Clinicopathologic factors influencing the screening accuracy of oral cytology: A retrospective cohort study

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Abstract. Cytology is a simple and non-invasive screening method for oral cancer. However, this method is not yet routinely used by clinicians because of its high false negative rate (FNR) and due to lack of sufficient studies examining the factors for high FNRs. The present retrospective study aimed to compare the screening performance of conventional cytology (CC) and liquid-based cytology (LBC) through histological validation, and to elucidate factors inducing false negative screening in oral cytology. Cytological specimens with histological examination and intraoral digital images of the lesion were retrospectively collected between January 2017 and December 2018 for CC and between October 2019 and September 2021 for LBC. Oral cytological screening was conducted based on the oral Bethesda system for oral cytology. Clinical subtypes were re-evaluated using intraoral digital images. The screening accuracy of oral cytology was calculated considering the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for detecting the malignant transformation of oral lesions. No statistically significant difference was noted in the inadequate rate between CC and LBC groups. For CC and LBC, the sensitivities were 60.9 and 59.2%, the specificities were 87.3 and 79.1%, the PPVs were 85.8 and 76.2%, and the NPVs were 63.9 and 63.2%, respectively. Thus, the screening accuracy was similar between methodologies. Among the clinicopathological factors investigated, histological diagnosis and cellularity contributed to false negative results. Homogeneous findings of oral epithelial dysplasia and the superficial growth of carcinoma *in situ*/squamous cell carcinoma resulted in false negative findings for CC and LBC. Furthermore, LBC samples with a lower cell number (<2,000 squamous cells) exhibited statistically significantly increased FNRs. The present study found that the cytological methods did not affect the inadequate rate and screening accuracy, whereas clinical subtype and cellularity decreased screening accuracy. Therefore, cytological screening and subsequent follow-up should be performed while considering clinical findings and the cellularity of cytology smears.

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Introduction

Oral squamous cell carcinomas (OSCCs) are assumed to develop from oral epithelial dysplasia (OED) into carcinoma *in situ* (CIS) and SCC through a multi-step carcinogenesis process (1,2). The current diagnostic methods for oral potentially malignant disorders (OPMDs) and OSCCs are inspection and palpation, followed by biopsy combined with histopathological assessment on suspecting malignancy. However, it is frequently the case that the disease

has reached an advanced stage at the time of initial diagnosis, often because the patient's physician or dentist has misdiagnosed this disease (3). Inspection and palpation are subjective and requires well-trained and experienced clinicians for accurate detection. There are conflicting results regarding the association relationship between treatment delays and the survival rates of patients with OSCC (4,5). Nevertheless, a consensus has been reached that early diagnosis and treatment of OSCC improve patients' quality of life after treatment (6). Therefore, early detection is critical for the treatment of OSCC.

Oral cytology is a simple, non-invasive, and relatively reliable OSCC screening method performed since 1951 and has been improved using state-of-the-art instruments and updated diagnostic criteria (7). In particular, the liquid-based cytology (LBC) technique can detect morphological changes as well as DNA, RNA, and protein expression changes in the same samples (8,9). Moreover, LBC samples have been reported to have lower inadequate rates and higher sensitivity and specificity than conventional cytology (CC) in the cervical and oral regions (10-14). However, very few studies have compared the application of CC and LBC in the oral cavity, and currently, there is no agreement regarding the screening accuracy and inadequate sample rates. Furthermore, oral cytology has not yet gained the trust of clinicians because several studies have reported a high false negative rate (FNR) (13,14). Since low cellularity increases the FNR of cytology in detecting squamous lesions, cellularity assessment prior to cytological screening is important (15,16).

For cervical cytology, the Bethesda System 2014 guidelines revealed a minimum of 8,000-12,000 and 5,000 squamous cells for CC and LBC, respectively (17). Oral cytology samples had lower cellularity than cervical cytology samples because of the presence of saliva and keratinization in oral squamous epithelium. Therefore, the criteria for cervical cytology are not applicable to oral cytology. To the best of our knowledge, no study has examined the appropriate cellularity for oral cytology and the research on factors influencing false negative findings is limited (13).

Hence, the primary goal of the present study was to evaluate differences in the inadequate rate and in screening accuracy between CC and LBC. The secondary goal of our study was to determine clinicopathological factors that may increase the FNR of oral cytology.

Materials and methods

Patients. We conducted a retrospective study evaluating cytology methodologies. Cytology as well as histology samples, and reports were obtained from the Oral and Maxillofacial Surgery Clinic at the Niigata University Medical and Dental Hospital (Niigata, Japan). The Human Research Ethics Committee of the university (2018-0228) approved the study. The requirement for informed consent was waived due to the retrospective study design and because the dataset was anonymized and de-identified. This work was conducted in accordance with the principles of the Declaration of Helsinki.

