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Original Research

Searching for new early detection markers of oral epithelial dysplasia and oral squamous cell carcinoma using oral liquid-based cytology

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ABSTRACT

Objective: Oral squamous cell carcinomas (SCCs) arise from oral potentially malignant disorders (OPMDs), including oral epithelial dysplasia (OED); however, useful detections markers for early detection of OED and oral SCC are lacking. In this study, we aimed to evaluate the sequential mRNA and protein expression patterns of BRD4, c-MYC, TP53, and MUC21 in human oral samples by liquid-based cytology (LBC) and determine whether these markers are useful for early detection of OED and oral SCC when combined with oral cytological screening.
Methods: A total 200 patients were obtained from our hospital between 2020 and 2022. These are samples from same cases with oral cytology and corresponding histological diagnosis. The expression of these markers was examined by immunocytochemistry and mRNA analysis. The percentages of cells with nuclear or cytoplasmic immunostaining were calculated using the labeling index (LI). BRD4, c-MYC, and TP53 mRNA expression levels were upregulated in tissue during progression from a status of negative for intraepithelial lesions or malignancy (NILM) to positive for SCC. BRD4-LI and c-MYC-LI significantly increased in low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL) and SCC samples, whereas the expression of *MUC21* mRNA and MUC21-LI gradually decreased. TP53-LI increased only in SCC samples.
Conclusions: We conclude that monitoring BRD4 and c-MYC expression with immunocytochemistry could improve the early detection of OED and oral SCC.

1. Introduction

According to Word Health Organization (WHO) data for 2020, the incidence of oral cavity cancer increased to more than 377,713 new patients and caused 177,757 deaths; it ranks as the sixth most common cancer worldwide [1–3]. The tongue, gingiva, and buccal mucosa are most common sites of oral cancer, accounting for more than half of all oral squamous cell carcinomas (SCCs) [1]. Most oral SCCs arise from oral potentially malignant disorders (OPMDs), including oral epithelial dysplasia (OED). Several studies have focused only on oral SCC detection; however, candidate markers for early detection of malignant transformation have not been identified [4]. This could be due to the lack of an oral cancer model that allows continuous observation of molecular changes that occur during the carcinoma sequence in the same oral cavity. We previously reported that Dark-Agouti (DA) rat is susceptible to 4-nitroquinoline 1-oxide (4NQO)-induced tongue cancer (TC) [5,6]. TC induced by 4NQO in DA rat is considered an experimental model for human TC because it share several morphological, molecular, and biological properties [7,8]. Using liquid-based cytology (LBC) techniques, malignant transformation events can be observed continuously in the same oral cavity in this model [9]. Our previous study identified candidate markers with which established a novel method to

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observe changes in the sequential expression patterns of mRNAs and proteins in our experimental TC model using LBC [9,10]. LBC had a higher screening accuracy than conventional cytology, and mRNA expression and immunocytochemistry (ICC) can be performed with residual LBC samples [11–13].

In our previous animal model studies [5–10], to identify candidate genes that are responsible for the effects of oral cancer, we performed a microarray analysis of TC and normal tongue tissues from TC model rats. Using the Rat Genome Database, we identified four candidate genes. The first, bromodomain protein 4 (*Brd4*), transcriptionally regulates *Myc* expression, and is expressed in the superficial or keratinized cell layers during early stages of carcinogenesis [14,15]. *Brd4* is a member of the bromodomain and extraterminal domain family of transcriptional regulatory proteins. It plays a key role in oral carcinogenesis [16]. The expression increases during 4NQO-induced tongue carcinogenesis in rat model, and it modulates tumorigenicity and tumor overgrowth [17]. Our previous oral cytology study revealed significantly higher levels of *Brd4* mRNA and protein expression in OED and oral SCC samples than in normal rat tissues [6–8,18]. Furthermore, *BRD4* binds to and activates the *c-MYC* promoter to induce MYC overexpression [19]. *c-MYC* plays a key role in the proliferation, metabolism, differentiation, promotion of *TP53* expression, and apoptosis. However, when *TP53* is mutated, cell death can be avoided, leading to the development of oral SCC [20,21]. MUC21 is a transmembrane-type mucin first cloned as the counterpart of mouse epiglycanin; our previous studies identified it as a candidate diagnostic gene for oral cancer [9–11]. Cells expressing MUC21 were significantly less adherent to each other and the extracellular matrix than cells that did not express *MUC21*; however, the function of MUC21 in oral mucosal epithelium has not been unknown [11]. *c-MYC* expression is associated with the late stage of OED [22,23], and *c-MYC* and *TP53* are overexpressed in the early stages of oral SCC. Therefore, these genes identified in our previous research may be useful markers for early detection of OED and oral SCC using immunohistochemistry (IHC) [24].

