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Original Article

Analysis of false-negatives in exfoliative cytology in oral potentially malignant disorders: A retrospective cohort study

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ABSTRACT

Introduction: Keratinized lesions have been a conceivable false-negative (FN) factor in oral exfoliative cytology (OEC); however, other factors are poorly analyzed. In this study, we aimed to identify the factors influencing the accuracy of OEC and FNs focusing on the lesion characteristics, patient background, and surgeon factors in oral potentially malignant disorders (OPMD).
Material and methods: We retrospectively studied 44 patients who underwent both OEC and histopathological diagnosis. Sensitivity, specificity, FN rate, false-positive (FP) rate, and prevalence of both methods were compared. Similarly, accuracy indices were compared among clinical diagnosis groups (leukoplakia vs. other diagnosis). The association between patient and surgeon-related factors influencing FN OEC results were investigated using Fisher's exact test and a multiple logistic regression analysis.
Results: Overall, the sensitivity; specificity; and FN, FP, and prevalence rates of OEC were 31.8%, 82.1%, and 68.8%, 17.9%, and 36.4%, respectively. Leukoplakia was significantly more common in clinical diagnosis ($P = 0.007$) with sensitivity, specificity, and FN rates of 20.0%, 95.2%, and 80.0%, respectively. Contrarily, non-keratinized lesions had sensitivity, specificity, and FN of 83.3%, 85.7%, and 16.7%, respectively. In the prevalent group, leukoplakia and anucleate squamous cells were significantly associated with FN cases ($P = 0.013$, $P = 0.050$). On multivariate analysis in OEC negative patients, age ≤64 ($P = 0.050$) and location on the tongue ($P = 0.047$) was independently associated with FNs.
Conclusion: FN of OEC was conceivable to be due to poor deep-seated cell sampling, which was associated with leukoplakia, age, and location. Therefore, these factors may be considered in the evaluation of OEC results.

1. Introduction

Squamous cell carcinoma (SCC) is one of the most common malignant neoplasms of the oral cavity [1]. Oral potential malignant disorder (OPMD) is a common precursor of SCC; clinical diagnoses include leukoplakia, erythroplakia, and erythroleukoplakia [2]. A recent review of leukoplakia reported that the malignancy rate of OPMD ranged from 1.1% to 40.8%, with an estimated proportion of 9.8% [3]. Furthermore, OPMD may be more widespread than obvious mucosal diseases such as localized leukoplakia and erythroplakia, which considerably increase the risk of developing SCC [4]. In particular, proliferative verrucous leukoplakia needs to be closely monitored as it is estimated that it starts with leukoplakia in multiple locations and progresses to malignancy in 49.5% of cases [5]. Hence, an early detection of oral precancerous lesions prevents the occurrence of malignancy and maintains patient survival and quality of life [6,7].

Previous studies have shown that oral exfoliative cytology (OEC) is a useful screening method for oral neoplasms and epithelial dysplasia [1,8–12]. The demand for OEC has increased in recent years due to the ease of cell collection, possibility of direct visualization, and low invasiveness [1,9,10,12]. Early oral epithelial dysplasia has few subjective symptoms and often exists for a long time before it is diagnosed [1,9]. Therefore, OEC plays an important role in identifying lesions that require biopsy or treatment despite their benign appearance [10,13]. There are many reports on the accuracy of OEC, including studies in high prevalence and high-grade populations. Generally,

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the accuracy of OEC was 70–90%, and the sensitivity and specificity were similar [1,8–10,12,13].

Previous studies have shown that the presence of keratinized lesions lead to sampling errors and decreased accuracy [1,8,9,12]. Reducing false-negative (FN) is crucial for preventing the occurrence of unexpected oral malignant diseases. Although the combined cytological and biopsy search is the gold standard for intraepithelial lesions, improving the accuracy of the primary search by OEC may help reduce FNs [1]. Thus, a certain degree of accuracy is also required for OEC in initial screening. However, although leukoplakia is the most common OPMD encountered in clinical practice [3,5,6,14,15], there are few reports of OEC targeting leukoplakia. Furthermore, factors other than histopathological findings that may influence the FN of OEC are unclear. In this study, we aimed to identify factors influencing accuracy of OEC in OPMD while focusing on lesion characteristics, patient background, and surgeon factors.

