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Immunohistological evaluation of Ki-67, p63, CK19 and p53 expression in oral epithelial dysplasias

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BACKGROUND: Oral squamous cell carcinoma develops through a multistep of genetic mutations, and the process can be morphologically recognized as oral epithelial dysplasia. To evaluate the hypothesis that distributional alterations of proliferating and stem cells may be a useful index to estimate the grading and development of epithelial dysplasia, we examined the distribution patterns according to stratified cell layers.

METHODS: Sixty-two oral dysplasia cases according to the histological grades were immunohistologically examined and the nuclear expression of Ki-67 and p63 antigens was counted according to epithelial layers as labeling index.

RESULTS: The Ki-67 labeling index in the basal and suprabasal layers and that of p63 in the basal layer showed a significant difference between low- and high-grade groups of epithelial dysplasia.

CONCLUSION: The architectural alteration of proliferating cell and stem cell distribution in the layers of epithelial dysplasias may provide useful information to evaluate the grading of oral epithelial dysplasias.

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Keywords: CK19; Ki-67; oral epithelial dysplasia; p53; p63

Introduction

It has been generally considered that oral squamous cell carcinoma (SCC) develops through a multistep process of the accumulation of genetic mutations related to cell proliferation and differentiation such as p53 (1, 2, 3), and the process can be morphologically recognized as cancer precursor lesions, although malignant transformation may rarely also develop directly from normal epithelium (NOE). The likelihood of oral epithelial precursor lesions progressing to SCC is designated as epithelial dysplasia. It is histopathologically classified depending on cellular atypia and architectural disturbances as the degree of mild, moderate and severe dysplasia (4), and has also been classified as squamous intraepithelial neoplasia in the upper aero-digestive tract (5). The standardization of the histopathological diagnosis and grading of epithelial dysplasia, however, remains subjective as there are many composite histological criteria of cellular atypia and architectural disturbances. Furthermore, it is rare for all criteria to be present in the same lesion (6), and the relationship of the grade to the subsequent development of cancer and to clinical observations has not yet been fully clarified (7, 8).

The oral mucosal epithelium is a stratified squamous epithelium that shows the typical stratification of cell layers such as basal, parabasal, prickle-cell and parakeratotic-cell layers, with the exception of specialized regional epithelia such as that in the dorsal tongue. The stratification of cellular layers results from the continuous cellular differentiation and proliferation of epithelial stem cells located in the basal layer (9). *p*63 is a candidate gene involved in the initiation and maturation of the stratification during embryogenesis and maturity (10, 11). The transcriptional factor *p63* belongs to the *p53* family expressed in at least six isoforms and suspected to play diverse roles through the counterbalanced expression of both isoforms TAp63 and ΔNp63 as a master molecular switch for the initiation of stratification and maintaining the proliferation potential of basal keratinocytes in mature epidermis (12). An experimental examination by the genetic elimination of *p63* disclosed that it plays an essential role in epithelial development to maintain keratinocyte stem cells (10, 11, 13), and the over expression of *p63* may play a role in the oncogenesis of head and neck tumors of squamous epithelial lineage (14, 15, 16). The altered expression of cytokeratin 19 (CK19), which is also regarded as a

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marker of epithelial stem cells (17) has been reported in oral epithelial dysplasia and SCC (18). It is therefore suggested that oral epithelial dysplasia may be histologically recognized as the distribution disturbance of proliferating cells and stem cells within the stratified layers of the epithelium.

The Ki-67 protein as a molecular marker of proliferating cells has been extensively examined in oral epithelial dysplasia and SCC, and the number of proliferating cells is increased according to the grade of dysplasia (19, 20). These histological examinations, however, for proliferating cells and alteration of stem cells have mainly focused on the total number of positive cells within the epithelium as an index of malignancy rather than for architectural distribution within the altered epithelium.

To evaluate the hypothesis that the distributional alteration of proliferating cells and stem cells within epithelial dysplasia may be a useful index to estimate the grading and development of epithelial precursor lesions, we immunohistologically examined the detailed distribution patterns of proliferating cells by an antibody for Ki-67 protein, stem cells by antibodies for p63, and CK19 proteins according to the layers within the epithelium, and an antibody for p53 protein to evaluate alteration of the tumor suppressor gene. We selected cases of different degrees of oral epithelial dysplasia based on the WHO criteria and a characteristic architectural pattern of moderate dysplasia with a two-phase appearance (21), and also NOE as a comparative purpose.

