Feasibility of HPV16, HPV18, and p16 Expression as Biomarkers for Distinguishing Normal Oral Epithelium From Oral Epithelial Dysplasia and Oral Intraepithelial Neoplasia

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This preliminary study evaluated the expression of human papilloma virus（HPV）16, HPV18, and p16 as possible biomarkers to distinguish normal oral epithelium（NOE）from oral epithelial dysplasia（OED）and oral intraepithelial neoplasia（OIN）. Subjects comprised 150 cases including 41 with OED, 30 with OIN, and 67 with oral squamous cell carcinoma（OSCC）. NOE was also taken from 12 healthy participants. Staining indices of HPV16, HPV18, and p16 were immunohistochemically examined using paraffin-embedded specimens. A statistically significant difference was seen in the expression of HPV18 and p16 in OED/OIN compared with NOE and OSCC. Furthermore, regression tree analysis of independent variables, including p16 and HPV18 expression and the participants' age, was performed to distinguish NOE from OED/OIN. In conclusion, this preliminary study demonstrated that p16 and HPV18 expression and participants' age（60 years）can be used to distinguish NOE from OED/OIN.

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INTRODUCTION

Many oral squamous cell carcinoma（OSCC）lesions develop from potentially malignant disorders（PMDs）[1]. The term "PMD" was defined by the World Health Organization（WHO）as the risk of malignancy being present in a lesion or condition either during the time of initial diagnosis or at a future date. The WHO also classified PMDs into two subgroups: a）precancerous lesions, benign lesions with morphologically altered tissue, which have a greater than normal risk of malignant transformation; and b）precancerous conditions, which are diseases or patients' habits that do not necessarily alter the clinical appearance of local tissue, but are associated with a greater than normal risk of precancerous lesions or cancer development in that tissue [2, 3].

Numerous criteria exist for the diagnosis of oral epithelial dysplasia（OED）, and there is not always a clear-cut distinction of what represents mild dysplasia— consisting of only focal atypia, moderate dysplasia, and severe dysplasia —which may present as carcinoma *in situ*（CIS）[4]. According to the general rules for clinical and pathological studies on oral cancer [5], mild and moderate dysplasia are defined as OED, while severe dysplasia is defined

as oral intraepithelial neoplasia (OIN). As for CIS and OIN, however, a definitive distinction cannot always be drawn between mild and moderate dysplasias and CIS/OIN. Dysplasia that encompasses an area greater than what could be considered "focal", when extremely severe in degree or when exhibiting a "top to bottom" change, particularly with respect to basilar hyperplasia, must be diagnosed as CIS, provided of course that it has not progressed to the point of true invasion of the connective tissue [4].

Although OED and CIS are defined by the presence of dysplastic cells in the epithelium, accurate clinical and histopathological diagnoses have been controversial [6]. The most important research focus must be on the development of molecular or histological markers that allow a stricter differentiation to be made between the diagnosis of normal tissue and OED/OIN, because histopathological diagnosis of OSCC has been established in routine paraffin-embedded specimens [3-5]. Recently, cytokeratin 13 and 17 are reportedly suitable for such a distinction between non-neoplastic tissue and a dysplastic or neoplastic (malignant) oral lesion; however, a clear distinction between OED/OIN and CIS is currently unavailable [7]. Furthermore, nucleus accumbens-associated protein 1 (NAC1) was reported to be a potential biomarker for distinguishing OED from CIS/OSCC, using a cut-off value of 50% with the NAC1 labeling index (LI) [8]. However, Ohira *et al.* [9] reported that NAC1 was not a definitive biomarker for distinguishing oral malignancies from non-malignancies.

The natural history of the human papilloma virus (HPV) has been characterized extensively in the uterine cervix [10-14], whereas much less data are available on the difference phases of HPV infection and oncogenesis in the oral, head, and neck regions [14]. The identification of HPV in oropharyngeal carcinoma has prognostic significance, with longer survival and a higher rate of response to therapy in cases positive for HPV [14-16]. In the field of OSCC, HPV infection was found to be present in 26.0% of cases, and high risk (HR)-HPV-positive cases have similar clinical characteristics as HR-HPV-negative cases, but had a significantly worse prognosis [17-20]. However the detail of HPV identification and the roles of these infections in

terms of the prognosis and carcinogenesis still remain unclear especially in OSCC [19-21].