2. Materials and methods

2.1. Study design and population

This retrospective cohort study included 73 patients who underwent histopathological diagnosis (HPD) after OEC at Kanagawa Dental University Yokohama Clinic between January 2018 and March 2021. We excluded patients with subepithelial lesions (such as fibroma, hemangioma, etc.), without a complete clinicopathological diagnosis, and with an interval of >100 days between cytology and HPD. Eligible patients were considered in the initial cohort.

2.2. Oral exfoliative and histopathological diagnoses

OEC was performed by five oral surgeons (with 2–25 years of experience) by swabbing with an interdental brush. The samples were stored in a cell specimen storage solution. Staining using the Papanicolaou technique and cytological diagnosis were performed at an external specialized laboratory facility. A cytology specialist classified samples according to four grades based on a Bethesda system [8] (modified for oral cytology was used to classify the diagnosis) as No Intraepithelial Lesion of Malignancy (NILM), Oral Low-grade Squamous Intraepithelial Lesion or Malignancy (OLSIL), Oral High-grade Squamous Intraepithelial Lesion or Malignancy (OHSIL), and SCC. Then, SCC, OHSIL, and OLSIL were defined as the OEC-positive group, and NILM was defined as the OEC-negative group. OEC and biopsy or excision surgeries were performed by the same surgeon in each case. HPD was performed by a specialist in oral pathology, and the lesion malignancy was graded into five levels (SCC, Severe, Moderate, Low, and Normal), and those above Low were considered dysplastic. In the initial cohort, patients in the OEC-positive group with dysplasia in HPD were defined as true positive (TP), those with no dysplasia in HPD as false-positive (FP), those in the OEC-negative group with dysplasia in HPD as FN, and those with no dysplasia as true negative (TN). The prevalent group included patients with TP and FN. The final cohort was the OEC-negative group, which included patients with FN and TN (Fig. 1).

2.3. Statistical analyses

In the initial cohort, we investigated OEC accuracy and its association with clinical diagnosis. The sensitivity, specificity, FP rate, FN rate, and prevalence of OEC were calculated with respect to HPD. The OEC accuracy of keratinized lesion and other lesion (non-keratinized lesion) were analyzed via comparison of the accuracy index between the leukoplakia group and other groups. Additionally, the relative frequency of leukoplakia and other clinical diagnoses in the initial cohort, OEC-negative group, and FN group were compared. Moreover, in the prevalent group, the associations and reproducibility