The aim of this study was to evaluate the sequential mRNA and protein expression patterns of BRD4, c-MYC, TP53, and MUC21 in human samples using LBC, to determine whether these markers are useful for the early detection of OED and oral SCC via oral cytological screening.

2. Methods

2.1. Patient samples

We obtained samples from 200 patients from the Oral and Maxillofacial Surgery unit at the Niigata University Medical and Dental Hospital. The patients who were involved underwent surgery at our institution between 2020 and 2022. We have oral cytology and corresponding histological diagnosis samples from same cases. This work was approved by the Human Research Ethics Committee of the university (2018–0228), and performed in accordance with the principles of the Declaration of Helsinki.

We collected the oral cells for cytology using an Orcellex® brush (Rovers Medical Devices, Oss, Netherlands) [9,10]. The LBC samples were transferred to SurePath vial (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA). Papanicolaou staining was performed at the Oral Pathology Section of the Department of Surgical Pathology at Niigata University Medical and Dental Hospital.

2.2. Clinicopathological data

We investigated clinicopathological data including patients sex, age, tobacco use, alcohol consumption, lesion subsite, histological diagnosis (normal epithelium, NOE; hyperplasia, HYP; OED; or SCC), and the cellularity of cytological samples.

Following cytological screening based on the Oral Bethesda System (the Japanese guidelines for oral cytology for diagnosis were published by the Japanese Society of Clinical Cytology: JSCC 2015), we categorized the samples according to the following classification scheme, based on reportes from oral pathologists: inadequate, negative for intraepithelial lesions or malignancy (NILM), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and SCC [25,26]. All histological diagnosis were made according to the WHO 2017 criteria [1]. The patient records are summarized in Table 1.

2.3. Immunostaining of BRD4, c-MYC, TP53, and MUC21 in cytological and histological specimens

We performed to observe the expression patterns of BRD4, c-MYC, TP53, and MUC21 in LBC and histological samples. All slides were subjected to antigen retrieval using 10 mM Tris-HCl, 1 mM EDTA-2Na (pH8.0) in a microwave oven of 1000 W for 20 min, followed by incubation with a rabbit polyclonal anti-TP53 antibody (1:50 dilution; clone ab131442; Abcam, Cambridge, MA, USA), a rabbit monoclonal anti-c-MYC antibody (1:100 dilution; clone ab32072; Abcam), a rabbit polyclonal anti-MUC21 antibody (1:200 dilution; clone NBP2–31023; Novus), or a rabbit monoclonal anti-BRD4 antibody (1:100 dilution; clone ab128874; Abcam). The slides were washed and incubated with the Envision+ /HRP system (Dako, Glostrup, Denmark). Immunoreactive cells were visualized using DAB (Dojindo, Kumamoto, Japan) and counterstained with hematoxylin.

2.4. Labeling index analysis for ICC

The percentages of cells with positively stained nuclei or cytoplasm in the samples incubated with each of antibodies was calculated as the labeling index (LI), using images captured at 200 × magnification and analyzed using e-Count2 cell counting software (e-Path, Kanagawa, Japan). Five random fields containing an average of 100 cells each were selected for analysis, and the average LI value was determined [9,10].