Materials and methods

Seventy-two biopsy or surgical cases, 36 male and 36 female aged 37–85 years (mean 64.1 years) were selected from surgical pathology files in the Department of Oral Pathology, Kagoshima University Graduate School of Medical and Dental Sciences, from 1999 to 2004 after a critical review of stored hematoxylin and eosin (HE)-stained sections according to the WHO criteria (4). The cases were classified as 10 hyperplasia (HYP), 10 mild dysplasia (MLD), 10 moderate dysplasia (MOD), 13 severe dysplasia (SED), 10 carcinoma *in situ* (CIS), and nine two-phase dysplasia (TPD) classified as MOD. Ten NOE cases were also added for comparative purposes. The intraoral sites of specimens were as follows: tongue (41 cases), gingiva (15 cases), buccal mucosa (eight cases), hard palate (four cases), oral floor (three cases) and lip (one case). All specimens were routinely fixed in buffered 10% formalin and embedded in paraffin. Serial 4 μm-thick sections were stained with HE to reevaluate histological diagnosis, and the other sets were used for immunohistochemical examinations.

The sections were dewaxed in xylene and rehydrated in graded ethanol. For antigen retrieval, the sections were placed in a pressure cooker containing 1 mM EDTA buffer (pH 8.0) heated to 130°C for 2.5 min and then cooled to room temperature. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min followed by washing in 0.05 mM Tris-buffered saline (TBS) at pH 7.4. The sections were incubated with precisely diluted mouse monoclonal antibodies against Ki-67 (MIB-1, 1:100), p63 (4A4, 1:100), p53 (DO-7, 1:50) and CK19 (RCK108, 1:50) (Dako, Kyoto, Japan) as primary antibodies overnight at 4°C. Subsequently, after washing in TBS, the sections were incubated with a secondary antibody conjugated with peroxidase-labeled dextran polymers (Envision+ Dual Link/HRP system, Dako) for 30 min at room temperature. After rinsing with TBS, they were treated with 0.5 mg/ml DAB solution containing 0.001% hydrogen peroxide to visualize reaction products, and counterstained with Mayer's hematoxylin for 3 min.

The nuclear expression of Ki-67 and p63 antigens was counted according to epithelial layers as the basal layer, nuclei positive just above the basement membrane; parabasal layer, nuclei positive within two layers above the basement membrane and next to the basal layer; and suprabasal layer, nuclei positive in a more upper layer above the parabasal layer, using a microscope at ×400 magnification. For p63 expression, nuclei showing a relatively strong signal were also selectively counted (Fig. 2).

The percentages of positively stained nuclei for antibodies in 500 epithelial cell nuclei in each layer were calculated as the labeling index (LI) of Ki-67 and p63. Statistically, one-way ANOVA test was used to evaluate the difference of mean LIs in each layer between histopathologically diagnosed groups by means of Stat View software package (SAS Institute Inc.) with significance at $P < 0.01$. The expression of p53 and CK19 antigens was histopathologically examined and evaluated in each section.

Results

The Ki-67 and p63 expressions were detected in all cases studied and restricted in the basal and parabasal layers of NOE (Figs 1 and 3a–f). There were no significant differences of LIs between groups by age, sex and region. The Ki-67 expression was mainly presented in the parabasal layer rather than in the basal layer of NOE (Fig. 3a). Ki-67-LI in the basal and suprabasal layers of epithelial dysplasias increased according to the grade of dysplasia, and up to CIS in the suprabasal layer, while it was constant through every grade in the parabasal layer. There were significant differences in mean Ki-67-LI between NOE and that in TPD, SED and CIS in both basal and suprabasal layers ($P < 0.01$; Fig. 4a), while there were no significant differences between that in HYP, MLD and MOD in both basal and suprabasal layers, and also between that in TPD, SED and CIS in the basal layer (Fig. 4a).