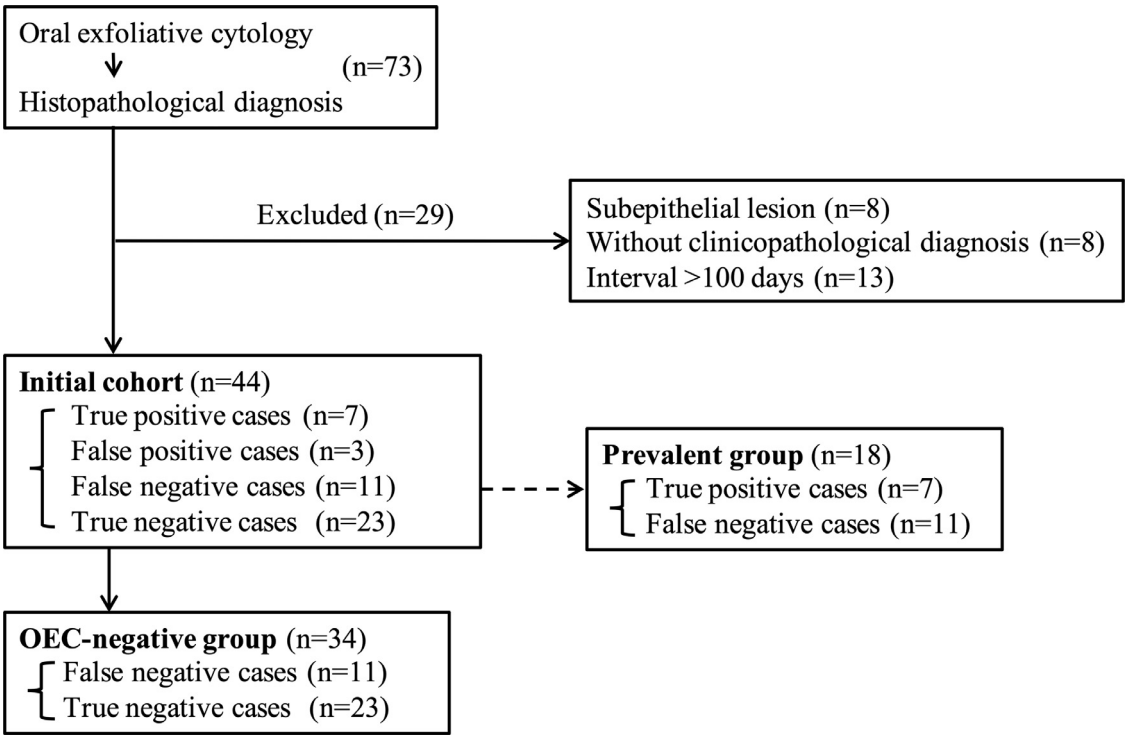

Fig. 1. Inclusion criteria and group definition.Patients who underwent oral exfoliation cytology followed by histopathological diagnosis were included. Patients with subepithelial lesions, those without a complete clinicopathological diagnosis, and those for whom >100 days had passed after cytology were excluded; patients eligible for analysis constituted the initial cohort. Those who were had "No intraepithelial lesion or malignancy" (NILM) in oral exfoliative cytology (OEC) were included in the OEC-negative group; this group included false-negatives and true-negatives. The prevalent group included true-positive and false-negative patients.

between leukoplakia or specimens containing anucleated squamous cells and FN results were analyzed.

In the OEC-negative group, each factor affecting the FN results was analyzed. The medical records of the patients were investigated to retrieve baseline characteristics that could constitute potential influencing factors (sex, age, site [tongue vs. other sites], lesion size, mucosal properties [homogeneous white lesion vs. other features], presence of pain, and smoking status). Surgeon factors included the clinical diagnosis, interval between OEC and biopsy or excision (interval), and years of experience. Since age, lesion size, and interval were not normally distributed (Shapiro–Wilk test, data not shown), we defined the two groups based on median age: 64 years, median size: 3 mm, and median interval: 40 days. Surgeon experience was divided into two groups based on a span of 10 years. The 2 × 2 tables were analyzed for association using Fisher's exact test; a P-value <0.05 was considered statistically significant. For statistically significant factors, the strength of the association was analyzed by calculating the φ coefficient. In addition, the degree of reproducibility between leukoplakia and anucleate squamous cells was determined using the κ coefficient. Finally, variables showing association in the χ^2 model were included in a multiple logistic regression analysis to identify independent factors influencing FN results. We used the E Z R software (version 1.54, Jichi Medical University Saitama Medical Center, Saitama, Japan) for all statistical analyses [16].

All procedures in this study were conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Research Ethics Board Committee of Kanagawa Dental University (No. 776). The need for informed consent was waived due to the retrospective study design, and the participants were provided the opportunity to opt-out.

3. Results

3.1. Patient and histopathological review results

A flowchart of patient selection is shown in Fig. 1. After applying the selection criteria, 44 patients were eligible for the analysis (initial cohort). The breakdown of clinical diagnosis and the result of OEC and HPD are shown in Table 1. In clinical diagnosis, leukoplakia was the most common (31 cases). Of the OEC-negative group, 11 were FN: 1 SCC, 1 Moderate, and 9 Low.