Also, detection of HPV DNA alone is not stringent proof of HPV-induced carcinogenesis [19-21]. Only transcriptionally active HPV DNA is biologically and clinically relevant for carcinogenesis [22]. HPV integration in the infected cell results in the deletion of the viral E2 gene promoter causing transcription of E6 and E7. Binding of the E7 oncoprotein to the Rb protein leads to Rb protein degradation and presumably to the compensatory overexpression of both cytoplasmic and nuclear p16 protein in HPV infected cells; hence, p16 is used widely as a surrogate biomarker for HPV infection [22, 23].

In this preliminary study, we thus evaluated the association between the expression of HPV16, HPV18, and p16 and various lesions derived from the oral epithelium, immunohistochemically, testing the hypothesis that the expression of HPV16, HPV18, and p16 could be feasible biomarkers to distinguish PMDs in the oral cavity. Furthermore, we evaluated the expressions of HPV16, HPV18, and p16 in OSCCs as an index of carcinogenesis to compare these with those found in PMDs.

METHODS

Participants and samples

All participants with clinically diagnosed OIN, OED, and OSCC underwent a preoperative biopsy at the Department of Oral and Maxillofacial Surgery, Shimane University Hospital, Japan from April 1980 to January 2014. Normal oral epithelium (NOE) was taken from healthy volunteers with no symptoms or medical history of any oral mucous disorder who provided consent for their samples to be used as standard controls.

Biopsy specimens taken from the margin of the oral mucosal lesions located on the tongue, gingiva, buccal mucosa, lip, and palate were fixed with 10% neutral buffered formalin for 24 h, processed as routine paraffin-embedded sections, stained with hematoxylin and eosin, and diagnosed by pathologists of the Department of Pathology. All cases were also diagnosed according to the WHO classification [5]. NOE samples taken from normal oral mucosa were

also processed as paraffin-embedded sections.

All participants provided informed consent to participate, following approval of the study protocol (approval no. 1676; November 12, 2014) by the ethics committee of Shimane University Hospital.

HPV16, HPV18, and p16 immunohistochemistry

HPV16 and HPV18 expression was determined immunohistochemically using an anti-HPV16 E1+E4 antibody (Abcam, Cambridge, UK; diluted at 1:100) and an anti-HPV18 E6 antibody (Abcam, Cambridge, UK; diluted at 1:500). As a surrogate marker of HPV presence, p16^{INK4a} (VENTANA, AZ, USA, ready to use) was also used.

After deparaffinization and rehydration, antigen retrieval was performed by autoclaving at 120°C for 20 min in citrate buffer solution (pH6.0). After autoclaving, the slides were allowed to cool to room temperature and the sections were incubated for 30 min in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. The sections were incubated sequentially with 10% rabbit blocking serum to block nonspecific reactions. Sequentially, the sections were incubated with each primary antibody overnight at 4°C. Immunoperoxidase staining was performed using an EnVision™+ Kit (Dako, CA, USA). Counterstaining was done with Mayer's hematoxylin (MUTO PURE CHEMICALS Co., Ltd., Tokyo, Japan). Negative controls for immunohistochemistry were incubated with phosphate-buffered saline instead of the primary antibodies and showed no positive reaction.

HPV16, HPV18, and p16 SIs

All sections were examined using a standard light microscope with a ×40 objective lens. An attached digital camera was used to capture images and estimate the number of HPV16-, HPV18-, and p16-positive cells (at least 100 cells/field) [24]. In OSCC, three high-power (×40 objective) fields in full sections were selected to represent the spectrum of staining seen on the initial overview of the whole section. The SI (stained cells / total cells counted ×100 [%]) was expressed as the percentage of positive cells among the total number of cells in the area scored [24]. In NOE, OED, and OIN, at least three high-power (×40 objective) fields were selected (which were always selected from whole epithelial layers to avoid errors for scoring a partial dense area), and the percentage of positive epithelial cells among the total number of epithelial cells was scored.