In total 562 CC and 739 LBC samples were obtained from our hospital from January 1, 2017, to December 31, 2018, and from October 1, 2019, to September 30, 2021, respectively.

All patients who had undergone histological diagnosis were enrolled in this study.

Cell collection was performed using an interdental brush for CC and an Orcellex® brush for LBC (Rovers Medical Devices B.V., Oss, The Netherlands). The LBC samples were transferred directly to a SurePath vial (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA). Papanicolaou staining was performed at the Department of Surgical Pathology at the Niigata University Hospital.

Clinicopathological data. Clinicopathological data, including data on sex, age, tobacco use, alcohol consumption, lesion subsite, clinical subtype (i.e., homogeneous, or non-homogeneous type for OED; exophytic, superficial, or endophytic type for CIS/SCC), histological diagnosis (no malignant lesion, OED, CIS, or SCC), and the cellularity of cytological samples (described in detail below) were evaluated.

We classified OED into two types: homogeneous type dysplasia, with a flat white patch or plaque without a red component and lacking a protrusion; and non-homogeneous type dysplasia, displaying other aspects/characteristics (e.g., white patch with red component or elevated with patch).

Clinical subtypes of CIS and SCC were classified into three types: exophytic type lesions, characterized as markedly protruding lesions from the surrounding mucosa; superficial type lesions, non-elevated or slightly elevated lesions relative to the surrounding mucosa; and endophytic type lesions, presenting with deep induration (for the main lesion).

Cytologic screening based on the oral Bethesda system was categorized according to the following classification scheme by oral pathologists: inadequate, negative for intraepithelial lesion or malignancy (NILM), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and squamous cell carcinoma (SCC) (18). All histological diagnoses were made in accordance with World Health Organization (WHO) 2017 criteria (19).

Cell counts for CC and LBC samples. Cytological samples were captured horizontally from left to right at x100 magnification using a microscope (Nikon Eclipse Ni; Nikon, Tokyo, Japan) equipped with a digital camera (Olympus DP74; Olympus Corporation, Tokyo, Japan). At least 100 well-isolated squamous cells from over three captured images were counted on each slide using e-Cynuc2 cell counting software (e-Path, Kanagawa, Japan). Subsequently, since the area of the x100 magnification fields seen through the eyepieces (field number=22) of the microscope was 3.9 mm^2 and the area of the captured image was 1.6 mm^2, the average number of cells per capture image was multiplied by 3.1 to estimate the average number of cells per x100 magnification fields.

Statistical analysis. To evaluate the screening accuracy of CC and LBC, we compared cytological screening against histological diagnosis, and calculated the respective sensitivities, specificities, positive predictive values, negative predictive values, false positive rates, FNRs, inadequate rates, and accuracies for these methodologies. Next, to determine clinicopathologic factors associated with a false negative

diagnosis within oral cytology, histological diagnoses were classified as negative (no malignant lesion) or positive (OED, CIS, and SCC), and cytological screening was classified into negative (NILM) and positive (LSIL, HSIL, and SCC) categories.

Categorical variables were compared using a Fisher's exact test. Continuous variables (e.g., the average number of cells per x100 magnification fields) were evaluated for normality using Shapiro-Wilk normality and the F tests and compared using a Mann-Whitney U-test. Multivariate logistic regression models were constructed using statistically significant factors detected on univariate analysis.

Finally, to evaluate the effect of cellularity in the cytology samples on screening accuracy, the area under the curve (AUC) was obtained using receiver operating characteristic (ROC) curve analysis, and the optimal cut-off value for the number of cells recommended for LBC techniques was determined using the Youden index.

All statistical comparisons were performed using R statistical software (version 4.0.2; The R Project for Statistical Computing, Vienna, Austria). Statistical significance was set at a two-sided P-value <0.05.

Results

Population characteristics. In total 562 CC and 739 LBC samples were collected at our hospital over a period of two years. Of these, 251 CC and 371 LBC samples were excluded because the histological analysis was not performed. In addition, 8 (1.4%) CC and 21 (2.8%) LBC samples with low cellularity were excluded as inadequate samples (Table I). Finally, a total of 303 (53.9%) CC and 347 (47.0%) LBC samples were evaluated (Table II).

The final screening included benign tumor in 44 cases of CC and 47 cases of LBC, oral lichen planus in 26 cases of CC and 42 cases of LBC, inflammatory disease in 16 cases of CC and 28 cases of LBC, and other diseases in 48 cases of CC and 46 cases of LBC (data not shown).

