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Immunohistochemical study of CK13, CK17, CK19, Ki-67, p53, p63, p21, p27 and Cyclin D1 in oral epithelial dysplasia

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In the current World Health Organization (WHO) classification, oral epithelial dysplasia (OED) is classified using a two- or three-grade system. However, no useful auxiliary diagnostic method for determining the OED grade of specimens by this system has yet been established. In this study, specimens of not only OED, but also epithelial hyperplasia (Hp) and oral squamous cell carcinoma (OSCC) were examined immunohistochemically to investigate expression levels/rates of various antibodies. Six specimens of Hp, 13 of mild epithelial dysplasia (MiD), 11 of moderate epithelial dysplasia (MoD), 11 of severe epithelial dysplasia (SD), and 9 of OSCC obtained from the First Department of Oral and Maxillofacial Surgery at Osaka Dental University Hospital were examined immunohistochemically to determine the expression levels of CK13, CK17, CK19, Ki-67, p53, p63, p21, p27 and cyclin D1. The dysplasia grade was negatively correlated with expression of CK 13 and CK17. As the dysplasia grade increased, expression rates of Ki-67, p53, p63, p21 and cyclin D1 increased, while the expression rate of p27 decreased. The expression rate of Ki-67 differed significantly between SD and OSCC, as did the expression of p53 between MoD and SD as well as between SD and OSCC. The expression rate of p63 differed significantly between MiD and MoD as well as between MoD and SD, as did the expression rate of p27 between SD and OSCC ($p < 0.05$). These results suggest that not only CK 13, CK17, Ki-67 and p53, but also p63, p21, p27 and cyclin D1 may be linked to dysplasia. The results also suggest that calculation of Ki-67, p53, p63 and p27 expression rates could be used for OED grading and auxiliary diagnosis of OSCC. (J Osaka Dent Univ 2023; 57: 107-117)

Key words: Oral epithelial dysplasia; Ki-67; p53; p63; p27

INTRODUCTION

In 1997 the World Health Organization (WHO) defined a precancerous lesion as “a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart”,[1] and in 2005 as “a generalized state associated with a significantly increased risk of cancer”.[2] 2017, the WHO proposed the new term, “oral potentially malignant disorders” (OPMDs),[3] which are defined as “clinical presentations that carry a risk of cancer development in the oral cavity, whether in a clinically definable precursor lesion or in clinically normal oral mucosa”.[4] OPMDs can progress to oral cancer, which makes histopathological grading of oral epithelial dysplasia (OED) important for their diagnosis.[3] WHO defines OED as “a spectrum of architectural and cytological epithelial changes caused by accumulation of genetic changes, associated with an increased risk of progression to squamous cell

carcinoma".[4] In the 2017 WHO classification of OED, the severity of dysplasia is graded using a three-grade (mild, moderate, or severe) or two-grade (low or high) classification system.[4] The malignant transformation rate is approximately 6% for mild, 18% for moderate, and 39% for severe dysplasia.[4] For low- and high-grade dysplasia, the rates are approximately 5% and 43%, respectively.[5] Because higher-grade dysplasia is associated with a higher risk of malignant transformation, accurate diagnosis of OED is important.

Histopathological diagnosis is generally performed using hematoxylin and eosin staining, but no useful auxiliary diagnostic method has yet been established for grading dysplasia in OED. Auxiliary diagnosis based on expression of proteins such as cytokeratins has been investigated extensively. Cytokeratins are cytoskeletal proteins that form intermediate filaments in epithelial cells. Expression levels of two of the 20 subtypes of cytokeratin (CK13 and CK17) are negatively correlated with dysplasia grade and are useful in the diagnosis of OED and oral squamous cell carcinoma (OSCC).[6] Ki-67 is a cell proliferation marker that is present in all active phases of the cell cycle from the G1 phase to the M phase, but not in the quiescent G0 phase, making it useful for determining the proliferation rate.[7] The tumor suppressor gene p53, related to apoptosis and the cell cycle,[8] is useful in the diagnosis of malignancy because it is highly expressed in malignant tumors and is involved in tumorigenesis and proliferation. A member of the p53 family that induces apoptosis by increasing p53 in DNA-damaged cells in order to inhibit cell proliferation is p63.[9] Other factors are p21 and p27 are cyclin-dependent kinase (CDK) inhibitors that bind to cyclin-CDK complexes to inhibit cell proliferation. Recent studies showing that both p21 and p27 are involved in apoptosis have generated renewed interest in these proteins.[10]

Many studies have investigated the diagnosis of OSCC and grading of OED, and some have shown that CK13, CK17, Ki-67 and p53 may be useful, but no clear index for grading has yet been developed. In this study, we determined the expression of cytoskeletal factors, cell proliferation markers, and cell cycle-related factors in hyperplasia (Hp), OED and OSCC by immunohistochemical analysis.

MATERIALS AND METHODS

Specimens

We examined 6 cases of Hp, 13 of MiD, 11 of MoD, 11 of SD, and 9 of OSCC of the oral mucosa handled by the First Department of Oral and Maxillofacial Surgery at Osaka Dental University Hospital. The patients were 22 males and 28 females between 31 and 82 years of age with a mean age of 60.0±15.2 years (Table 1). The lesions were located on the tongue (25 cases), buccal mucosa (9 cases), mandibular gingiva (9 cases), hard palate (5 cases), floor of the mouth (1 case), and maxillary gingiva (1 case) (Table 2). Specimens were fixed in 10% formalin solution, dehydrated in a graded ethanol series, and embedded in paraffin. We clas-

Table 1 Age and gender of subjects

Lesion	Age (yrs)		Gender		Total
	Average	Range	Male	Female	
Hp	51.7	32-80	3	3	6
MiD	64.9	53-82	4	9	13
MoD	62.8	49-79	6	5	11
SD	57.5	38-77	6	5	11
OSCC	58.0	31-76	3	6	9
Total	60.0	31-82	22	28	50

Hp: Hyperplasia, MiD: Mild dysplasia, MoD: Moderate dysplasia, SD: Severe dysplasia, OSCC: Oral squamous cell carcinoma.