The intensity of p63 immunostaining was heterogeneous in each nucleus (Figs 2 and 3d–f), and there was no significant difference in mean p63-LI to any degree in cell layers (data not shown). Selectively counting the strong signal (Fig. 2) of p63-LI decreased according to the severity of dysplasia from NOE to TPD, SED and CIS in the basal layer ($P < 0.01$; Fig. 4b). In the suprabasal layer, p63-LI slightly increased with the severity of dysplasia, while it was constant in the parabasal

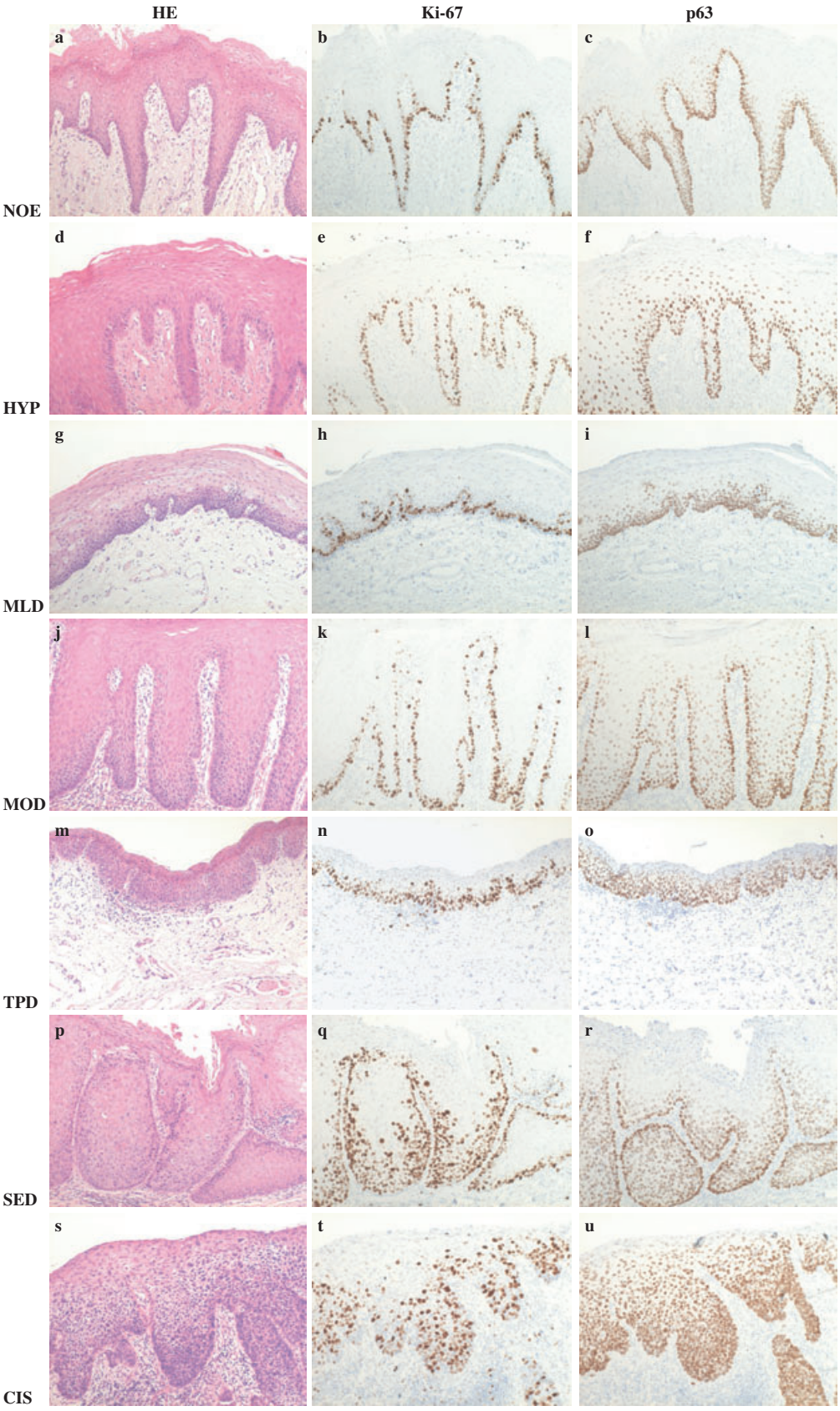

Figure 1 Histopathological and immunohistological findings of Ki-67 and p63 expression pattern in oral epithelial dysplasias. (a–c): normal epithelium (NOE), (d–f): hyperplasia (HYP), (g–i): mild dysplasia (MLD), (j–l): moderate dysplasia (MOD), (m–o): two-phase dysplasia (TPD), (p–r): severe dysplasia (SED), (s–u): carcinoma *in situ* (CIS), and (a–s): hematoxylin and eosin (HE), (b–t): Ki-67, (c–u): p63 (×20).