The median age of the CC and LBC samples was 70.0 years (interquartile range [IQR]: 61.0-78.0) and 68.0 years (IQR: 58.0-78.0), respectively. The male to female ratio was 1:1.1 and 1:1.4 for CC and LBC, respectively. The most common site for conducting CC was the gingiva (226/562, 40.2%), followed by the tongue (169/562, 30.1%), buccal mucosa (90/562, 16.0%), and palate (46/562, 8.2%) (Table I). In contrast, the most common site for conducting LBC was the tongue (246/739, 33.3%), followed by the gingiva (244/739, 33.0%), buccal mucosa (151/739, 20.4%), and palate (59/739, 8.0%) (Table I). There was no statistically significant difference in the inadequate rate between CC and LBC (Table I, P=0.09).

Diagnostic accuracy of CC and LBC in detecting OED, CIS, and OSCC. Table II shows the results of conventional cytological and liquid-based cytological screening with histological diagnoses. NILM was the most frequently screened category (183/303, 60.4% for CC; 204/347, 58.8% for LBC), followed by LSIL (57/303, 18.8% for CC; 89/347, 35.6% for LBC), HSIL (47/303, 15.5% for CC; 28/347, 8.0% for LBC), and SCC (16/303, 5.3% for CC; 26/347, 7.5% for LBC).

Table I. Comparison of inadequate rate for CC and LBC.

Sites	Total number		Inadequate rate, n (%)	
	CC	LBC	CC	LBC
Tongue	169	246	1 (0.6)	3 (1.2)
Buccal mucosa	90	151	3 (3.3)	2 (1.3)
Gingiva	226	244	3 (1.3)	8 (3.3)
Labial mucosa	13	24	0 (0.0)	5 (20.8)
Floor of mouth	18	15	1 (5.6)	0 (0.0)
Palate	46	59	0 (0.0)	3 (5.1)
Total	562	739	8 (1.4)	21 (2.8)

No statistically significant difference (P=0.09; Fisher's exact test) in the inadequate rate was found between the CC and LBC groups. CC, conventional cytology; LBC, liquid-based cytology.

Table III shows the screening accuracies for CC and LBC. The sensitivities of CC and LBC were 60.9 and 59.2%, respectively, and the respective specificities were 87.3 and 79.1%. These results indicate that both cytological methods demonstrated similar diagnostic accuracy (Table III). Moreover, the FNRs were 39.1% for CC and 40.8% for LBC; specifically, FNRs were 90.0% (45/50) for CC and 78.7% (48/61) for LBC in OED cases only (Tables II and III).

Clinicopathological factors influencing the FNR. The detailed clinicopathological factors associated with FNR, as detected in this study, are listed in Table IV. Although clinical factors did not affect the FNR, a statistically significant difference in the FNRs for CC and LBC was observed between the OED and CIS/SCC cases regarding pathological factors on both univariate and multivariate Cox regression analyses (P<0.01, Table IV). More specifically, both univariate and multivariate analyses revealed that the average number of cells per x100 magnification fields was statistically significantly associated with the FNR in LBC samples (P<0.01, Table IV). However, there was no statistically significant difference in the average number of cells in the CC samples (P=0.17, Table IV).

Assessment of cellularity in CC and LBC samples. To determine the appropriate cellularity for cytological screening, we assessed the number of squamous cells in oral cytology samples in which cytological screening was either consistent or inconsistent with the histological diagnosis. Analysis of the ROC curve revealed that the average number of cells was associated with a high screening accuracy for LBC, with an AUC of 0.621 (minimum 0.541; maximum 0.691). The optimal cut-off value, as determined by the Youden index was 22.6 cells per capture image (Fig. 1). Based on these data, we estimated the cutoff value for low cellularity to be an average of 70.1 cells per x100 magnification fields.

Relationship between clinical classifications and FNRs. A statistically significant difference in the FNR was observed between the homogeneous and non-homogeneous types of

Table II. Relationship between the cytological screening and the histological diagnosis by CC and LBC.

Histological diagnosis	Cytological screening, n				
	Negative	Positive			
	NILM	LSIL	HSIL	SCC	Total, n
CC					
Total	183	57	47	16	303
No malignancy	117	17	0	0	134
OED and malignancy	66	40	47	16	169
OED	45	5	0	0	50
CIS	11	9	6	0	26
SCC	10	26	41	16	93
LBC					
Total	204	89	28	26	347
No malignancy	129	32	2	0	163
OED and malignancy	75	57	26	26	184
OED	48	13	0	0	61
CIS	14	12	3	2	31
SCC	13	32	23	24	92

NILM, negative for inadequate lesion or malignancy; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma; CC, conventional cytology; OED, oral epithelial dysplasia; CIS, carcinoma *in situ*; LBC, liquid-based cytology.

