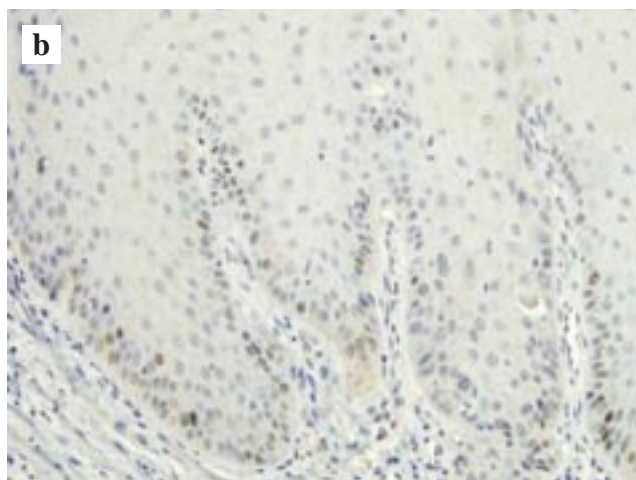
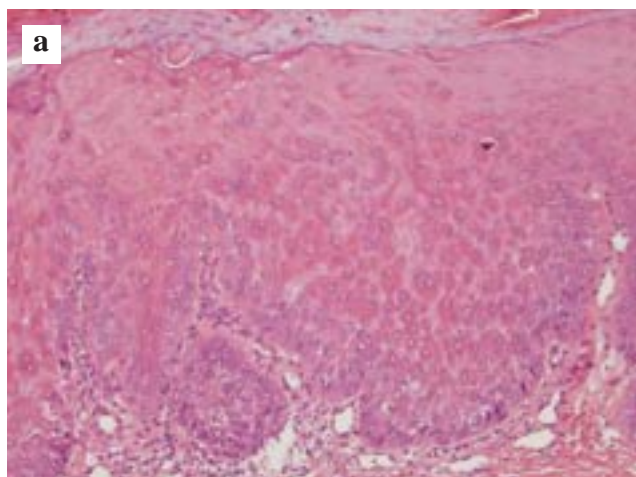


Figure 8. Patient 10 (postoperative, 1 year 10 months), histopathology of the recurrent lesion. (a) Histology of surgical margin (haematoxylin and eosin, $\times 200$). (b) p53 Immunostaining (haematoxylin and eosin, $\times 200$). (c) Ki-67 immunostaining (haematoxylin and eosin, $\times 200$).

itself,^{13,14} this may not accurately predict malignant transformation of epithelial dysplasia or the clinical prognosis.

Immunohistochemical analyses show a correlation between the degree of epithelial dysplasia and p53- and Ki-67-positive rates, with the highest expression rates in severe dysplasia.²⁻⁴ Expression of p53 and Ki-67 at an early stage

are important in prognosis. Thus, the presence of p53- and Ki-67-positive cells in epithelial dysplasia suggests early malignant potential.¹⁻⁴

Studies of head and neck cancers report that p53 immunostaining is useful in evaluating recurrence and prognosis, and that the rate of local tumour recurrence is about 50% when surgical margins are p53-positive.⁵⁻⁷ The 2 patients with recurrent epithelial dysplasia showed p53-positive findings both at initial resection and recurrence in this study too, thus suggesting an association. However, the data regarding p53 positivity in about half of patients with local tumour recurrence seems to differ from our results.

A genetic analysis study by this group, using an early tongue cancer model in rats,^{14,15} found high p53 and Ki-67 expressions in epithelial dysplasia when compared to early invasive cancer; this suggests p53 and Ki-67 involvement at an early stage in the malignant transformation process. This indicates the need for careful examination of p53 and Ki-67 positivity in tumour cells and epithelial dysplasia of tissue margins contiguous with the tumour, and for additional surgical resection when necessary.

Our results showed p53 and/or Ki-67 expression even in mild-to-moderate epithelial dysplasia of surgical margins. This suggests that genetic analysis of p53 and Ki-67 does not necessarily correlate with the histological degree of epithelial dysplasia, but rather is associated with early morphologic changes in the epithelium. Our study failed to show any correlation between p53 or Ki-67 expression and the histological degree of epithelial dysplasia. This may be because patients with surgical margins showing severe epithelial dysplasia usually undergo additional resection at our hospital; thus, most had mild-to-moderate dysplasia. The tongue cancer model suggested by this group also showed no clear relationship between the degree of histological findings and the genetic analyses results.^{14,15}

The site and pattern of p53 and Ki-67 expressions are similar, with positive findings in the basal and upper basal layers of normal epithelium. In epithelial dysplasia, depending on the degree of atypia, in addition to the basal layer, p53- and Ki-67-positive cells are also seen in the prickle and granular cell layers.^{2,4}

With regard to the molecular-pathological diagnostic criteria for Ki-67 immunostaining of oral mucosal epithelium, the following characteristic findings have been suggested for Ki-67-positive cells: large nuclei with a distinct nucleolus in the basal layer; usual presence in the parabasal and prickle cell layers; both characteristics at certain stages.¹⁶ In our study, the pattern of expression of Ki-67 exhibited similar positive findings in initially resected lesions and recurrent epithelial dysplasia.