Table 2 Site of the lesions

Lesion	Site						Total
	Tongue	Maxillary gingiva	Mandibular gingiva	Buccal mucosa	Floor of the mouth	Hard palate	
Hp	1	0	4	1	0	0	6
MiD	6	1	0	4	0	2	13
MoD	5	0	1	3	1	1	11
SD	8	0	2	0	0	1	11
OSCC	5	0	2	1	0	1	9
Total	25	1	9	9	1	5	50

sified epithelial dysplasia based on the 2017 WHO classification of tumours of the head and neck.[4] Age, sex, and site distribution are shown in Tables 1 and 2.

This research was approved by the Ethics Committee of Osaka Dental University (Approval No.111090).

Immunohistochemical staining

Sections (4 μm thickness) were deparaffinized in Hemo-De® (Falma, Tokyo, Japan) and rehydrated through a graded ethanol series. Antigen retrieval to anti-human CK13 mouse monoclonal antibody (DakoCytomation, Glostrup, Denmark) was carried out by autoclaving at 121°C for 15 min in 10 mM citrate buffer at pH 6.0. Antigen retrieval to anti-human Ki-67 mouse monoclonal antibody (Leica Biosystems Newcastle, Newcastle, UK), anti-human p21 mouse monoclonal antibody (DakoCytomation), anti-human p27 mouse monoclonal antibody (DakoCytomation), anti-human p53 mouse monoclonal antibody (DakoCytomation), anti-human p63 mouse monoclonal antibody (Nichirei Biosciences, Tokyo, Japan), and anti-human cyclin D1 mouse monoclonal antibody (DakoCytomation) was carried out by

Table 3 Clones and dilutions of the primary antibodies

Antibody	Source	Clone	Dilution
CK13	Dako	DE-K13	1 : 100
CK17	Leica	E3	1 : 20
CK19	Leica	b170	1 : 100
Ki-67	Leica	MM1	1 : 200
p53	Dako	DO-7	1 : 50
p63	Nichirei	4A4	1 : 1
p21	Dako	SX118	1 : 50
p27	Dako	SX58G8	1 : 50
cyclin D1	Dako	DCS-6	1 : 200

Dako: Dako Cytomation, Glastrup, Denmark, Leica: Leica Biosystems Newcastle, Newcastle, UK, Nichirei: Nichirei Biosciences, Tokyo, Japan.

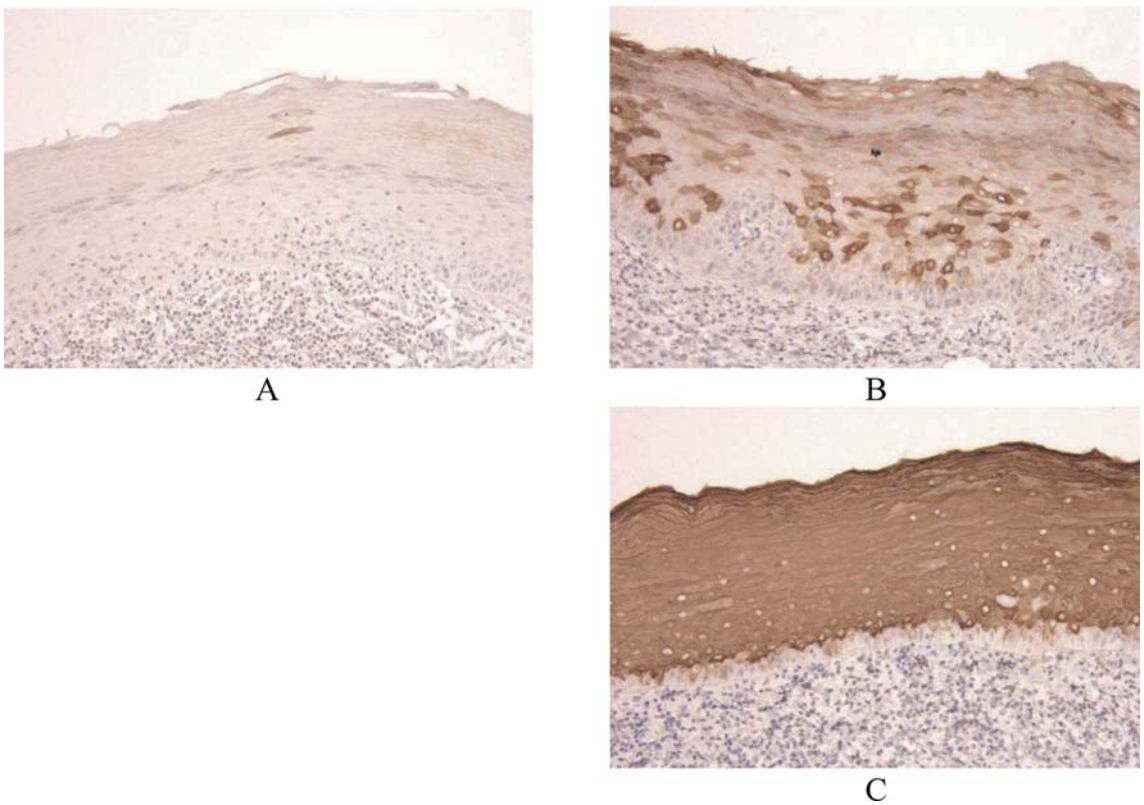

Fig. 1 Immunohistochemical evaluation of CK13, where (A) is valued 0 (no expression), (B) is valued 1 (weak and/or patchy expression), and (C) is valued 2 (strong and diffuse expression (×100)).

autoclaving at 121°C for 15 min in 10 mM citrate buffer at pH 7.0. Antigen retrieval to anti-human CK 17 mouse monoclonal antibody (Leica Biosystems Newcastle) was carried out by autoclaving at 121°C for 15 min in Target Retrieval Solution (DakoCytomation) at pH 9.0. Antigen retrieval to anti-human CK19 mouse monoclonal antibody (Leica Biosystems Newcastle) was performed at room temperature for 5 min in Histofine protease solution (Nichirei Biosciences). Endogenous peroxidase was blocked by 3% H_2O_2. These antibodies were diluted with Antibody Diluent with Background Reducing Components (DakoCytomation). CK13, CK17, Ki-67, p21, p27, p53 and cyclin D1 were reacted at room temperature for 30 min, while CK19 and p63 were reacted at room temperature for 60 min. Sections were incubated with Histofine Simple Stain Max-PO (M) (Nichirei Biosciences) at room temperature for 30 min. The sections were visualized by 3,3′-diaminobenzidine tetrahydrochloride (Nichirei Biosciences) and counterstained with hematoxylin. The original sources, clones, dilutions, and manufacturers of all the antigens used are summarized in Table 3.