Table 1
Correlation between oral LBC and clinicopathological variables in oral cancer patients.

Oral liquid-based cytology (LBC)			
Variable	NILM	LSIL/HSIL/SCC	P value
Sex, n (n = 200)			
Male	35	70	0.65
Female	35	60	
Age, n (n = 200)			
<65 years	28	40	0.21
≥65 years	42	90	
Tobacco use, n (n = 150)			
Yes	35	70	<0.05
No	25	20	
Alcohol use, n (n = 155)			
Yes	38	75	<0.05
No	22	20	
Site, n (n = 200)			
Tongue	25	65	0.13
Buccal mucosa	10	25	
Gingiva	25	30	
Palate	6	5	
Floor of mouth	3	3	
Labial mucosa	1	2	
Histological diagnosis, n (n = 200)			
NOE	35	0	<0.001
HYP	20	0	
OED	14	10	
CIS	1	35	
SCC	0	85	

Abbreviations: NILM, negative for inadequate lesion or malignancy; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous cell intraepithelial lesion; SCC, squamous cell carcinoma; NOE, normal epithelium; HYP, hyperplasia; OED, oral epithelial dysplasia; CIS, carcinoma in situ.

2.5. RNA isolation and quantitative real-time PCR

LBC samples in RNAlater reagent (Thermo Fisher, Vilunus, Lithuania) were processed using the RNAqueous kit (Ambion, Grand Island, NY, USA). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). cDNA was amplified by TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) for *BRD4* (Hs04188087_m1), *c-MYC* (Hs00153408_m1), *TP53* (Hs01034249_m1), and *MUC21* (Hs01379324_g1); *18 S* (Hs99999901_s1) was used as the internal standard [8–10]. The $2^{-\Delta\Delta Cq}$ method was used for comparing the target gene transcripts levels in LSIL, HSIL, and SCC samples with the level in NILM samples and to calculate mRNA levels [26,27].

2.6. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) and R version 4.0.2 (R Foundation, Vienna, Austria). Experimental data were analyzed using one-way analysis of variance (ANOVA) with post hoc Tukey test or Dunn's test after Kruskal-Wallis test. Association between the LIs for *BRD4*, *c-MYC*, *TP53* and *MUC21*-LI and patient characteristics were studied using Fisher's exact test or the chi squared test ($n = 200$). We evaluated the diagnostic ability of these biomarkers using receiver operating characteristic (ROC) curves [28]. As a global measure of diagnostic accuracy, and were performed as described previously [10, 11]. The threshold for statistical significance was set at $p < 0.05$.

3. Results

3.1. Relationship between LBC screening and clinicopathologic features in oral SCC and OPMDs

The clinicopathologic features of 200 cases organized by screening status are shown in Table 1. Statistically significant differences were associated with history of smoking ($p < 0.05$), alcohol consumption ($p < 0.05$), and histological diagnosis ($p < 0.001$). No significant correlation was apparent between LBC screening and the parameters age, sex or tumor site.

3.2. Papanicolaou staining

Papanicolaou staining of NILM samples reveled keratinized and non-keratinized squamous epithelial cells in the superficial to deeper layers (Fig. 1). Although the nucleus of the light-green stained cell is smaller than that of orange-G stained cell, no cellular pleomorphism was observed in the specimen (Fig. 1A). LSIL samples showed hyper parakeratinized superficial layer of squamous epithelial cells forming scattered and small clusters. The superficial layer cells were characterized by cellular atypia including increased of nuclear size, hyperchromasia and dyskeratosis (Fig. 1B). HSIL samples showed keratinized and/or non-keratinized superficial to middle layer of squamous epithelial cells in scattered and small to large clusters. The middle layer cells featured cellular atypia such as nuclear pleomorphism, increased nuclear-cytoplasmic (N/C) ratio, and enlarged nuclei (Fig. 1C). The SCC samples showed small to large cellular clusters composed of atypical epithelial cells. The deeper layer cells showed prominent cellular atypia such as nuclear pleomorphism, increased N/C ratio, and enlarged nuclei. The superficial layers of squamous epithelial cells also showed marked cellular atypia such as hyperchromasia and enlarged nuclei (Fig. 1D).