Statistical analysis

The participants were stratified according to a pathological classification with four levels: NOE, OED, OIN, and OSCC. In addition to analysis by all participants, subgroup analysis in participants with NOE, OED, and OIN was performed. Continuous and categorical variables were summarized as the mean ± standard deviation (SD) and frequency (percentage), respectively. To explore an increasing or decreasing trend of variable distribution in the order of NOE, OED, OIN, and OSCC (NOE, OED and OIN), the Jonckheere-Terpstra test and Cochran-Armitage test were used for continuous and categorical variables, respectively. In addition, to construct a clinically useful decision tool for the diagnosis of NOE or OED/OIN, regression tree analysis was performed using a conditional inference method with a splitting criterion of $p < 0.05$. Age, sex, positive/negative, and SIs for HPV16, HPV18, and p16 were used as candidate predictors in the regression tree analysis. A p-value < 0.05 was considered significant. All statistical analyses were performed using SAS™ version 9.3 (Cary, NC, USA) and R version 3.2.2 (R Foundation, Vienna, Austria) with "ctree" in "party" library.

RESULTS

Participants' backgrounds

The participants comprised 150 cases (age range: 29-91 years), 12 with NOE (48-76 years), 41 with OED (39-86 years), 30 with OIN (29-91 years), and 67 with OSCC (30-90 years). Sex and age distributions are shown in Table 1. The participants' age increased in the order of NOE (mean ± SD: 60.7 ± 10.1 years), OED (65.2 ± 12.6 years), and OIN (71.7 ± 10.8 years; trend test: $p = 0.002$, Table 1). However, there was no significant trend when the OSCC group (63.8 ± 15.4 years) was included in the analysis ($p = 0.823$). Sex distribution of the lesions had no significant trend in the

Table 1. Participants' information

Category		NOE	OED	OIN	*p*-value of trend test	OSCC	*p*-value of trend test
		n = 12	n = 41	n = 30		n = 67	
Sex	Men	5	20	12	0.755	47	0.010
	Women	7	21	18		20	
Age	Mean ± SD	60.7 ± 10.1	65.2 ± 12.6	71.7 ± 10.8	0.002	63.8 ± 15.4	0.823
WHO classification	Normal	12	0	0	NE	0	NE
	Dysplasia mild	0	31	0		0	
	Dysplasia moderate	0	10	8		0	
	Dysplasia severe	0	0	12		0	
	CIS	0	0	10		0	
	SCC	0	0	0		67	

Abbreviations:

NOE: normal oral epithelium, OED: oral epithelial dysplasia, OIN: oral intraepithelial neoplasia, OSCC: oral squamous cell carcinoma, CIS: carcinoma *in situ*, SCC: squamous cell carcinoma, WHO: World Health Organization, NE: not evaluated
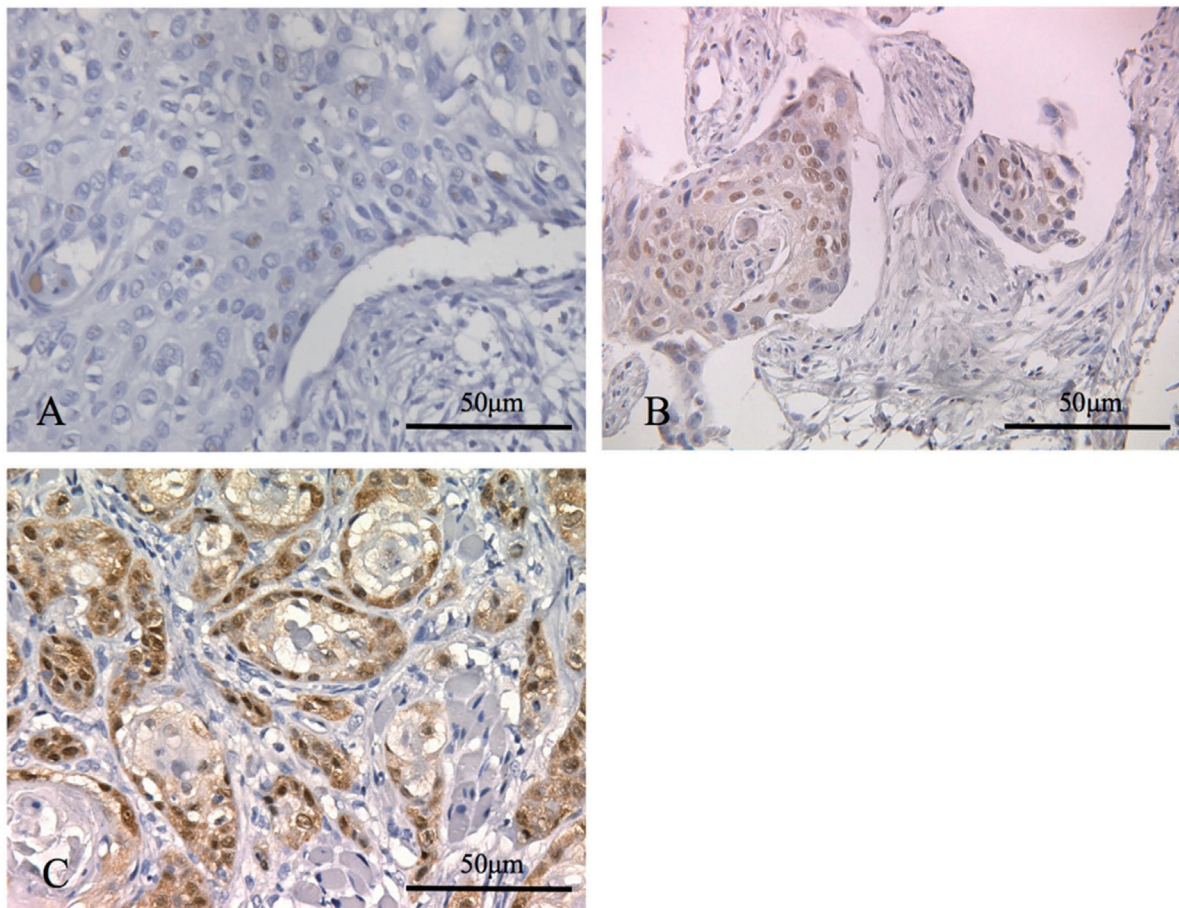

Fig. 1. Expression of HPV16, HPV18, and p16 in oral squamous cell carcinoma（OSCC）. Abbreviations are those used in Table 1. In OSCC, staining for HPV16-positive cells was distributed in the nucleus of dysplastic or tumor cells（A, ×40）. Staining for HPV18-positive cells was distributed predominantly in the nucleus of dysplastic or tumor cells（B, ×40）, and staining for p16-positive cells was distributed predominantly in the nucleus and/or cytoplasm of dysplastic or tumor cells（C, ×40）

Table 2. Expression of HPV16, HPV18, and p16 in participants

Category		NOE	OED	OIN	p-value of trend test	OSCC	p-value of trend test
		n = 12	n = 41	n = 30		n = 67	
HPV16	Expression (%)	0 (0.0%)	3 (7.3%)	0 (0.0%)	0.572	3 (4.5%)	0.974
	SIs (mean ± SD)	0.0 ± 0.0	0.4 ± 1.7	0.0 ± 0.0	0.815	0.2 ± 1.2	0.989
HPV18	Expression (%)	6 (50.0%)	26 (63.4%)	22 (73.3%)	0.145	46 (68.9%)	0.257
	SIs (mean ± SD)	30.2 ± 32.7	56.1 ± 36.0	47.1 ± 41.3	0.043	29.9 ± 31.4	0.271
p16	Expression (%)	0 (0.0%)	29 (70.7%)	18 (60.0%)	<0.001	21 (31.3%)	0.152
	SIs (mean ± SD)	0.0 ± 0.0	22.0 ± 22.1	22.5 ± 26.8	0.027	8.7 ± 20.5	0.053