Immunohistochemical evaluation

Immunohistochemical expression of CK13 and CK 17 was evaluated according to Yagyuu *et al.*[11]: (0) no expression, (1) weak and/or patchy expression, (2) strong and diffuse expression (Figs. 1 and 2). Immunohistochemical expression of CK19 was evaluated according to Kale *et al.*[12]: (0) no expression, (1) positive staining for less than one-third of the tissue section, (2) positive staining for between one-third and two-thirds of the tissue section, (3) positive staining for more than two-thirds of the tissue section (Fig. 3). Expression of Ki-67, p53, p63, p21, p27, and cyclin D1 was evaluated by counting positive cells. We counted more than 500 epithelial cells in the three high-power fields in the epithelium

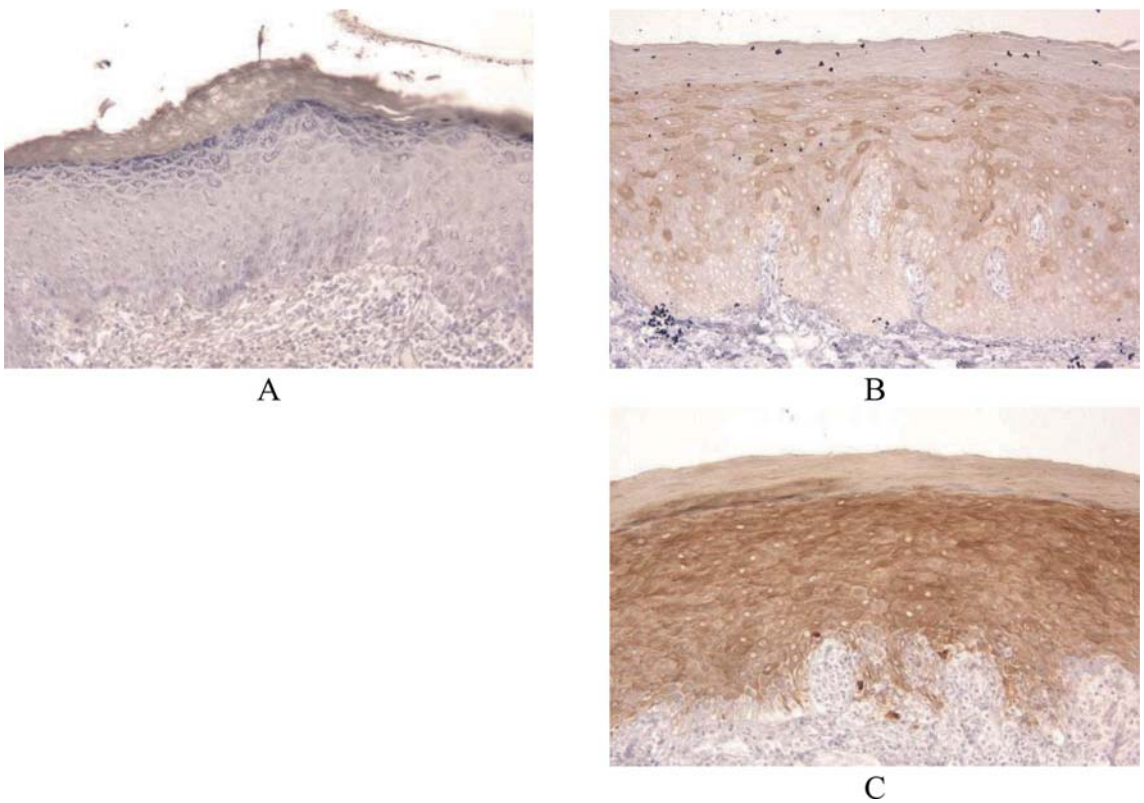

Fig. 2 Immunohistochemical evaluation of CK17, where (A) is valued 0 (no expression), (B) is valued 1 (weak and/or patchy expression), and (C) is valued 2 (strong and diffuse expression (×100)).