Table III. Screening accuracy of CC and LBC.

Accuracy index	CC, % (95% CI)	LBC, % (95% CI)
Sensitivity	60.9 (53.6-68.3)	59.2 (52.1-66.3)
Specificity	87.3 (81.7-92.9)	79.1 (72.9-85.4)
False positive rate	12.7 (7.1-18.3)	20.9 (14.6-27.1)
False negative rate	39.1 (31.7-46.4)	40.8 (33.7-47.9)
Positive predictive value	85.8 (79.6-92.1)	76.2 (69.2-83.2)
Negative predictive value	63.9 (57.0-70.9)	63.2 (56.6-69.9)

CC, conventional cytology; LBC, liquid-based cytology.

OED in both univariate Cox regression analyses (P<0.01, see Table V). Among the cytology samples of homogeneous OED, 35/36 (97.2%) of the CC samples and 35/39 (89.7%) LBC of the samples showed false negative findings (Table V).

In CIS and SCC cases, the clinical subtype statistically significantly affected the FNR for both CC and LBC (P<0.01, Table V). Regarding CC, the FNR for superficial type CIS/SCC was 62.5% (10/16), whereas the FNR was only 15.9% (7/44) for the exophytic type and 6.8% (4/59) for endophytic type CIS/SCC. Similarly, regarding LBC, the FNR was 48.4% (15/31) for the superficial type CIS/SCC, 13.5% (7/52)
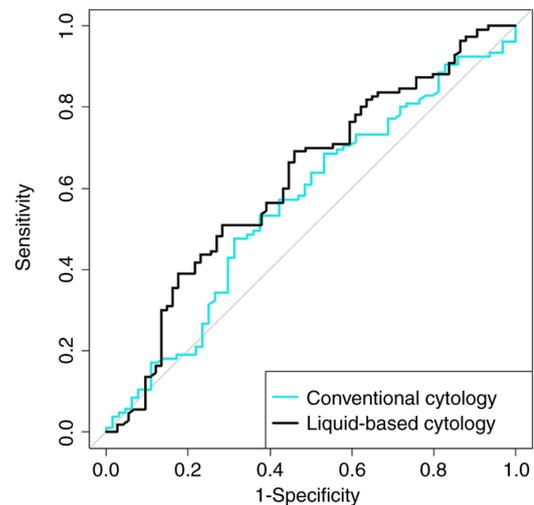
Parameter	AUC	95% CI	P value
Conventional cytology	0.563	0.472–0.654	0.168
Liquid-based cytology	0.621	0.537–0.705	<0.01

Figure 1. Receiver operating curve analyses for the mean cellularity of conventional (blue line) and liquid-based (black line) cytology samples, differentiating false negative (OED/CIS/SCC with negative for epithelial lesions or malignancy) from true positive groups (OED/CIS/SCC with low or high grade squamous intraepithelial lesion or SCC). The AUCs for conventional cytology and liquid-based cytology were 0.563 (P=0.168; 95% CI, 0.472-0.654) and 0.621 (P<0.01; 95% CI, 0.537-0.705), respectively. AUC, area under the curve; CIS, carcinoma *in situ*; OED, oral epithelial dysplasia; SCC, squamous cell carcinoma.

for the exophytic type, and 10% (4/40) for the endophytic type CIS/SCC (Table V).

Discussion

To the best of our knowledge, the current study is the largest conducted to date within the oral cytology literature. We found that there was no difference in inadequate rates between the 739 LBC and 562 CC samples and that the screening accuracy of oral cytology was similar for the 347 LBC and 303 CC samples with a histopathological diagnosis, indicating that collection techniques and collection devices may not affect the diagnostic accuracy of oral cytology. Moreover, it should be noted that clinical subtype, OED, and the cellularity of cytological smears are factors that can contribute to false negative results (i.e., false-negative factors) in oral cytology.