Abbreviations:

NOE: normal oral epithelium, OED: oral epithelial dysplasia, OIN: oral intraepithelial neoplasia, OSCC: oral squamous cell carcinoma,

SIs: staining indices

order of NOE, OED, and OIN (p = 0.755), but had a significant trend when the OSCC group was included (p = 0.010).

According to the general rules for clinical and pathological studies on oral cancer, cases with mild dysplasia were classified into OED (31 cases), and cases with severe dysplasia and CIS were classified into OIN (severe dysplasia: 12 cases; CIS: 10 cases). In the cases with moderate dysplasia, 10 were classified into OED and 8 were classified into OIN (Table 1).

Immunohistochemical findings for HPV16, HPV18, and p16

Staining in HPV16-positive cells in OED and OSCC was distributed in the nucleus of dysplastic or tumor cells (Fig. 1A). In addition, in NOE, staining in HPV18-positive cells was distributed predominantly in the nucleus of parabasal cells and prickle cells. Staining in HPV18-positive cells in OED, OIN, and OSCC was distributed predominantly in the nucleus of dysplastic or tumor cells (Fig. 1B). Staining in p16-positive cells in OED, OIN, and OSCC was distributed predominantly in the nucleus and/or cytoplasm of dysplastic or tumor cells (Fig. 1C).

Expression and SIs
HPV16

Although no HPV16 expression was seen in NOE and OIN, the proportions of HPV16-positive cases were 7.3% (3/41 cases) in OED and 4.5% (3/67 cases) in OSCC (Table 2). The SIs (%) of HPV16 were 0.4 ± 1.7% (mean ± SD) and 0.2 ± 1.2% in OED and OSCC, respectively (Table 2). There was no trend for HPV16 status and SIs in all cases (p = 0.974 and p = 0.989, respectively) or in the subgroup including NOE, OED, and OIN (p = 0.572 and p = 0.815, respectively; Table 2).

HPV18

The proportions of HPV18-positive cases were 50.0% (6/12 cases) in NOE, 63.4% (26/41 cases) in OED, 73.3% (22/30 cases) in OIN, and 68.9% (46/67 cases) in OSCC (Table 2). The SIs of HPV18 were 30.2 ± 32.7% (mean ± SD), 56.1 ± 36.0%, 47.1 ± 41.3%, and 29.9 ± 31.4% in NOE, OED, OIN, and OSCC, respectively (Table 2). There was no trend for HPV18 status in all cases (p = 0.145) or in the subgroup including NOE, OED, and OIN (p = 0.257, Table 2). Although no trend for HPV18 SIs was provided in all cases (p = 0.271), an increasing trend was observed in the subgroup including NOE, OED, and OIN (p = 0.043, Table 2 and Fig. 2).