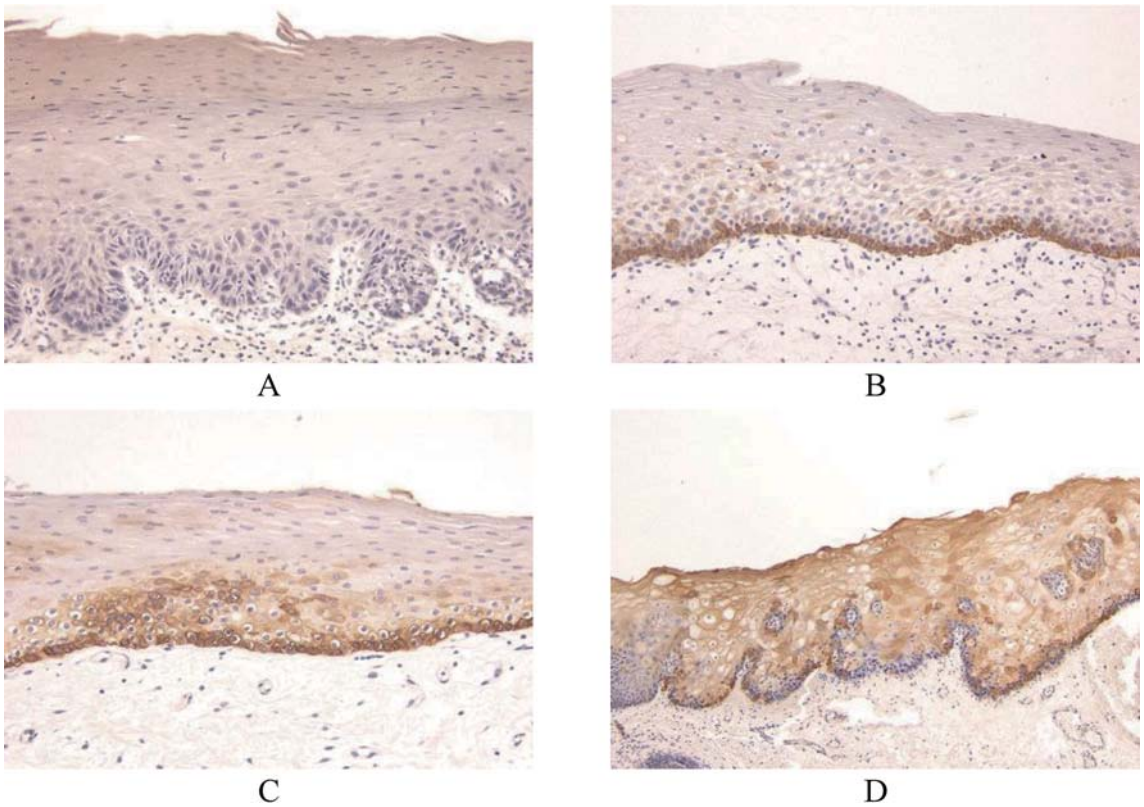

Fig. 3 Immunohistochemical evaluation of CK19, where (A) is valued 0 (no expression), (B) is valued 1 (positive staining for less than one-third of the tissue section), (C) is valued 2 (positive staining area ranging from one-third to two-thirds of the tissue section), and (D) is valued 3 (positive staining for more than two-thirds of tissue section (× 100)).

and calculated the percentage positive for Ki-67, p 53, p63, p21, p27 and cyclin D1. The results of CK 13, CK17 and CK19 were examined by the Yates m × n Chi square test. The results of Ki-67, p53, p63, p21, p27, and cyclin D1 were examined by the Mann-Whitney *U*-test. A *p*-value of < 0.05 was considered statistically significant.

RESULTS

Expression of CK13, CK17 and CK19 was detected in the cytoplasm, while expression of Ki-67, p53, p63, p21, p27 and cyclin D1 was detected in the nuclei.

CK13

Most specimens of CK13 (Hp: 5/6, 83.3%; MiD: 11/13, 84.6%) had a score of 2. Among MoD specimens, 3 of 11 (27.3%) had a score of 2, and 4 of 11 (36.4%) had a score of 0 or 1. Most SD specimens (8/11, 72.7%) and all OSCC specimens had a score of 0. The CK13 score decreased as the dysplasia grade increased. Scores differed significantly between SD and Hp, SD and MiD, as well as between OSCC and Hp, OSCC and MiD (Fig. 4).

CK17

Among the Hp specimens of CK17, 4 of 6 (67%) had a score of 0, and 2 of 6 (33%) had a score of 1. About half of the MiD specimens (6/13, 46%) had a score of 0. About half of the MoD specimens (6/13, 46%) had a score of 2. Most SD specimens (10/11, 91%) and all OSCC specimens had scores of 2. The scores increased as the dysplasia grade increased. Scores differed significantly between SD and Hp, SD and MiD, OSCC and Hp, and OSCC and MiD (Fig. 4).

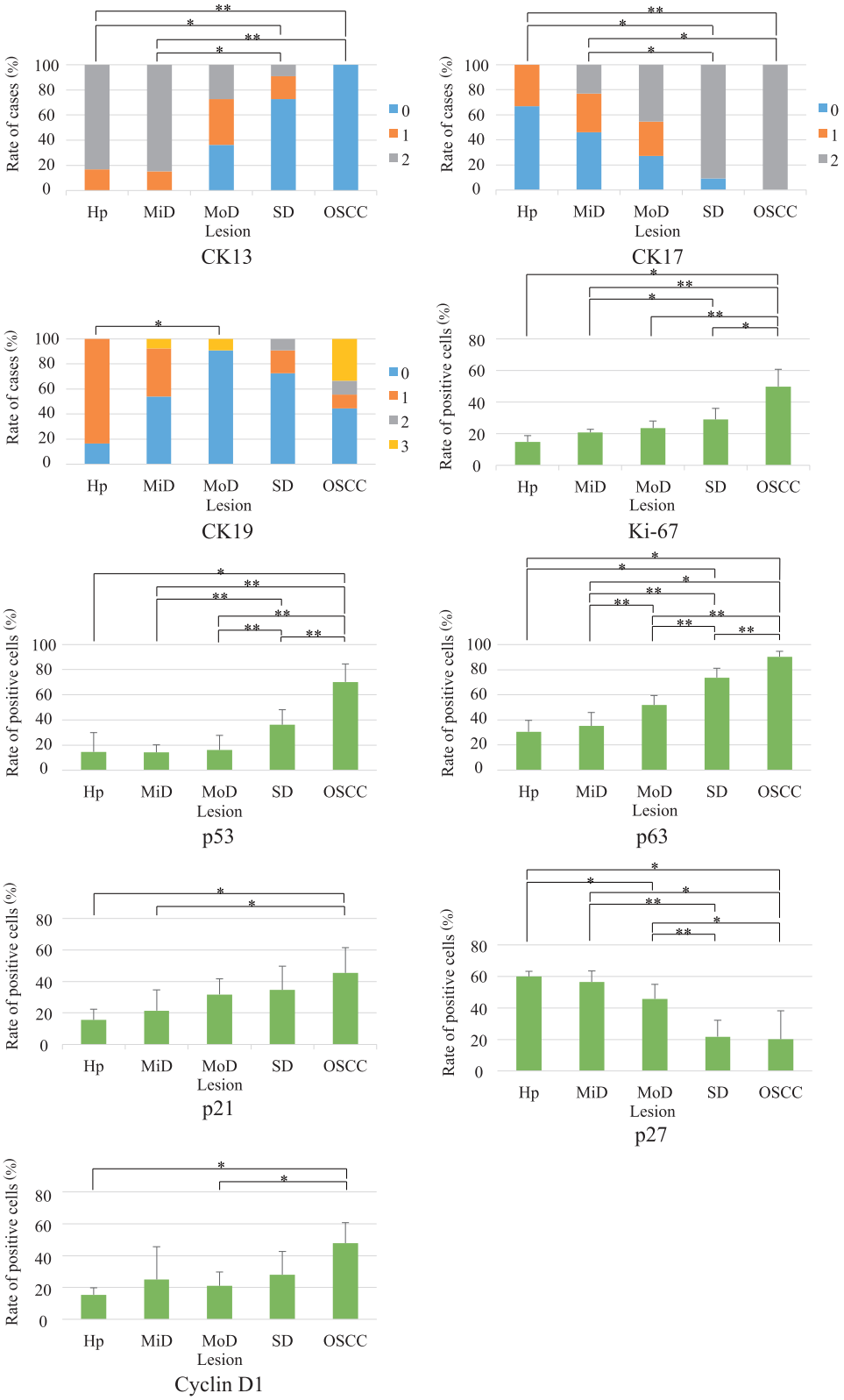

Fig. 4 Results of immunohistochemical stain.
Hp: Hyperplasia, MiD: Mild epithelial dysplasia, MoD: Moderate epithelial dysplasia, SD: Severe dysplasia, OSCC: Oral squamous cell carcinoma, $*p<0.05$, $**p<0.01$.