3.2. Accuracy index

The accuracy index of OEC for HPD in the initial cohort group, sensitivity and prevalence rate were low, with an FN rate of 68.8%. The leukoplakia group showed a lower sensitivity and higher FN rate (80%) than the other diagnosis groups. Sensitivity was >80% and FN rate was 17% in the other diagnosis group. Specificity was high for both groups (Table 2).

3.3. Clinical diagnosis of leukoplakia

Number of clinical diagnoses of leukoplakia in the initial cohort, OEC-negative, and FN groups were significant; the rate of leukoplakia increased as the flowchart progressed, the initial cohort group; 31/44, $P = 0.007$, the OEC-negative group; 27/34, $P < 0.001$, the FN group; 10/11, $P = 0.007$ (Fig. 2). The prevalent group showed significantly higher FNs in leukoplakia and samples containing anucleate squamous cells ($P < 0.05$) with strong association ($0.4 \leq \varphi < 0.7$). In addition, there was a high reproducibility between leukoplakia and anucleate squamous cells ($\kappa \geq 0.6$) (Table 3).

Table 1
Breakdown of clinical diagnosis, exfoliative cytology, and histopathological grade.

Clinical diagnosis		
		Case (n)
Leukoplakia		31
Other	SCC	1
	Mucosal lesion	2
	Benign tumor	4
	Lichen planus	4
	Melanosis	1
	Ulcer lesion	1
Total		44

Histopathological review		
OEC (n)	HPD (n)	
OEC-positive (10)	***Positive (7)***	
		Negative (3)
SCC (1)	SCC (3)	
OHSIL (2)	Severe (1)	Normal (3)
OLSIL (7)	Low (3)	
OEC-negative (34)	***Positive (11)***	***Negative (23)***
	SCC (1)	
NILM (34)	Moderate (1)	Normal (23)
	Low (9)	
Total (44)	***(18)***	***(26)***

HPD, histopathological diagnosis; OEC, oral exfoliative cytology; OHSIL, Oral high-grade squamous intraepithelial lesion or malignancy; OLSIL, Oral low-grade squamous intraepithelial lesion or malignancy; NILM, No intraepithelial lesion of malignancy; SCC, Squamous cell carcinoma.

3.4. False-negatives influencing factors in the OEC-negative group

The OEC-negative group consisted of 34 patients (Fig. 1). The detailed patient and surgeon factors associated with the FN results are shown in Table 4. Regarding patient characteristics, age ≤64 years and lesion site on the tongue showed a strong correlation with FN results; no significant association was observed for sex, lesion size, mucosal properties, presence of pain, and smoking status. Among surgeon factors, the interval between OEC and HPD showed a strong relationship with FN results; the clinical diagnosis and years of experience had no influence in this regard. Multiple logistic regression analysis revealed age ≤64 and tongue location to be independently associated with FN results. However, the interval between the OEC and HPD was not significant as an independent factor associated with FN.

4. Discussion

We retrospectively analyzed OECs and histopathological analyses performed during a 3- year period, investigated the influencing factors of the OEC accuracy and FN results focusing on the lesion characteristics, patient's background, and surgeon factors and found that FNs of OEC were common in leukoplakia, age ≤64, and tongue lesions.

Previous OEC studies have shown that poor sampling may lead to a lower diagnostic accuracy. Generally, most dysplastic lesions arise in the basal and parabasal layer initially, and in most cases, the oral mucosa is well-differentiated superficially in the form of keratinization; therefore, the collection of deep-seated cells is crucial for accurate diagnosis using OEC [8,12]. However, low-grade lesions, especially those with strong keratinization, make collection of deep-seated cells challenging [8,10,12]. In this study, OEC sensitivity decreased as the proportion of leukoplakia increased, and eventually almost all FNs were clinically diagnosed as leukoplakia. Moreover, the fact that leukoplakia and the presence of anucleate squamous cells were equally associated with FN and reproducibility of the two

Table 2
Accuracy index of oral exfoliative cytology.