p16

In NOE, no p16-positive cells were found. The proportions of p16-positive cases were 70.7% (29/41 cases) in OED, 60.0% (18/30 cases) in OIN, and 31.3% (21/46 cases) in OSCC (Table 2). The SIs of p16 were 22.0 ± 22.1% (mean ± SD), 22.5 ±
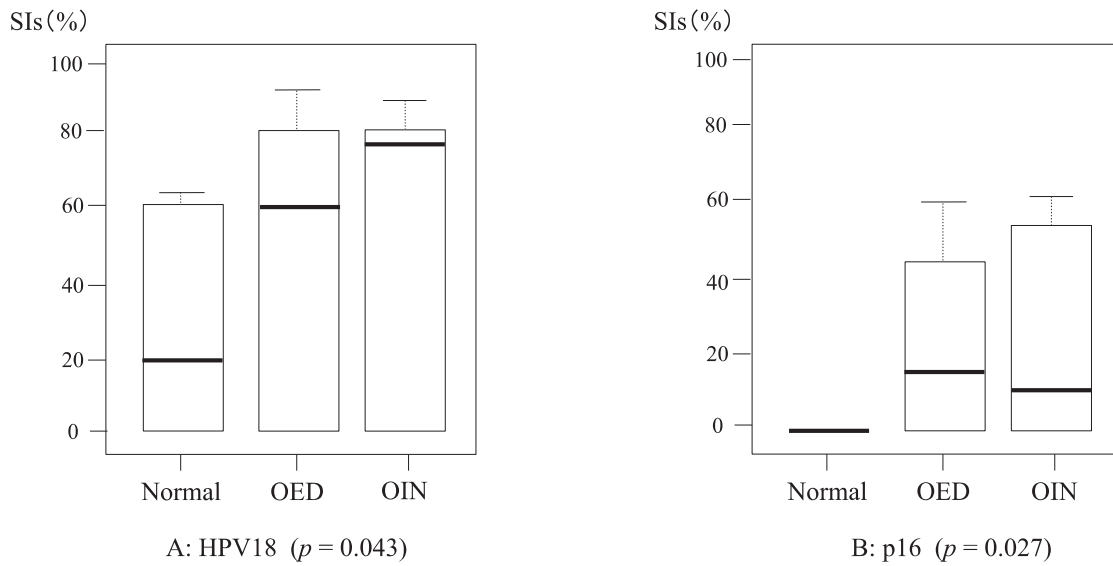
Fig. 2. Boxplot stratified by pathological classification of staining indices of HPV18 and p16
SIs: staining indices; other abbreviations are those used in Table 1. SIs of A: HPV18 and B: p16. NOE showed a lower value than OED/OIN for expression of HPV18 and p16 ($p = 0.043$, and 0.027, respectively, Jonckheere-Terpstra test)

26.8%, and 8.7 ± 20.5% in OED, OIN, and OSCC, respectively (Table 2). Although there was no trend for p16 status and SIs in all cases ($p = 0.152$ and $p = 0.053$, respectively), significant trends were found in the subgroup including NOE, OED, and OIN ($p < 0.001$ and $p = 0.027$, respectively; Table 2 and Fig. 2).

Discrimination between NOE and OED/OIN

Age, the SIs of HPV18, and the status and SIs of p16 had increasing trends in the order of NOE, OED and OIN (Tables 1, 2 and Fig. 3); therefore, age and the variables related to p16 and HPV18 were used further as covariates in regression tree analysis for predicting OED/OIN against NOE. As a result, 4 stratified groups (100.0%, 93.8%, 60.0%, and 30.0% of OED/OIN) by age, p16 status, and HPV18 status were provided. The p16-positive group included all OED/OIN cases; the p16-negative, age > 60 years, and HPV18-positive group included 93.8% of OED/OIN cases; the p16-negative, age > 60 years, and HPV18-negative group included 60.0% of OED/OIN cases; and the p16-negative and age ≤ 60 years group included 30.0% of OED/OIN cases (Fig. 3).

DISCUSSION

HVP detection strategies vary not just in design, but in their detection targets. These targets have included HPV DNA, HPV RNA, viral oncoproteins, cellular proteins and HPV-specific serum antibodies [25]. Recently, multimodality methods of HPV detection using p16 immunohistochemistry and HPV DNA detection such as DNA in situ hybridization and PCR based technique is recommended [25].

There are a few limitations of this study that merit consideration. This study was retrospective, some of the materials were preserved for a long period of time, and the expression of HPV16, HPV18, and p16 was evaluated in formalin-fixed and paraffin-embedded tissue. Formalin fixation now appears to have a less damaging effect on the quality of the proteomic analysis obtained from formalin-fixed and paraffin-embedded tissue than had been assumed [26]. The time in storage of formalin-fixed and paraffin-embedded tissue was initially also thought to be a potential variable factor in protein extraction yield [27, 28]. Several studies have reported that storage of unstained slides long term may lead to false-negative immunostaining for antigens [29]. Recent studies have however suggested that there is no significant difference in the number of proteins iden-

tified from fixed tissues even with prolonged storage [30, 31]. And the antigen retrieval (AR) could obtain the highly accurate results in this study. The AR technique is a non-enzymatic antigen unmasking method that is utilized prior to immunohistochemical staining of formalin-fixed, paraffin-, celloidin- or plastic-embedded tissue sections [25, 32]. In this study, a non-enzymatic pretreatment based on high-temperature heating was applied.