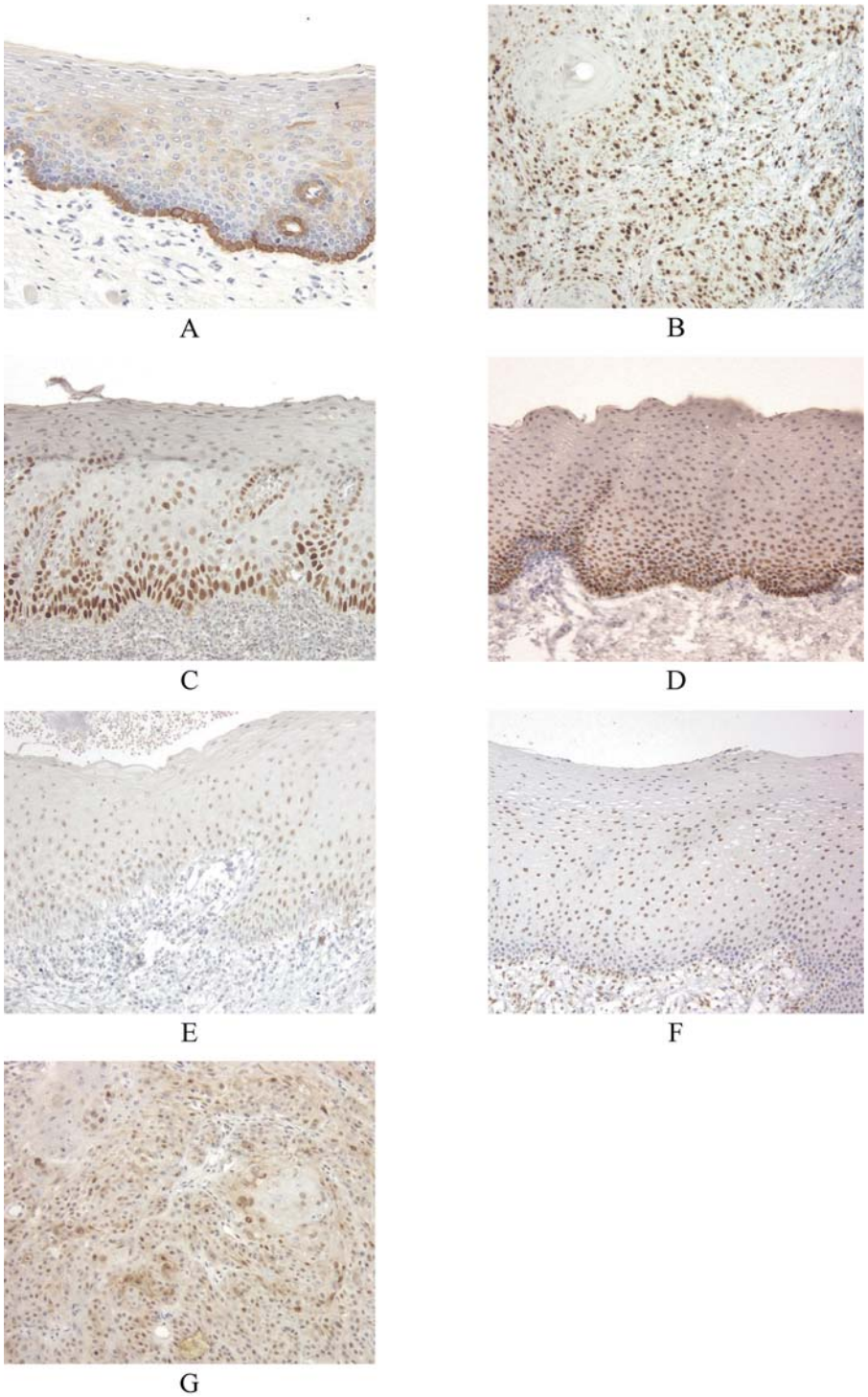

Fig. 5 Immunohistochemical staining (×100). (A) Expression of CK19 in Hp was observed in the cytoplasm of basal cells. (B) Overexpression of Ki-67 in OSCC was observed in the nuclei of about half of cancer cells. (C) Overexpression of p53 in SD was observed in the nuclei of cells in the stratum basale and the lower half of the stratum spinosum. (D) Overexpression of p63 in SD was observed in the nuclei of cells in the stratum basale and the lower half of the stratum spinosum. (E) Overexpression of p21 in MoD was observed in the nuclei of cells in the lower half of the stratum spinosum. (F) Expression of p27 in MiD was observed in the nuclei of cells in the stratum spinosum. (G) Overexpression of cyclin D1 in OSCC was observed in the nuclei of about half of the cancer cells.

CK19

Most Hp specimens of CK19 (5/6, 83%) had a score of 1 (Fig. 5 A), and expression was observed in the deepest third of the epithelium, particularly in the stratum basale. About half of the MiD specimens (7/13, 54%) and almost all the MoD (10/11, 91%) and SD (8/11, 73%) specimens had a score of 0. About half of the OSCC specimens (4/9, 44%) had a score of 0, but 3 of 9 (33%) had a score of 3. Scores were low in Hp to MoD, but tended to be higher in SD and OSCC. Scores differed significantly between MoD and Hp (Fig. 4).