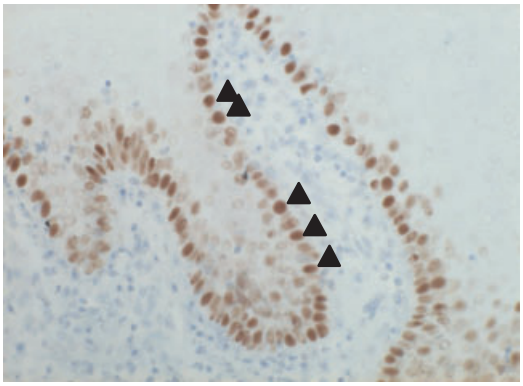

Figure 2 Immunohistological findings of p63 in normal epithelium. Intensity of p63 immunostaining was heterogeneous in each nucleus. Strongly positive staining (arrow heads) nuclei were selectively counted for p63-labeling index (×100).

layer. There was no significant difference of mean p63-LI between TPD, SED and CIS in the basal layer (Fig. 4b).

Based on the above analysis for each layer and histological group, the analysis of both LIs in each layer between tentatively combined with MLD and MOD as a low-grade group and that of TPD and SED as a high-grade group showed a significant difference of Ki-67-LI in basal and suprabasal layers and that of p63-LI in the basal layer of both groups ($P < 0.01$; Table 1).

The CK19-positive cells were restricted in the basal layer of NOE (Fig. 3g) but the staining disappeared in MLD adjacent to NOE (Fig. 3h). CK19-positive cells were found heterogeneously in every layer of CIS. Positive staining of p53 was detected in 15.4% cases of SED and 70% cases of CIS with a mainly focally restricted pattern (Fig. 3i).

Discussion

This study has demonstrated different expression patterns of Ki-67 as a marker of proliferating cells in different degrees of oral epithelial dysplasia. In NOE, the proliferating cells were restricted mainly in the parabasal rather than basal layer, in agreement with a previous report (22). The expression pattern of CK19 and p63 protein as markers of stem cells suggests that epithelial stem cells might be present in the basal layer. Therefore, it could be considered that transient