The inadequate rate of oral cytology found in the present study was comparable to that reported in the previous literature (12,14). The only study that compared CC and LBC at the same institution and found a statistically significant difference in the inadequate rate by methodology was reported by Sukegawa *et al* (12). Generally, an interdental brush or special brushes (such as the Orcellex® brush and the Oral CDx® brush; Oral CDx Laboratories, Inc. Suffern, NY, USA) are used in oral cytology because cell collection is unfavorable within oral cytology specifically due to the presence of saliva and a strong tendency for keratinization in the oral cavity (20). However, the report by Sukegawa *et al* (12) found a statistically significant difference in the inadequate rate between CC and LBC using cotton swabs, which tend to lead to low cellularity in cytology

Table IV. Clinicopathological characteristics of the cytology samples from OED, CIS and SCC cases, and the results of the univariate statistical analyses.

Variable	CC (n=169)			LBC (n=184)		
	NILM	LSIL/HSIL/SCC	P-value	NILM	LSIL/HSIL/SCC	P-value
Sex, n						
Male	32	55	0.87	37	59	0.65
Female	32	50		37	51	
Age, n						
<65 years	17	29	>0.99	29	31	0.15
≥65 years	47	76		45	79	
Tobacco use, n (n=154[a])						
Yes	24	49	0.74	27	54	0.87
No	29	52		27	49	
Alcohol use, n (n=154[a])						
Yes	22	38	0.73	27	36	0.09
No	31	63		27	67	
Site, n						
Tongue	22	50	0.10	23	44	0.16
Buccal mucosa	6	10		14	14	
Gingiva	29	36		27	35	
Labial mucosa	0	1		2	6	
Floor of mouth	0	4		1	7	
Palate	7	4		7	4	
Histological diagnosis, n						
OED	43	7	<0.01	48	13	<0.01
CIS	11	15		12	21	
SCC	10	83		14	76	
Mean number of cells per x100 magnification field (range)	17.5 (7.1-715.0)	22.0 (4.2-759.1)	0.17	59.0 (4.1-988.0)	123.5 (9.3-630.0)	<0.01

[a]Data on alcohol intake and years of smoking were not available for some cases. CC, conventional cytology; LBC, liquid-based cytology; NILM, negative for inadequate lesion or malignancy; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous cell intraepithelial lesion; SCC, squamous cell carcinoma; OED, oral epithelial dysplasia; CIS, carcinoma *in situ*.

samples (21). Therefore, it is possible that cell collection using cotton swabs may have affected the detected inadequate rates between CC and LBC. Based on the current results, we believe that cell collection using an interdental brush or special brushes provides a stable preparation of cytology specimens regardless of the employed CC or LBC techniques.

Several studies comparing the screening accuracy between CC and LBC have reported varying results (12,14,22,23). This discrepancy is attributed to the distribution of histopathological diagnoses in the target cases. To be more concrete, those reports that indicated high sensitivity of cytology included a low proportion of OED cases, while those including a high proportion of OED cases tended to show a lower sensitivity (Table VI). Therefore, OED may be a factor that decreases the sensitivity of screening; that is, it may increase the FNR.

Moreover, several studies have detected a similar screening accuracy for CC and LBC, which is consistent with the present results. More specifically, our results indicated that among NILM cases, 36.1% (66/183) of the cases evaluated using CC and 36.8% (75/204) of the cases evaluated using LBC had diagnoses of OED, CIS, and SCC. Among the OED cases, we also found 90.0% (45/50) for CC and 78.7% (48/61) for LBC to be false-negative results.

Few statistical studies have been conducted on the clinicopathological factors affecting the screening accuracy of oral cytology. To date, only one statistical study investigating false negative factors in oral cytology has been reported (13). These researchers reported that leukoplakia, an age of ≤64 years and tongue lesions were contributing factors to false negative findings. The present study revealed that OED (especially the homogeneous type) and superficial CIS/SCC were clinical factors inducing false negative screening on using oral cytology. Additionally, no statistically significant differences in the cellularity of cytology samples between cases

Table V. Relationship between cytological screening and clinical subtypes of (A) OED, and (B) CIS and SCC.

A, OED

Clinical subtype	CC (n=50)			LBC (n=61)		
	NILM, n	LSIL/HSIL/SCC, n	P-value	NILM, n	LSIL/HSIL/SCC, n	P-value
Homogeneous	35	1	<0.01	35	4	<0.01
Non-homogeneous	8	6		13	9	

B, CIS/SCC

Clinical subtype	CC (n=119)			LBC (n=123)		
	NILM, n	LSIL/HSIL/SCC, n	P-value	NILM, n	LSIL/HSIL/SCC, n	P-value
Exophytic	7	37	<0.01	7	45	<0.01
Superficial	10	6		15	16	
Endophytic	4	55		4	36	

CC, conventional cytology; LBC, liquid-based cytology; OED, oral epithelial dysplasia; NILM, negative for inadequate lesion or malignancy; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous cell intraepithelial lesion; SCC, squamous cell carcinoma; CIS, carcinoma *in situ*.