Although our study did not contain cases with oropharyngeal carcinoma, limited to OSCC lesions, HPV16 was detected in 4.5% of OSCC cases and HPV18 was observed in 68.8% of OSCC cases in formalin-fixed and paraffin-embedded sections of OSCC. Krüger M et al. described in his up-to-date review of 136 literatures of HPV infection in OSCC that the prevalence of OSCC related HPV infection including low risk (LR)-HPV to HR-HPV varies from 0% to 100% and possible reasons for the different prevalence rates could be the detection method used for HPV infection, ethnos, geographical site and collective size [33].

In this study, HPV16 was also detected in OED (7.3%), while HPV18 was observed in OED (63.4%) and OIN (73.3%) in the oral cavity, including tongue, gingiva, buccal mucosa, lip, and palate samples. HPV causes a range of epithelial lesions from common warts to neoplasia and cancer [19]. The main criticism of studies that concentrate on the possible premalignant characteristics of OED and OIN is the lack of sufficient clinical and histological data to support the initial diagnosis in patients who eventually develop OSCC [5]. Agrawal et al. [34] reported that the SI of HPV16 was 10% in OED, and 60% of HPV16-positive OED were positive for p16^{INK4a}, suggesting a role for HPV16 in the pathogenesis of OED. As for the association of the expression of HPV and PMDs, although LR-HPV-positive lesions result in papillary and atypical proliferation [35], HR-HPV is not always associated with atypical oral lesions [36] and/or OSCC [37].

Conversely, our study showed the expression of HPV18 in NOE (50.0%). According to the detection of HPV by sensitive PCR-based sequencing analysis, HPV18 was detected in 86.7% of adult NOE cases, suggesting that subclinical and latent
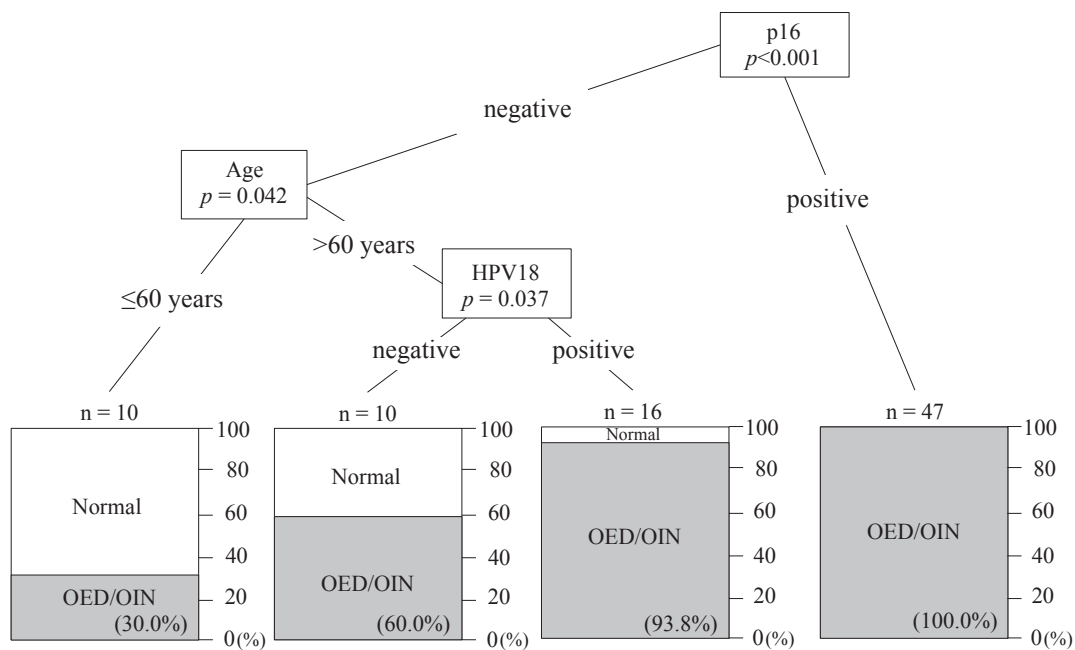