Ki-67

Expression rates for Ki-67 were as follows: Hp 14.9 ±4.0%, MiD 20.8±2.0%, MoD 23.5±4.6%, SD 28.9±7.2%, and OSCC 49.8±10.8% (Fig. 5 B). The rates increased as the dysplasia grade increased. Rates differed significantly between OSCC and Hp, MiD, MoD and SD, as well as between SD and MiD (Fig. 4).

p53

Expression rates for p53 were as follows: Hp 14.6 ±15.2%, MiD 14.2±6.1%, MoD 16.0±11.6%, SD 36.0±12.2% (Fig. 5 C), and OSCC 70.2±14.2%. The expression rates did not differ between Hp, MiD and MoD, but were higher in SD and OSCC. Rates differed significantly between OSCC and Hp, MiD, MoD and SD, as well as between SD and MiD, and SD and MoD (Fig. 4).

p63

Expression rates for p63 were as follows: Hp 30.6 ±9.2%, MiD 35.2±10.7%, MoD 52.0±7.4%, SD 73.7±7.4% (Fig. 5 D), and OSCC 90.4±4.2%. The rates increased as the dysplasia grade increased. Rates differed significantly between OSCC and Hp, MiD, MoD and SD; between SD and Hp, MiD and MoD; and between MoD and MiD (Fig. 4).

p21

Expression rates for p21 were as follows: Hp 15.5 ±6.8%, MiD 21.45±13.1%, MoD 31.7±9.9% (Fig. 5 E), SD 34.5±15.2%, and OSCC 45.4±15.9%. The rates increased as the dysplasia grade increased. Rates differed significantly between OSCC and Hp, OSCC and MiD (Fig. 4).

p27

Expression rates for p27 were as follows: Hp 59.8 ±3.4%, MiD 56.5±6.9% (Fig. 5 F), MoD 45.7± 9.2%, SD 21.7±10.5%, and OSCC 20.1±18.1%. The rates decreased as the dysplasia grade increased. Rates differed significantly between OSCC and Hp, MiD and MoD; between SD and MiD, SD and MoD; and between MoD and Hp (Fig. 4).

Cyclin D1

Expression rates for cyclin D1 were as follows: Hp 15.5±4.3%, MiD 24.9±20.6%, MoD 21.0±8.7%, SD 28.1±14.4%, and OSCC 47.9±12.8% (Fig. 5 G). The rates increased as the dysplasia grade increased. Rates differed significantly between OSCC and Hp, OSCC and MoD (Fig. 4).

DISCUSSION

In this study, we used immunohistochemical methods to analyze the expression of cytoskeletal factors, cell proliferation markers, and cell cycle-related factors in Hp, OED and OSCC. CK13 is an acidic cytokeratin that is a component of the stratified squamous epithelium of mucous membranes.[6, 7] In normal oral mucosal epithelium, CK13 is expressed from the stratum spinosum to the stratum corneum, excluding the stratum basale in normally differentiated cells and keratinized cells.[6] Nobusawa *et al.* found that decreased expression of CK13 reflects the presence of atypical cells because changes in the cytoskeleton alter the cell shape in atypical cells.[6] Sun *et al.* also found that the absence of CK13 in OSCC suggests that cancer cells may have lost keratin differentiation potential.[7] In the present study, while CK13 expression was observed in all Hp and MiD specimens, it was lower in the MoD, SD and OSCC specimens. CK13 is also said to be a marker of prickle cells.[13] As the dysplasia grade increased, atypical cells proliferated from the parabasal layer into the stratum spinosum, thereby decreasing the number of differenti-

ated spinous cells. This likely explains the reduced expression of CK13.

CK17 is an acidic cytokeratin that is not expressed in normal oral mucosal epithelium, but is expressed in cells with high proliferative potential, such as in OED and OSCC.[14] In normal tissues, CK 17 is expressed in basal cells of the trachea, pharynx, and bronchi as well as in myoepithelial cells of the salivary and sweat glands. Its expression has also been observed in SCC of the oral cavity, esophagus, lungs, and uterine cervix.[6, 14] Nobusawa *et al.* found strong expression in MoD and SD, which suggests that CK17 expression is a specific marker for neoplastic changes.[6] In the present study as well, although CK17 expression was not detected in the majority of Hp specimens, its rate of expression increased as the dysplasia grade increased, and it was detected in all SCC specimens. This is likely because the number of CK17-expressing atypical cells with neoplastic changes increased as the dysplasia grade increased. Mikami *et al.* found that CK13 expression is accompanied by reduced CK17 expression and that CK13−/CK17 ＋is an important immunohistochemical feature of OSCC.[15] The present study also showed a negative correlation between CK13 and CK17, which may aid in differentiation between OED and OSCC.

CK19 is an acidic cytokeratin and epithelial stem cell marker that is expressed by basal cells in most secretory and squamous epithelia.[14, 16] It is also expressed in cervical cancer, and is used as a peripheral blood marker in breast, lung, stomach, and colon cancers.[14, 17] Findings regarding its expression have been inconsistent. One study showed that the expression rate increased as the dysplasia grade increased,[17] another that expression was low in MiD and MoD but increased sharply in SD,[18] and yet another showed that expression was low in MiD, MoD and SD but was present in all epithelial layers in carcinoma *in situ* (CIS).[16] The results of the present study were similar to those of Rajeswari *et al.*[18] No previous study has mentioned the cause of the increased expression in SD nor CIS, and we were also unable to observe it. Further research in a larger number of patients is warranted. CK19 expression differed significantly only between Hp and MoD in the present study, which indicates that CK 19 might aid in differentiation between Hp and MoD.

Ki-67 is a cell proliferation marker that is primarily expressed in the highly proliferative parabasal layer.[8] It is expressed in all cells in the G1, S, G2 and M phases, but not in quiescent cells in the G0 phase. Overexpression of Ki-67 indicates active cell proliferation.[7] Gonzalez-Moles *et al.* found that Ki-67 expression increases significantly with an increasing dysplasia grade.[19] In the present study as well, the expression rate increased as the dysplasia grade increased. In addition, the Ki-67 expression rate was significantly higher in OSCC than in SD Suggesting that it could be used to differentiate between SD and OSCC.

The tumor marker p53 is a protein encoded by tumor suppressor genes that is associated with apoptosis, the cell cycle, and DNA repair.[8] It inhibits cell proliferation by regulating the G1/S transition in the cell cycle. Fernando *et al.* found that p53 is an important indicator of mutation from normal epithelium to neoplastic epithelium.[20] Another study also showed that the p53 expression rate increased as the dysplasia grade increased.[21] In the present study, the expression rate was consistently low in Hp, MiD and MoD (at about 15%) and did not differ significantly among them, but was significantly higher in SD and OSCC. This indicates that the expression rate of p53 may aid in differentiation between MoD and SD as well as between SD and OSCC.