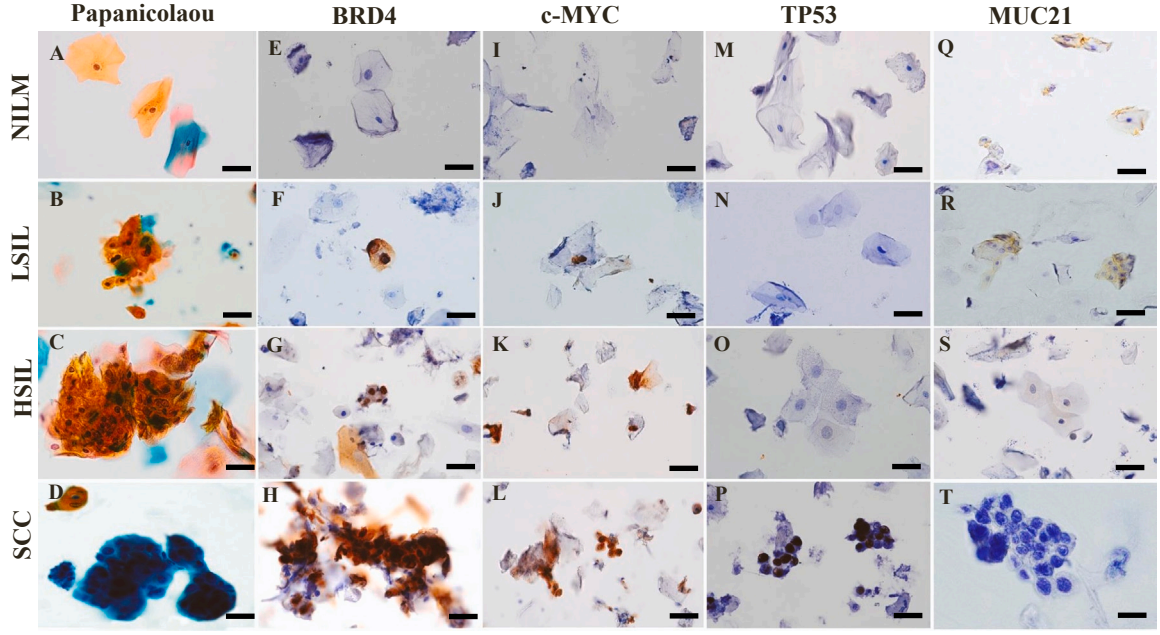

Fig. 1. Papanicolaou staining in liquid-based cytology (LBC) samples and BRD4, c-MYC, TP53, and MUC21 immunohistochemical study in oral cytological samples: (A) NILM, (B) LSIL, (C) HSIL, and (D) SCC. (E-H) BRD4 immunocytochemical staining. Although BRD4 staining was generally negative in (E) NILM samples, (F) positive nuclear staining was observed in LSIL, (G) HSIL, and (H) SCC samples. (I-L) c-MYC immunocytochemical staining. Although c-MYC staining was generally negative in (I) NILM samples, positive nuclear staining was observed in (J) LSIL, (K) HSIL, and (L) SCC samples. (M-P) TP53 immunocytochemical staining. Although TP53 staining was generally negative in (M) NILM, (N) LSIL, and (O) HSIL samples, positive nuclear staining was observed in (P) SCC samples. (Q-T) MUC21 immunocytochemical staining. Although MUC21 staining was generally positive in (M) NILM and (N) LSIL, negative cytoplasmic staining was observed in (O) HSIL and (T) SCC samples. Original magnification, 400 x. Scale bars, 20 μm. NILM, negative for intraepithelial lesion or malignancy; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma.