Fig. 3. Result of regression tree analysis for discrimination between NOE and OED/OIN
Abbreviations are those used in Table 1. The independent variables were p16, HPV16, HPV18, and age. To predict OED/OIN, the primary variable for the hierarchical tree was p16 status, the secondary variable was age (60 years), and the tertiary variable was HPV18 status

HPV infection of NOE is common and HPV18 would be the predominant genotype [38]. Furthermore, a low level HPV infection would also suggest that the oral cavity could be a reservoir of HPV, and this infection in combination with other factors is associated with the later development of PMDs, including OED/OIN [34].

To the best of our knowledge, these preliminary results obtained here might shed light on the first attempt in terms of HPV infection to distinguish NOE from OED/OIN in consideration of carcinogenesis of OSCC [8, 9].

In this study, p16 was used as a surrogate marker for the expression of HPV, as p16 is a surrogate marker for HPV infection in the cervix [39]. It has been observed that HPV16 disrupts the regulation of p16^{INK4a} suppressor protein and its overexpression can be used as surrogate marker for the detection of HPV16 in association with oral SCC [34]. Similarly, the value of immunostaining for p16^{INK4a} was shown for the identification of oral dysplastic lesions [34]. Combined p16 and HPV testing is also reported to be useful for the prediction of head and neck cancer survival [40, 41] as well in a cohort study on oropharyngeal tumors [42].

There have been no reports so far comparing the prevalence of HPV infection in PMDs with that in OSCC by the same detection methods. In our study, we thus compared the expressions of HPV16, HPV18, and p16 in OSCCs with those in PMDs, obtained from the identical immunohistochemistry study as described here. There were no significant trends for HPV16, HPV18, and p16 status and SIs in all study groups. These results might suggest that HPV infections would be related to the initial phase of HPV-induced carcinogenesis.

In this study, a statistical trend test of each variable was first performed, then followed by the manifest confirmation of statistically significant values, which revealed putative feasible candidates for biomarkers or factors, namely, age, p16, and HPV18, to distinguish NOE from OED/OIN. Further regression tree analysis considering the participants' age revealed that p16 and HPV18 expression and the participants' age (60 years) are feasible biomarkers to distinguish NOE and OED/OIN, as summarized in Table 2 and Fig. 3. With respect to the biomark-

ers and factors of aging in this study, their role in the carcinogenesis of oral, head, and neck squamous cell carcinoma remains unclear. In cancer of the cervix uteri, HPV infection, such as HR-HPV, together with the senescence programs mediated by HPV infections, repeatedly cause genetic changes in cervical cells that play important roles in carcinogenesis from normal cervical epithelium or precancerous cervical lesions, such as cervical intraepithelial neoplasia, to SCC transformation, if the virus is not cleared [43]. Further, it has been proposed recently that epigenetic variations and environmental factors, including chronic inflammation and immune evasion of infected cells, are related to persistent HPV infection. These factors could contribute to the high risk of complex genetic diseases such as carcinogenesis [44-46]. Therefore, the aging variable identified in this study is a possible factor for analysis. In other words, detailed investigation of cases of persistent HPV infection using regression tree analysis is reasonable, although further investigations, both in vivo and in vitro, are necessary to determine its role in carcinogenesis of oral, head, and neck squamous cell carcinoma.

In conclusion, the expression of HPV18 and p16 and participants' age are factors that distinguish NOE from OED/OIN.

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Conflicts of Interest: None

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