		Clinical diagnosis	
n (%)	Initial cohort (*n* = 44, 100%)	Leukoplakia (*n* = 31, 71%)	Other (*n* = 13, 29%)
Accuracy Index (%)			
Sensitivity	31.3	20.0	83.3
Specificity	82.1	95.2	85.7
False-negative rate	68.8	80.0	16.7
False-positive rate	17.9	4.8	14.3
Prevalence	36.4	32.3	46.2

Accuracy indexes of oral exfoliative cytology were calculated and compared between the initial cohort, leukoplakia group, and other diagnosis group.

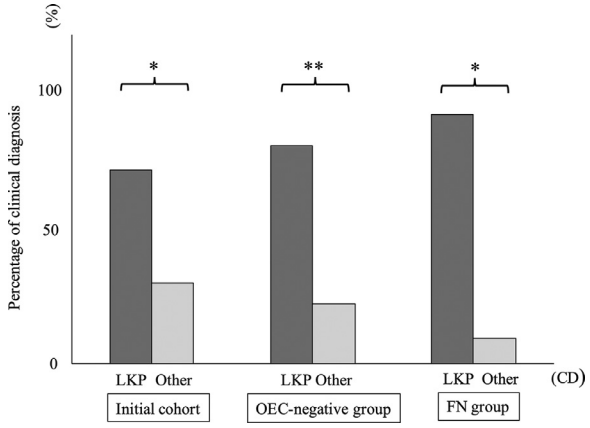

Fig. 2. Relative frequency of leukoplakia.Prevalence of leukoplakia and other clinical diagnoses were compared in the initial cohort, OEC-negative group, and FN group. * $P < 0.05$, ** $P < 0.001$. CD, clinical diagnosis; FN, false-negative; LKP, leukoplakia; OEC, oral exfoliative cytology.

factors was obtained, strongly suggesting that the superficial anucleate squamous cells were harvested from leukoplakic lesions. This may support previous studies that the misdiagnosis was due to sampling error where deep-seated cells were not collected. Evidence of this was the high sensitivity and specificity of the non-keratotic lesion group in this study, which was consistent with the accuracy levels of other OEC studies [1,8–10,12,13]. Of note, the inclusion of one case of SCC among those diagnosed as OEC-negative (NILM) should be considered a serious matter.

Regarding sampling methods, in recent years, liquid-based cytology has been used to improve the accuracy of cytology. This method may be superior to conventional smears because it removes unwanted cells and bacteria and reduces artifacts [1,9,10,12]. However, the low accuracy despite the use of liquid-based cytology in this study may be attributed to problems with cell collection. Cervical cytology and urine cytology have been shown to have high specificity but low sensitivity for low prevalence setting and low malignancy

Table 3
Associated factors of false-negative in the prevalent group.

Prevalent group (*n* = 18)	Fisher's exact test			
Factors	Properties	*P* value	φ coefficient	κ coefficient
Clinical diagnosis	leukoplakia Other	0.013 *	0.64 †	0.67‡
Anucleate squamous cell	+ –	0.050 *	0.57 †	

Association between leukoplakia or specimens containing anucleate squamous cells and false-negative results were analyzed using Fisher's exact test and the φ coefficient. The reproducibility of the two factors was evaluated by the κ coefficient. * $P < 0.05$, † $0.4 \leq \varphi < 0.7$, ‡ $\kappa \geq 0.6$.