Table VI. Review of the literature on comparison studies of CC and LBC (including this study).

First author/s, year	Method	Brush type	Inadequate sample rate, % (n)	Sensitivity, % (n)	Specificity, % (n)	OED, % (n)	(Refs.)
Remmerbach *et al*, 2017	CC	No data	No data	96.3 (78/81)	90.6 (29/32)	No data	(22)
	LBC	No data	No data	97.5 (79/81)	68.8 (22/32)	No data	
Jajodia *et al*, 2017	CC	Toothbrush	4.2 (2/48)	95.5 (42/44)	50.0 (1/2)	21.3 (10/47)	(23)
	LBC	Toothbrush	12.5 (6/48)	92.5 (37/40)	50.0 (1/2)	21.3 (10/47)	
Kondo *et al*, 2020	CC	Cervix brush	0.8 (2/241)	71.1 (32/45)	100 (11/11)	32.8 (19/58)	(14)
	LBC	Cervix brush	1.2 (4/341)	61.3 (38/62)	91.7 (38/39)	50.0 (37/74)	
Sukegawa *et al*, 2020	CC	Cotton brush	3.5 (3/85)	96.6 (28/29)	41.5 (28/59)	2.4 (2/82)	(12)
	LBC	Cotton brush	0.0 (0/169)	95.5 (63/66)	55.2 (63/114)	3.0 (5/169)	
Present study	CC	Interdental brush	1.4 (8/562)	60.9 (103/169)	87.3 (117/134)	16.5 (50/303)	-
	LBC	Orcellex brush	2.7 (19/715)	58.6 (102/174)	78.6 (120/152)	17.6 (61/347)	

CC, conventional cytology; LBC, liquid-based cytology; OED, oral epithelial dysplasia.

with homogeneous and non-homogeneous OED or exophytic, superficial, and endophytic CIS/SCC, were noted indicating the clinical subtype did not affect the number of cells in the cytology samples (data not shown). Most cytology samples were collected from the superficial or keratinized cell layers.

We noted that, in SCC and two-phase OED/CIS presenting without atypical superficial keratinocytes on the surface, it is occasionally difficult to collect atypical cells using oral cytology. It is also easier to obtain samples from deeper layers of non-homogeneous OED and endophytic CIS/SCC because these lesions have thinner keratinized layers than other clinical lesion subtypes. These factors may have affected the

false negative results of the oral cytology in the present study. Therefore, to improve the screening accuracy of oral cytology, it is important to identify useful biomarkers that can detect neoplastic changes in keratinized cell layers, and to perform immunocytochemistry, genetic, and/or proteomic analyses using LBC residual specimens.

In a previous study, we established a novel experimental animal model that allowed our research group to observe the carcinoma sequence of OSCC and reported that Brd4 and c-Myc could be useful biomarkers for the early detection of oral cytology (9). Moreover, several prospective biomarkers have been reported in previous studies. However, these

markers are yet to be used in routine clinical practice due to a lack of sufficient validation (24-26). Additional studies should be conducted to assess the effects of these candidate markers on the diagnostic performance of oral cytology.

The average number of cells per x100 magnification fields was a pathological factor inducing false negative screening within oral cytology in the current study. In cervical cytology, there is a consensus that low cellularity compromises screening performance in detecting squamous lesions, and the Bethesda System 2001 guidelines specify a minimum of 8,000-12,000 squamous cells for CC and 5,000 squamous cells for LBC (15-17). However, studies examining the appropriate cellularity for oral cytology are insufficient, and the criteria for inadequate samples are unclear.

In the current study, although cellularity did not meaningfully affect screening accuracy for CC, a statistically significant difference was detected for LBC. This is because the average number of cells per magnification field reflects the total number of cells in the LBC specimens with a defined smear area, but this is not the case for CC specimens with varying smear areas. Moreover, for LBC, the ROC analysis revealed an AUC of 0.621 (P<0.01), indicating a high likelihood of a false negative diagnosis when the average number of cells was less than 22.6 cells per x100 field. Moreover, because the diameter of the LBC specimen was 13.0 mm, the smeared area was approximately 132.7 mm^2 (consulting the BD Totalys™ SlidePrep User Manual p. 27, 1.6.8). The average cell count of the captured image multiplied by 82.9 is considered the total cell count of the LBC specimen since the area of the captured image is 1.6 mm^2. Therefore, we consider that screening LBC samples with <2,000 squamous cells as inadequate samples may reduce the false-negative rate of oral cytology. Furthermore, re-sampling should be recommended for cases with inadequate samples to reduce under-diagnosis.