3.3. Analysis of BRD4, c-MYC, TP53, and MUC21 expression using ICC and IHC

NILM, LSIL, HSIL, and SCC samples were evaluated by ICC staining for BRD4, c-MYC, TP53, and MUC21 expression. NILM samples were negative for three markers (Fig. 1E, I, and M), but expressed MUC21 (Fig. 1Q). In LSIL and HSIL samples, BRD4 and c-MYC expression was observed in the atypical nuclei of the superficial and intermediate cells (Fig. 1F, G, J, and K). In SCC samples, BRD4 and c-MYC were expressed in parabasal/basal cells that showed nuclear changes, such as nuclear shape abnormalities, and increased N/C ratios (Fig. 1H and L). In contrast, TP53 was only expressed in SCC samples with atypical nuclei in parabasal/basal cells (Fig. 1M-P). MUC21 was not expressed in HSIL and SCC samples (Fig. 1Q-T).

Samples that had been histologically diagnosed as NOE (Fig. 2A), HYP (Fig. 2B), OED (Fig. 2C), and SCC (Fig. 2D) after H&E staining were analyzed for BRD4, c-MYC, TP53, and MUC21 expressions by IHC staining (Fig. 2Q-T). BRD4 expression was positive in the basal layer to the superficial layer of HYP, OED, and SCC samples (Fig. 2F-H), whereas c-MYC expression was limited to the basal/parabasal layers of HYP samples. c-MYC-positive cells were found from the basal layer to the superficial layers in OED and SCC samples (Fig. 2I-L). TP53 nuclear positivity was only detected in SCC samples, but not in NOE and HYP samples (Fig. 2M-P). The cytoplasm in the prickle cell layers of NOE to OED samples was positive for MUC21. Consistent expression patterns were observed using of ICC and IHC analyses.

3.4. mRNA expression of candidate markers in LBC samples

We observed statically significant differences in the mRNA expression, *BRD4*, *c-MYC*, *TP53,* and *MUC21* among the samples organized by Oral Bethesda System ($p < 0.01$; Fig. 3A-D) [10,11]. The mRNA levels of *BRD4*, *c-MYC,* and *TP53* were significantly higher in SCC samples than in NILM, LSIL, and HSIL samples ($p < 0.01$; Fig. 3A-D). Our results suggested that the mRNA expression of *BRD4*, *c-MYC*, and *TP53* was upregulated during the carcinoma sequence, whereas that of *MUC21* was downregulated.

3.5. Protein expression of candidate markers in LBC samples

To identify BRD4, c-MYC, TP53, and MUC21 protein expression patterns in different sample grades based on the Oral Bethesda System [25], we performed ICC on NILM, LSIL, HSIL, and SCC samples. We observed significant differences in the LIs of BRD4 (BRD4-LI), c-MYC (c-MYC-LI), and TP53 (TP53-LI) in the samples at different stages ($p < 0.01$). The BRD4-LI was significantly higher in the HSIL and SCC samples than in the NILM and LSIL samples ($p < 0.01$) (Fig. 3E). Consistent with BRD4 expression, c-MYC-LI was significantly higher in the HSIL and SCC samples than in the NILM and LSIL samples ($p < 0.01$) (Fig. 3F). Similarly, TP53–LI was significantly higher in the SCC samples than in the NILM, LSIL, and HSIL samples ($p < 0.01$) (Fig. 3G). In line with MUC21 mRNA expression, the MUC21–LI was significantly higher in the NILM and LSIL samples than in the HSIL and SCC samples ($p < 0.01$) (Fig. 3H). These results showed that the expression of BRD4, c-MYC, and TP53 increased with disease severity. The protein expression levels of BRD4 and c-MYC were particularly high in the LSIL and HSIL samples.