In clinical practice, p63 is used in the diagnosis of prostate cancer.[22] It is a member of the p53 family that activates p53 in DNA-damaged cells to arrest the cell cycle and inhibit cell proliferation.[9] It has also attracted attention as a keratinocyte stem cell marker involved in the regulation of epithelial cell proliferation and differentiation, and several studies of its role in OED have been published in recent years.[16] Chen *et al.*[23] and Matsubara *et al.*[5] found that the p63 expression rate increased with increasing dysplasia grade in OED. In the present study as well, expression rate correlated with in-

creasing dysplasia grades. The expression rate also increased significantly between MiD and OSCC. This indicates that the p63 expression rate may aid in differentiation of MiD from MoD, MoD from SD, and SD from OSCC.

When p53 expression increases in response to cellular DNA damage, it activates the cyclin-dependent kinase inhibitor (CKI) p21, which then binds to the complex formed by the cell cycle regulator cyclin D1 and cyclin-dependent kinase 4. This process causes cell cycle arrest in the G1 phase, which inhibits cell proliferation by inducing DNA repair and apoptosis.[24, 25] Baghaei *et al.* found that the expression rate of p21 is significantly higher in OSCC than in Hp.[26] However, findings have been inconsistent, as Queiroz *et al.* found no significant difference in the expression rate of p21 between normal oral mucosal tissue and OSCC, and concluded that p21 is not useful for predicting proliferative potential or malignancy.[25] In the present study, significant differences in p21 expression were observed between Hp and OSCC as well as between MiD and OSCC. The expression rate increases correlated with increasing dysplasia grades, suggesting that p21 expression may be correlated with the dysplasia grade in OED. In addition, the finding that the expression rate of p21 tended to increase as the expression rate of p53 increased indicates that p21 was activated by p53.

Similar to p21, p27 is a CKI that binds to the complex formed by cyclin E and cyclin-dependent kinase 2 (CDK 2) in response to growth inhibitors, and arrests the cell cycle at the G_1/S checkpoint.[25, 27] Decreased p27 expression has been reported in many cancers, including prostate, breast, and parathyroid cancer; it is also said to be more abundant in quiescent cells and less abundant in proliferating cells.[28] Queiroz *et al.* found that p27 is highly expressed in normal oral mucosal epithelium and is downregulated in OSCC, which suggests its involvement in abnormal proliferation of cancer cells.[26] In a study of MiD and MoD, Guan *et al.* found that p27 expression decreased as the dysplasia grade increased.[28] In the present study as well, the expression rate of p27 decreased with increasing dysplasia grade, suggesting that a decrease in growth inhibitory factors as dysplasia increased led to the decrease in the p27 expression rate. In addition, the expression rate decreased significantly between MoD and SD. This suggests that it could be used to differentiate between MoD and SD.

Cyclin D1, a cell cycle regulator that is synthesized by stimulation of cell proliferation, promotes progression of the cell cycle from the G1 phase to the S phase.[22, 29] It also has many other functions, including regulation of mitochondrial metabolism, cell differentiation, and DNA repair.[30] In esophageal squamous cell carcinoma and bladder cancer, cyclin D1 expression is significantly correlated with prognosis.[29] Ramasubramanian *et al.* found that cyclin D1 expression increased significantly with increasing dysplasia grade.[31] In the present study, significant differences were observed between Hp and OSCC as well as between MoD and OSCC. The expression rate increased as the dysplasia grade increased, indicating that dysplasia grade may be correlated with cyclin D1 expression in OED. This increased rate of expression was likely caused by increased activation of cyclin D through increased stimulation of cell proliferation as dysplasia grades increased.

These results suggest that not only CK13, CK17, Ki-67 and p53, which have been previously identified as potentially useful indicators of dysplasia, but also p63, p21, p27 and cyclin D1 may be linked to dysplasia. The results also suggest that calculation of Ki-67, p53, p63 and p27 expression rates might be used for OED grading and auxiliary diagnosis of OSCC.

REFERENCES

1. Pindborg JJ, Reichart PA, Smith CJ, Waal I. Histological typing of cancer and precancer of the oral mucosa. 2nd ed. Heidelberg: Springer, 1997: 21-27.
2. Slootweg PJ, Eveson JW. Tumours of the oral cavity and oropharynx. In: Barnes L, Eveson JW, Reichart P, Sidransky D. Pathology and genetics of head and neck tumours. WHO classification of tumours. 3ed ed. Lyon: IARC Press, 2005: 163-208.
3. Speight PM, Khurram SA, Kujan O. Oral potentially malignant disorders: risk of progression to malignancy. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2018; **125**: 612-627.