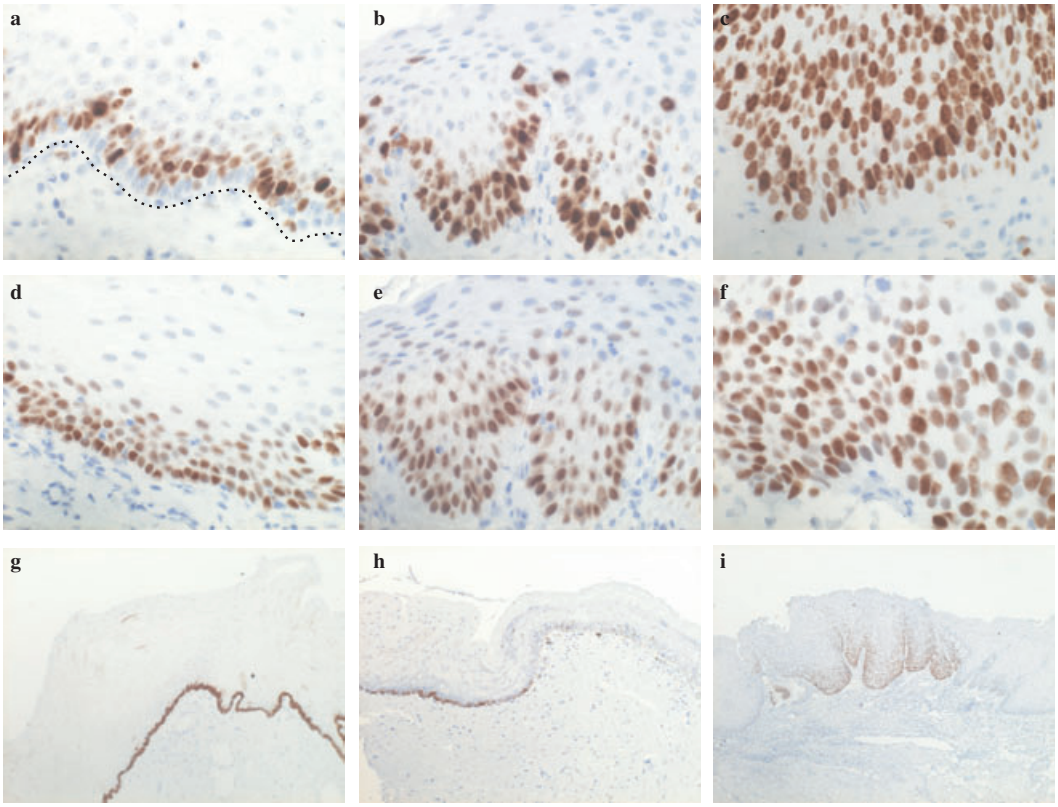

Figure 3 Immunohistological findings of Ki-67, p63, CK19 and p53 in epithelial dysplasias. (a): Ki-67-positive cells were mainly present in the parabasal rather than the basal layer in normal epithelium. The dotted line indicates the basal lamina. (b): Ki-67-positive cells were increased in the basal layer and also in the suprabasal layer in two-phase dysplasia. (c): Marked increase of Ki-67-positive cells in the suprabasal layer in severe dysplasia. (d): Strongly positive cells for p63 were mainly present in the basal layer. (e): p63-positive cells were present in the basal and parabasal layer but also occasionally in the suprabasal layer in two-phase dysplasia. (f): p63-positive cells were increased in the suprabasal layer but the strongly positive cells in basal layer were decreased. (g): CK19-positive cells were restricted to the basal layer in normal epithelium. (h): CK19-positive cells disappeared in mild dysplasia adjacent to normal epithelium. (i): p53-positive cells were detected in the basal to suprabasal layer but mainly in a focally restricted pattern in severe dysplasia (a–f: ×100, g–i: ×20).

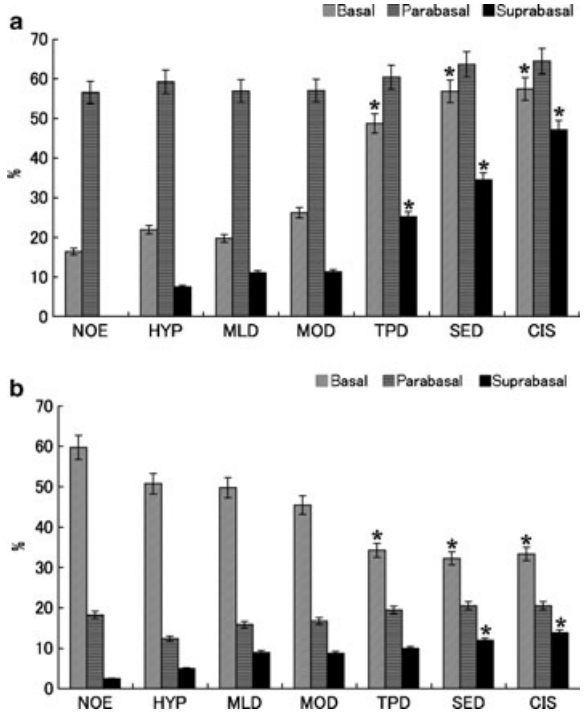

Figure 4 Ki-67-labeling index (a) and p63-labeling index (b) in each epithelial layer of epithelial dysplasias. (mean with SE, $*P < 0.01$). NOE, normal epithelium; HYP, hyperplasia; MLD, mild dysplasia; MOD, moderate dysplasia; TPD, two-phase dysplasia; SED, severe dysplasia; CIS, carcinoma *in situ*.

Table 1 Mean values of Ki-67- and p63-labeling index (LI) in epithelial layers of low-grade and high-grade epithelial dysplasias (mean/SD)

Layers	*Normal (10)*	*Low-grade (20)*	*High-grade (22)*
Ki-67-LI			
Basal	16.4/4.8	22.9/5.9	53.5/11.9*
Parabasal	56.5/5.6	57.0/8.2	62.3/7.5
Suprabasal	0.0/0.0	11.1/3.4	30.7/9.2*
p63-LI			
Basal	59.7/5.2	47.5/8.5	33.0/5.9*
Parabasal	18.2/5.1	16.2/6.6	20.1/5.8
Suprabasal	2.4/1.7	8.8/4.8	11.1/5.6*

*Significant ($P < 0.01$).