populations, and a high FN rate is a concern. Particularly, sampling error has been associated with FN results [17–20]. These results are consistent with our findings. However, it should be considered that OEC as a screening tool should have high sensitivity and low FN rate even for low grade and low prevalence populations. Currently, various OEC cell collection tools are being used, including dental curettes, cotton swabs, nylon brushes, toothbrushes, and interdental brushes; however, they are yet to be unified [8,9,21]. For accurate collection of deep-seated cells, the use of the Orcellex® brush, EndoCervex-brush® (Rovers Medical Devices B.V., the Netherland), which is specially designed for cytological studies, is recommended [10,12]. However, this specialized equipment is expensive for routine usage [21]. Nevertheless, clinicians should closely and continuously monitor OPMD. This is because the malignant transformation of OPMD is a progressive process that can regress or progress, and the diagnosis at a given point in time is not definitive or permanent [22]. Hence, for this purpose, further prospective study is required to reconsider and standardize less expensive and more accurate cell collection methods and instruments.

In our data, age ≤64 years was independently associated with FN. There has not yet been a report analyzing the relationship between age and OEC accuracy, and this is the first report of its kind. Previous studies on malignant transformation of leukoplakia and OPMD have shown that malignant transformation progresses with age [3,4,7]. Furthermore, from a histological point of view, age-related changes in the physiological oral mucosa include decreased keratinization and epithelial atrophy [23,24]. Particularly, the tongue epithelium is the thickest in the 4th decade and thinner thereafter [24]. These reports suggest that the older the patient, the easier it may be to harvest cells that characterize the lesion condition. In addition, the thinning of the epithelium with age may make it easier to distinguish the extent of the lesion from the extent of healthy mucosa. Regarding lesion sites, we observed that tongue lesions showed considerably higher FN results than lesions in other sites. Alsarraf et al. [10] reported that sampling of the tongue margin was associated with a higher number of cells retrieved and more superficial cells compared with other oral cavity sites. The tongue has a thick, highly keratinized epithelium that may be subject to diagnostic interference due to several factors such as exfoliated keratin, tongue coating, and bacteria [1,25]. Moreover, it is suggested that sampling may be difficult in areas where there is no bone lining and pressure application is difficult [10]. Thus, collecting deep-seated cells from the tongue may be challenging and should be considered a factor for FNs. Many studies have suggested that the tongue has a distinctly high incidence of epithelial dysplasia and intraepithelial carcinoma and is the most likely site for malignant transformation of leucoplakia [3, 4,14,15,26]. Therefore, extreme care should be taken and accurate sampling methods should be used when diagnosing white lesions of the tongue in younger patients. For the tongue or keratinized lesions, opting for biopsy at the initial diagnosis or removing keratin and other debris before cell collection may also be effective in increasing sensitivity. Nevertheless, our findings may reinforce preferential OEC screening

Table 4
Independent factors of false-negative in the OEC group.

OEC-negative group (n = 44)		Fisher's exact test		Multiple logistic regression 95% CI			
Properties		*P* value	φ coefficient	OR	Low	High	*P* value
Sex	Male Female	0.46					
Age	≤64 64<	0.03 *	0.40 †	9.53	1.00	90.84	0.050*
Site	Tongue other	0.0048 *	0.52 †	7.75	1.03	58.23	0.047*
Lesion size (mm)	≤3 >3	0.64					
Mucosa properties	Homogeneous Other	0.14					
Pain	+ –	0.41					
Smoking	+ –	0.68					
Clinical diagnosis	Leukoplakia Other	0.38					
Interval (days)	≤40 >40	0.026 *	0.44 †	7.52	0.86	65.54	0.068
Surgeon experience (years)	≤10 >10	0.15					

Association between factors and false-negative results was analyzed using Fisher's exact test and the φ coefficient. Multiple logistic regression analysis was performed to identify factors independently associated with false-negative results. Odds ratios, 95% confidence intervals, and *P*-values are shown. * $P < 0.05$, † $0.4 \leq \varphi < 0.7$. CI, confidence interval; OEC, oral exfoliative cytology; OR, odds ratio.

for patients over 65 years of age and for lesions in sites other than on the tongue.