4. Reibel J, Gale N, Hille J, eds Oral potentially malignant disorders and oral epithelial dysplasia. In: El-Naggar AK, Chan JKC, Grandis JR, eds. WHO classification of tumours of the head and neck. 4th ed. Lyon: IARC Press, 2017: 112-115.
5. Matsubara R, Kawano S, Kiyosue T, Goto Y, Hirano M, Jinno T, Toyoshima T, Kitamura R, Oobu K, Nakamura S. Increased ΔNp63 expression is predictive of malignant transformation in oral epithelial dysplasia and poor prognosis in oral squamous cell carcinoma. *Int J Oncol* 2011; **39**: 1391-1399.
6. Nobusawa A, Sano T, Negishi A, Yokoo S, Oyama T. Immunohistochemical staining patterns of cytokeratins 13, 14, and 17 in oral epithelial dysplasia including orthokeratotic dysplasia. *Pathol Int* 2014; **64**: 20-27.
7. Sun Y, Kuyama K, Burkhardt A, Yamamoto H. An immunohistochemical study of oral carcinoma cuniculatum. *Int J Oral-Med Sci* 2012; **10**: 336-343.
8. Matsuhira A, Noguchi S, Sato K, Tanaka Y, Yamamoto G, Mishima K, Katakura A. Cytokeratin 13, Cytokeratin 17, Ki-67 and p53 expression in upper layers of epithelial dysplasia surrounding tongue squamous cell carcinoma. *Bull Tokyo Dent Col* 2015; **565**: 223-231.
9. Murray-Zmijewski F, Lane DP, Bourdon JC. p53/p63/p 73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. *Cell Death Differ* 2006; **13**: 962-972.
10. Zhang M, Li J, Wang L, Tian Z, Zhang P, Xu Q, Zhang C, Wei F, Chen W. Prognostic significance of p21, p27 and survivin protein expression in patients with oral squamous cell carcinoma. *Oncol Lett* 2013; **6**: 381-386.
11. Yagyuu T, Obayashi C, Ueyama Y, Takano M, Tanaka Y, Kawaguchi M, Takeda M, Kasai T, Kirita T. Multivariate analyses of Ki-67, cytokeratin 13 and cytokeratin 17 in diagnosis and prognosis of oral precancerous lesions. *J Oral Pathol Med* 2015; **44**: 523-531.
12. Kale AD, Mane DR, Babji D, Gupta K. Establishment of field change by expression of cytokeratins 8/18, 19, and MMP-9 in an apparently normal oral mucosa adjacent to squamous cell carcinoma: a immunohistochemical study. *J Oral Maxillofac Pathol* 2012; **16**: 10-15.
13. Kobayashi T, Maruyama S, Cheng J, Ida-Yonemochi H, Yagi M, Takagi R, Saku T. Histopathological varieties of oral carcinoma in situ: diagnosis aided by immunohistochemistry dealing with the second basal cell layer as the proliferating center of oral mucosal epithelia. *Pathol Int* 2010; **60**: 156-166.
14. Tsuji K, Wato M, Hayashi T, Yasuda N, Matsushita T, Ito T, Gamoh S, Yoshida H, Tanaka A, Morita S. The expression of cytokeratin in keratocystic odontogenic tumor, orthokeratinized odontogenic cyst, dentigerous cyst, radicular cyst and dermoid cyst. *Med Mol Morphol* 2014; **47**: 156-161.
15. Mikami T, Cheng J, Maruyama S, Kobayashi T, Funayama A, Yamazaki M, Adeola HA, Wu L, Shingaki S, Saito C, Saku T. Emergence of keratin 17 vs. loss of keratin 13: their reciprocal immunohistochemical profiles in oral carcinoma in situ. *Oral Oncol* 2011; **47**: 497-503.
16. Takeda T, Sugihara K, Hirayama Y, Hirano M, Tanuma JI, Semba I. Immunohistological evaluation of Ki-67, p63, CK19 and p53 expression in oral epithelial dysplasias. *J Oral Pathol Med* 2006; **35**: 369-375.
17. Yoshida K, Sato K, Tonogi M, Tanaka Y, Yamane GY, Katakura A. Expression of cytokeratin 14 and 19 in process of oral carcinogenesis. *Bull Tokyo Dent Coll* 2015; **56**: 105-111.
18. Rajeswari P, Janardhanan M, Suresh R, Savithri V, Aravind T, Raveendran GC. Expression of CK 19 as a biomarker in early detection of oral squamous cell carcinoma. *J Oral Maxillofac Pathol* 2020; **24**: 523-529.
19. 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; **22**: 658-661.
20. Sousa FACG, Paradella TC, Carvalho YR, Rosa LEB. Immunohistochemical expression of PCNA, p53, bax and bcl-2 in oral lichen planus and epithelial dysplasia. *J Oral Sci* 2009; **51**: 117-121.
21. Tsuji K, Wato M, Morita S. Detection of human papillomavirus and abnormal expression of tumor suppressor genes in epithelial dysplasias and squamous cell carcinomas of the oral mucosa. *J Osaka Dent Univ* 2009; **43**: 63-69.
22. Kövesi G, Szende B. Prognostic value of cyclin D1, p27, and p63 in oral leukoplakia. *J Oral Pathol Med* 2006; **35**: 274-277.
23. Chen YK, Hsue SS, Lin LM. Expression of p63 protein and mRNA in oral epithelial dysplasia. *J Oral Pathol Med* 2005; **34**: 232-239.
24. Okazaki Y, Tanaka Y, Tonogi M, Yamane G. Investigation of environmental factors for diagnosing malignant potential in oral epithelial dysplasia. *Oral Oncol* 2002; **38**: 562-573.
25. Queiroz AB, Focchi G, Dobo C, Gomes TS, Ribeiro DA, Oshima CTF. Expression of p27, p21(WAF/Cip1), and p 16 (INK4a) in normal oral epithelium, oral squamous papilloma, and oral squamous cell carcinoma. *Anticancer Res* 2010; **30**: 2799-2803.
26. Baghaei F, Shojaei S, Afshar-Moghaddam N, Zargaran M, Rastin V, Nasr M, Moghimbeigi A. Study of P 21 expression in oral lichen planus and oral squamous cell carcinoma by immunohistochemical technique. *J Dent (Shiraz)* 2015; **16**: 156-161.
27. Wato M, Watanabe Y, Hida T, Tanaka M, Tanaka A. Immunohistochemical expression of P-cadherin and cyclin D1 in oral epithelial dysplasia. *J Osaka Dent Univ* 2005; **39**: 97-101.
28. Guan G, Bakr MM, Firth N, Love RN. Expression of cyclin D1 correlates with p27 KIP1 and regulates the degree of oral dysplasia and squamous cell carcinoma differentiation. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2018; **126**: 174-183.
29. Vallonthaiel AG, Singh MK, Dinda AK, Kakkar A, Thakar A, Das SN. Prognostic significance of cytoplasmic p27 in oral squamous cell carcinoma. *J Oral Pathol Med* 2016; **45**: 475-480.
30. Ramos-García P, González-Moles MÁ, González-Ruiz L, Ayén Á, Ruiz-Ávila I, Bravo M, Gil-Montoya JA. Clinicopathological significance of tumor cyclin D1 expression in oral cancer. *Arch Oral Biol* 2019; **99**: 177-182.
31. Ramasubramanian A, Ramani P, Sherlin HJ, Premkumar P, Natesan A, Thiruvengadam C. Immunohistochemical evaluation of oral epithelial dysplasia using cyclin-D1, p27 and p63 expression as predictors of malignant transformation. *J Nat Sci Biol Med* 2013; **4**: 349-358.