Amagasa et al¹⁷ have reported cases of multiple recurrence after excision of leukoplakia, including malignant transformation after several years of observation. As the time

to malignant transformation also decreases with increasing severity of epithelial dysplasia, long-term follow-up for any malignant changes will be required for patients with recurrent epithelial dysplasia.

Immunohistochemical analysis of p53 and Ki-67 at the surgical margins were performed in 14 patients with early tongue carcinoma and the results evaluated for correlation with pathological changes. Two of the 14 patients studied had recurrent epithelial dysplasia, and both p53 and Ki-67 were positive at initial resection and at recurrence.

Findings of this study suggest that epithelial dysplasia at the surgical margins, with p53- and Ki-67-positive cells, is more likely to undergo morphological changes, with an increased risk for malignant transformation. Thus, long-term follow-up for local recurrence is mandatory in these patients. Immunohistochemical analysis of p53 and Ki-67 positivity at the surgical margins is useful in detecting early pathological changes, regardless of the severity of epithelial dysplasia.

References

1. Kövesi G, Szende B. Changes in apoptosis and mitotic index, p53 and Ki67 expression in various types of oral leukoplakia. *Oncology*. 2003;65:331-6.
2. Kurokawa H, Matsumoto S, Murata T, Yamashita Y, Tomoyose T, Zhang M, et al. Immunohistochemical study of syndecan-1 down-regulation and the expression of p53 protein or Ki-67 antigen in oral leukoplakia with or without epithelial dysplasia. *J Oral Pathol Med*. 2003;32:513-21.
3. Piattelli A, Rubini C, Fioroni M, Iezzi G, Santinelli A. Prevalence of p53, bcl-2, and Ki-67 immunoreactivity and of apoptosis in normal oral epithelium and in premalignant and malignant lesions of the oral cavity. *J Oral Maxillofac Surg*. 2002;60:532-40.
4. Kushner J, Bradley G, Jordan RC. Patterns of p53 and Ki-67 protein expression in epithelial dysplasia from the floor of the mouth. *J Pathol*. 1997;183:418-23.
5. van Houten VM, Leemans CR, Kummer JA, Dijkstra J, Kuik DJ, van den Brekel MW, et al. Molecular diagnosis of surgical margins and local recurrence in head and neck cancer patients: a prospective study. *Clin Cancer Res*. 2004;10:3614-20.
6. Nathan CO, Amirghahri N, Rice C, Abreo FW, Shi R, Stucker FJ. Molecular analysis of surgical margins in head and neck squamous cell carcinoma patients. *Laryngoscope*. 2002;112:2129-40.
7. Ball VA, Righi PD, Tejada E, Radpour S, Pavelic ZP, Gluckman JL. p53 immunostaining of surgical margins as a predictor of local recurrence in squamous cell carcinoma of the oral cavity and oropharynx. *Ear Nose Throat J*. 1997;76:818-23.
8. Tanaka Y, Yamane G, Asanami S. Pathological study of tongue carcinoma using Breadloaf step sectioning and the cooperation of clinic and pathology [article in Japanese]. *J Jpn Soc Oral Tumors*. 2001;13:217-21.
9. Leemans CR, Tiwari R, Nauta JJ, van der Waal I, Snow GB. Recurrence at the primary site in head and neck cancer and the significance

- of neck lymph node metastases as a prognostic factor. *Cancer*. 1994;73:187-90.
10. Silverman S Jr, Gorsky M, Lozada F. Oral leukoplakia and malignant transformation. A follow-up study of 257 patients. *Cancer*. 1984;53:563-8.
 11. Pindborg JJ, Reichart PA, Smith CJ, van der Waal I, editors. *Histological typing of cancer and precancer of the oral mucosa*. 2nd ed. Berlin: Springer-Verlag; 1997.
 12. Kurokawa H, Yamashita Y, Matsumoto S, Fukuyama H, Takahashi T. Pathological study of epithelial dysplasia on surgical margin in squamous cell carcinoma of the tongue [article in Japanese]. *J Jpn Soc Oral Tumors*. 2002;14:89-93.
 13. Partridge M, Emilion G, Pateromichelakis S, Phillips E, Langdon J. Field cancerisation of the oral cavity: comparison of the spectrum of molecular alterations in cases presenting with both dysplastic and malignant lesions. *Oral Oncol*. 1997;33:332-7.
 14. Okazaki Y, Tanaka Y, Tonogi M, Yamane G. Investigation of environmental factors for diagnosing malignant potential in oral epithelial dysplasia. *Oral Oncol*. 2002;38:562-73.
 15. Sato K, Okazaki Y, Tonogi M, Tanaka Y, Yamane GY. Expression of beta-catenin in rat oral epithelial dysplasia induced by 4-nitroquinoline 1-oxide. *Oral Oncol*. 2002;38:772-8.
 16. Gonzalez-Moles MA, Ruiz-Avila I, Rodriguez-Archilla A, Martinez-Lara I. Suprabasal expression of Ki-67 antigen as a marker for the presence and severity of oral epithelial dysplasia. *Head Neck*. 2000:658-61.
 17. Amagasa T, Fujii E, Suzuki T, Yamashiro M, Ogura I, Miyakura T, et al. Clinical characteristics of precancerous lesions and early squamous cell carcinoma in the oral cavity [article in Japanese]. *J Jpn Soc Oral Tumors*. 1999;11:357-63.