An interval of more than 40 days between the OEC and HPD was the only surgeon-related factor associated with FN results. Malignant transformation of OPMD, such as leukoplakia, is generally thought to occur over several years, but the tendency for malignant transformation in a short period of time has also been reported [14,26]. Oral epithelial lesions are superficially differentiated, and as the lesions progress, atypical cells proliferate from the basal to the superficial layers [8]. Therefore, even if the OEC is initially negative (NILM), repeated examinations at regular intervals, or the biopsy of clinically suspicious intraepithelial lesions may enable more accurate diagnosis and avoid overlooking malignant transformation. On conducting multiple logistic regression analysis, the interval between the OEC and HPD was not significant as an independent factor associated with FN. There were many OEC-negative lesions, and there was a period of time during which mechanical irritants that could affect the intraepithelial lesions were eliminated and improvement of the lesions was expected. In particular, the time to HPD might have been significantly longer in the tongue than in the other sites because a more conservative non-surgical approach was initially chosen to improve the lesion, since postoperative impairment was expected to be greater ($P < 0.05$, Mann-Whitney U test, data not shown). Therefore, this might have been a confounding factor in the multivariate analysis. Additionally, for the same reason, the median of the interval may have become larger. Since few studies have examined the time from cytology to biopsy, we believe that further prospective studies are needed on this topic [Supplementary data is available].

The accurate diagnosis of OPMD is influenced by several factors, including the choice of examination site and the subjectivity of the pathologist. Extensive lesions may show different grades of epithelial dysplasia within the same lesional area [3]. In this connection, Amagasa et al. [15] reported the usefulness of a colorimetric method using 3% Lugol's solution staining for non-invasive determination of the degree of dysplasia in OPMD. Additionally, toluidine blue staining has been used as an important stain to highlight potentially malignant oral lesions because of its ability to recognize mucosal changes [6]. Okamoto et al. [27] reported that by comparing the surface features of leukoplakia using dermoscopy, important indicators for the presence of dysplasia and cancer may be found non-invasively. The combination of these methods may be useful in determining the location of cell collection for OEC. Singh et al. [28] conducted a histopathological study comparing direct examination, such as visual examination, and cytopathological diagnosis, and suggested the need to use more quantitative and objective indicators to determine the benignity or malignancy of mucosal lesions. Therefore, more careful evaluation is necessary to determine the extent and grades of the lesion by direct visual examination and staining methods.

For histopathological evaluation of oral epithelial dysplasia, there are known methods such as the binary method introduced by Kujan et al. [29] in 2006, which is a good predictor of malignant changes in oral intraepithelial lesions because it complements the WHO classification 2005. However, since this study was not designed to predict the prognosis of malignant transformation of OPMD, we did not use this binary method. The cytology guideline introduced in Japan in 2015 (Cytology Guidelines 5, Digestive system, 2015. Edited by the Japanese Society of Clinical Cytology. KANEHARA & CO. Tokyo, Japan. Publication only in Japanese) uses the modified Bethesda system, which is based on the Bethesda system, a classification for cervical cancer cytology [30], for the evaluation of oral epithelial dysplastic lesions. Although this classification provides a simple and high accuracy diagnosis of intraepithelial lesions, it is not yet standardized in the world [8]. In 2018, Alsarraf et al. [10] attempted to apply their own modified version of this classification to OEC, and further research, utilization, and standardization are expected in the future.

One important limitation of this study was that the disease duration was unknown. Furthermore, due to the retrospective nature of the study, inaccurate patient information might have been included. Moreover, the number of anucleate squamous epithelium was not quantitatively examined. At our hospital, lesions that were clearly high-grade by visual examination or OEC were promptly referred to higher medical institutions. Hence, several patients underwent only cytological examination and lacked a definite HPD. Therefore, we might have underestimated the prevalence of intraepithelial dysplasia or SCC in our study. Finally, because of the small sample size of this study, further investigation in a larger population may be necessary for generalization. The same reason may have contributed toward the wide confidence interval.

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Data availability

As the data contains the patient's medical information, it will not be disclosed.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jormas.2022.02.001.

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