amplifying cells derived from stem cells might be present in the parabasal layer of NOE (9). Asymmetric cell division in epithelial stem cells located in the basal layer may produce transient amplifying cells located in the parabasal layer in oral epithelium. Furthermore, loss of asymmetry cell division may lead to increasing numbers of stem cells in the parabasal layer as increasing p63-positive cells in the parabasal and suprabasal layers of oral dysplasias presented in this study and also previous studies (23, 24). The distributional disturbance of p63-positive cells in oral epithelial dysplasia according with grade of the dysplasia might be play a role in oral tumorigenesis (23). The mechanism of asymmetrical cell division of stem cells and its relevance in cancer stem cells for oncogenesis remains obscure; however, the possibility of a control mechanism of cellular polarity may relate to asymmetrical cell division in stem cells and cancer stem cells (25, 26). It is well recognized that the dispolarity of basal cells is a histological criterion and a hallmark of cellular atypia in epithelial dysplasia (4). The distributional disturbance of proliferating cells and suspected stem cells in oral epithelial dysplasia presented in this study suggest loss of function of stem cells, as cancer stem cells could contribute to the development of oral epithelial dysplasias and oncogenesis of oral SCC.

Although previous studies have described increases in the total number of proliferating cells according to the degree of epithelial dysplasia and SCC (19, 20), that increase in the suprabasal layer has also been suggested as a useful marker of high-grade dysplasia (27, 28), as confirmed in this study. The increased proliferating cell population in both basal and suprabasal layers of epithelial dysplasia in this study suggest that proliferating cells might increase not only in a superficial direction but also downward to the basal layer in epithelial dysplasias. The decrease of CK19 expression and p63-LI in the basal layer of epithelial dysplasias suggests an alteration of stem cell function, and the stem cells could be replaced by proliferating cells in the basal layer of epithelial dysplasias. The architectural disorganization of proliferating cells and stem cells in oral epithelium could be a useful index to estimate the grading of epithelial dysplasias if added to histomorphological examinations in HE staining sections. Regional differences of epithelial morphology and cellular kinetics have been suggested in different sites of oral mucosa (22, 29). Although there were no site-related differences of LIs in this study, further studies including increased cases for each region are required for precise assessment.

The antibody for p63 (4A4) used in this study recognized all six *p63* isoforms that produce different functional proteins and might have an opposite or similar function to p53 protein (30). The intensity of immunostaining using the p63 antibody was heterogeneous and if all positive cells were counted there was no significant difference between dysplasia groups in this study as previously suggested (31). Therefore, in this study we also selectively counted only intensely stained nuclei. The intensity of the immunostaining might be related with maintain of stem cell function as the intensely stained cells were mainly present in the basal layer of NOE. The decrease of p63-LI in the basal layer of SED and CIS suggests that the function of *p63* in oral epithelium might be to maintain stem cell function rather than the direct correlation of oncogenesis and malignant transformation. However, detailed molecular mechanisms of how the expression of isoforms of *p63* contributed to oncogenesis remain to be elucidated (32, 33, 34). Although it has been suggested that the functional correlation of *p63* overexpression to oncogenesis is based on balanced expression levels of different isoforms (33, 35), examinations using antibodies specific for the isoforms and RT-PCR analysis in epithelial dysplasias should be performed (31, 36, 37).

Immunostaining for p53 protein is generally accepted as a marker of malignant transformation, and p53 overexpression was observed only in SED and CIS in this study, showing the pattern of a focal compact area as previously reported (38). In this study there were no positive cases in NOE, HYP, MLD and MOD that included TPD. Cellular atypia in TPD was not so extensive and was classified as MOD (21), but the patterns of Ki-67- and p63-LIs suggest that TPD might belong to a high-grade group rather than a low-grade group. The LI pattern examined in this study was considered as a useful index to estimate the degree of epithelial dysplasia such as the determination of low-grade and high-grade groups of epithelial dysplasia. The relationship between the degree of precursor lesions and occurrence of malignant transformation to SCC remains controversial. Furthermore, p53 immunostaining could not detect its nonsense mutation. The correlation between histological grading and molecular alterations should be clarified by accumulating information about gene alterations using a microdissection-based PCR method that is now being prepared for examination based on the results of this study. It seems important to accumulate evidence by detailed and long-term clinicopathological examinations about the relationship between the degree of epithelial dysplasia and its clinical features by retrospective and prospective studies (39, 40).

In conclusion, examinations for the architectural alteration of proliferating cell and stem cell distribution in the layers of epithelial dysplasias may provide useful information to evaluate the grading of oral epithelial dysplasias when it is combined with conventional histomorphological criteria for oral epithelial precursor lesions. Furthermore, the results suggest that *p63* may contribute to the development of epithelial dysplasias through the alteration of stem cell function in the basal layer, resulting in an increased number of proliferating cells, and its altered distribution in basal and suprabasal layers within oral epithelial dysplasias.

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