There are some limitations of this study. First, we excluded cytological samples without histopathological examination. NILM cases that clearly showed no malignancy on visual examination did not undergo biopsy. Moreover, the diagnostic accuracy of this study focused on cytological samples with clinically suspected malignancy. Thus, bias may have occurred as a result. If the cytology samples that did not undergo histological examination are assumed to contain no malignant lesions, we can infer that the probability of OED, CIS, and SCC in NILM cases is 14.9-36.1% for CC and 12.6-36.8% for LBC. Second, the brush type used for oral cytology differs between CC and LBC cases. Although no study has compared the cellularity and quality of cytology smears between interdental and Orcellex® brushes, we cannot rule out the possibility that the brush type may have affected the inadequate rate as well as the diagnostic accuracy. However, based on current evidence, it is reasonable to say that the interdental brush and the Orcellex® brush have similar effects on diagnostic accuracy since the results of this study resemble those reported in similar studies (12,14,22,23).

In conclusion, we observed no statistically significant differences in the screening accuracy and inadequate rates of CC and LBC in performing oral cytology. Furthermore, we conclude that cytological screening must be interpreted with consideration that clinical findings and the cellularity of cytology samples can affect screening performance. Clinicians should convey detailed clinical findings to pathologists and must not forget that the cytological screening of OED (especially the homogeneous type) and superficial CIS/SCC is associated with a high false negative rate. Pathologists should also need to consider the relevant clinical findings and the cellularity of cytology specimens at the time of screening.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MK, SM, MY, TAb, NNC and JIT designed the study outline and performed the data analysis. MK wrote the manuscript. All authors performed the literature search and drafted the manuscript. AF, AU, TAk and KT supervised the study and contributed to data interpretation and manuscript revision. MK, SM and JIT confirmed the authenticity of all raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the ethics committee of Niigata University (Niigata, Japan). The Ethics Board of the Niigata University Graduate School of Medicine and Dental Sciences (Oral Life Science; Niigata, Japan) reviewed and approved the experimental protocol for analyzing surgical materials (approval no. 12-10-13). This work was conducted in accordance with the principles of the Declaration of Helsinki. The requirement for informed consent was waived due to the retrospective nature of the study and the anonymization/de-identification of the study data.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Imai FL, Uzawa K, Shiiba M, Watanabe T, Miya T, Kubosawa H, Kondo Y and Tanzawa H: Evidence of multi-step oncogenesis of oral squamous cell carcinoma. Oncol Rep 5: 1489-1491, 1998.
2. Mascolo M, Siano M, Ilardi G, Russo D, Merolla F, De Rosa G and Staibano S: Epigenetic disregulation in oral cancer. Int J Mol Sci 13: 2331-2353, 2012.
3. Grafton-Clarke C, Chen KW and Wilcock J: Diagnosis and referral delays in primary care for oral squamous cell cancer: A systematic review. Br J Gen Pract 69: e112-e126, 2019.
4. Gigliotti J, Madathil S and Makhoul N: Delays in oral cavity cancer. Int J Oral Maxillofac Surg 48: 1131-1137, 2019.
5. Jensen JS, Jakobsen KK, Mirian C, Ghanizada M, Håkansson K, Wessel I, Grønhøj C, Rasmussen JH and von Buchwald C: Impact of time to treatment initiation for patients with oral cavity squamous cell carcinoma: A population-based, retrospective study. Acta Oncol 60: 491-496, 2021.
6. Tirelli G, Gatto A, Bonini P, Tofanelli M, Arnež ZM and Piccinato A: Prognostic indicators of improved survival and quality of life in surgically treated oral cancer. Oral Surg Oral Med Oral Pathol Oral Radiol 126: 31-40, 2018.
7. Montgomery PW and Von Haam E: A study of the exfoliative cytology in patients with carcinoma of the oral mucosa. J Dent Res 30: 308-313, 1951.
8. Fujii T, Asano A, Shimada K, Tatsumi Y, Obayashi C and Konishi N: Evaluation of RNA and DNA extraction from liquid-based cytology specimens. Diagn Cytopathol 44: 833-840, 2016.
9. Kawaharada M, Yamazaki M, Maruyama S, Abé T, Chan NN, Kitano T, Kobayashi T, Maeda T and Tanuma JI: Novel cytological model for the identification of early oral cancer diagnostic markers: The carcinoma sequence model. Oncol Lett 23: 76, 2022.
10. Strander B, Andersson-Ellström A, Milsom I, Rådberg T and Ryd W: Liquid-based cytology versus conventional Papanicolaou Smear in an organized screening program: A prospective randomized study. Cancer 111: 285-291, 2007.
11. Cheung AN, Szeto EF, Leung BS, Khoo US and Ng AW: Liquid-based cytology and conventional cervical smears: A comparison study in an Asian screening population. Cancer 99: 331-335, 2003.
12. Sukegawa S, Ono S, Nakano K, Takabatake K, Kawai H, Nagatsuka H and Furuki Y: Clinical study on primary screening of oral cancer and precancerous lesions by oral cytology. Diagn Pathol 15: 107, 2020.
13. Ishii S, Sakaguchi W, Sugai M, Nagumo T, Koeda S, Ozawa M, Kitamura T, Yamamura M, Akiyama H, Tsukinoki K and Nakamura A: Analysis of false-negatives in exfoliative cytology in oral potentially malignant disorders: A retrospective cohort study. J Stomatol Oral Maxillofac Surg: Feb 5, 2022 (Epub ahead of print).
14. Kondo Y, Oya K, Sakai M, Fujiwara C, Tojo F, Usami Y, Fukuda Y, Kogo M and Kishino M: Accuracy of liquid-based cytology (LBC) in the oral mucosa according to novel diagnostic guidelines in Japan: Classification of cytology for oral mucosal disease (JSCC, 2015). Oral Sci Int 17: 22-28, 2020.
15. Studeman KD, Ioffe OB, Puszkiewicz J, Sauvegeot J and Henry MR: Effect of cellularity on the sensitivity of detecting squamous lesions in liquid-based cervical cytology. Acta Cytol 47: 605-610, 2003.
16. Siebers AG, van der Laak JA, Huberts-Manders R, Vedder JE and Bulten J: Accurate assessment of cell density in low cellular liquid-based cervical cytology. Cytopathology 24: 216-221, 2013.
17. Nayar R and Wilbur D: The Bethesda system for reporting cervical cytology: Definitions, criteria, and explanatory notes, 3rd edition. Springer, New York, 2015.
18. Suzuki T, Isaka E, Hiraga C, Akiyama Y, Okamura M, Oomura Y, Hashimoto K, Sato K, Tanaka Y and Nomura T: New oral cyto-diagnostic criteria predict change to oral epithelial dysplasia (OED) and cancerization. Oral Sci Int 18: 203-208, 2021.
19. El-Naggar AK, Chan JKC, Grandis JR, Takata T and Slootweg PJ (eds.): World Health Organization classification of Head and Neck Tumours. Vol. 9, 4th edition. IARC, Lyon, pp109-111, 2017.
20. Reboiras-López MD, Pérez-Sayáns M, Somoza-Martín JM, Gayoso-Diz P, Barros-Angueira F, Gándara-Rey JM and García-García A: Comparison of the Cytobrush®, dermatological curette and oral CDx® brush test as methods for obtaining samples of RNA for molecular analysis of oral cytology. Cytopathology 23: 192-197, 2012.
21. Cocchia N, Paciello O, Auletta L, Uccello V, Silvestro L, Mallardo K, Paraggio G and Pasolini MP: Comparison of the cytobrush, cotton swab, and low-volume uterine flush techniques to evaluate endometrial cytology for diagnosing endometritis in chronically infertile mares. Theriogenology 77: 89-98, 2012.
22. Remmerbach TW, Pomjanski N, Bauer U and Neumann H: Liquid-based versus conventional cytology of oral brush biopsies: A split-sample pilot study. Clin Oral Investig 21: 2493-2498, 2017.
23. Jajodia E, Raphael V, Shunyu NB, Ralte S, Pala S and Jitani AK: Brush cytology and AgNOR in the diagnosis of oral squamous cell carcinoma. Acta Cytol 61: 62-70, 2017.
24. He Q, Chen Z, Cabay RJ, Zhang L, Luan X, Chen D, Yu T, Wang A and Zhou X: microRNA-21 and microRNA-375 from oral cytology as biomarkers for oral tongue cancer detection. Oral Oncol 57: 15-20, 2016.
25. Noda Y, Kondo Y, Sakai M, Sato S and Kishino M: Galectin-1 is a useful marker for detecting neoplastic squamous cells in oral cytology smears. Hum Pathol 52: 101-109, 2016.
26. Kujan O, Huang G, Ravindran A, Vijayan M and Farah CS: CDK4, CDK6, cyclin D1 and Notch1 immunocytochemical expression of oral brush liquid-based cytology for the diagnosis of oral leukoplakia and oral cancer. J Oral Pathol Med 48: 566-573, 2019.