3.6. Between mRNA expression and LI of BRD4, c-MYC, TP53 and MUC21

To identify the relationship between the mRNA expression and LI by ICC, we performed a correlation analysis for each candidate marker. Fig. 4 shows the relationship between *BRD4*, *c-MYC*, *TP53*, and *MUC21* mRNA expression and the corresponding LI values. We detected significant correlations between the expression levels of BRD4 ($R=0.781$, $p < 0.01$; Fig. 4A), c-MYC ($R=0.807$, $p < 0.01$; Fig. 4B), TP53 ($R=0.606$; $p < 0.01$; Fig. 4C), and MUC21 ($R=0.514$; $p < 0.05$; Fig. 4D).

3.7. Screening accuracy of candidate markers

To evaluate the screening accuracy of increased LI for candidate markers, we stratified the LI of each marker as positive (LI below the cut-off) or negative (LI above the cut-off). We calculated the sensitivity and specificity for detecting LSIL or a higher category by performing ROC analysis (Fig. 5). The areas under the curves were 0.881 (standard error

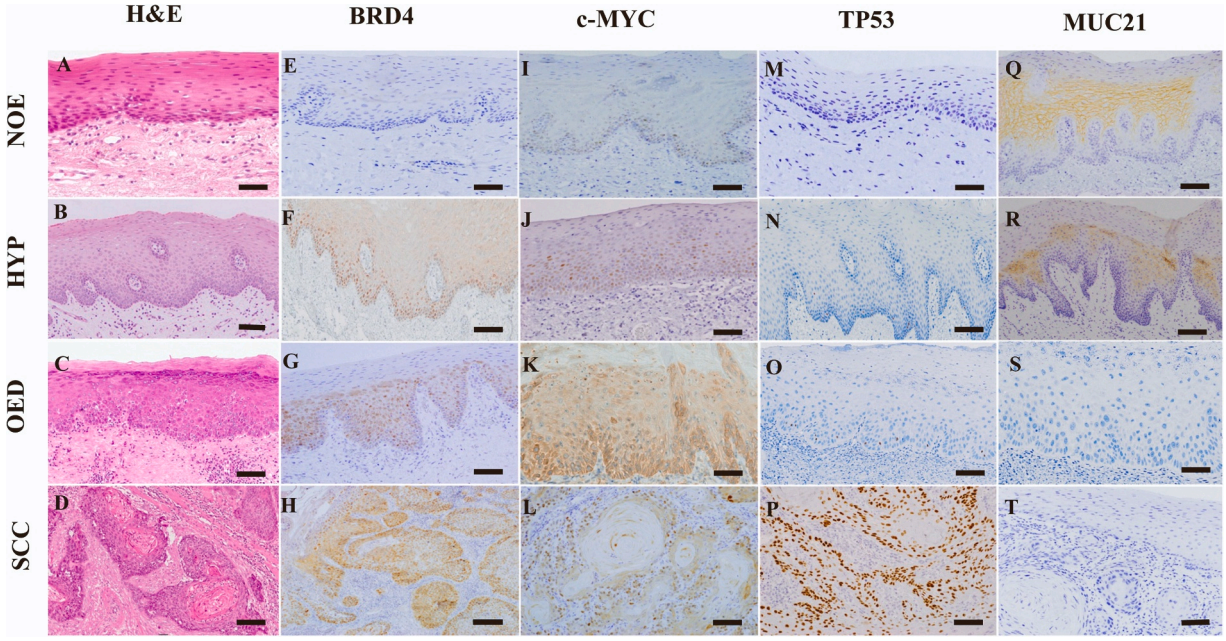

Fig. 2. Histopathological and immunohistochemical expressions patterns of BRD4, c-MYC, TP53, and MUC21 in normal epithelium (NOE), hyperplasia (HYP), oral epithelial dysplasia (OED), and squamous cell carcinoma (SCC) samples. (A, E, I, M, Q) NOE, (B, F, J, N, R) HYP, (C, G, K, O, S) OED and (D, H, L, P, T) SCC. (A-D) Hematoxylin and eosin (H&E), (E-H) BRD4, (I-L) c-MYC, (M-P) TP53, and (Q-T) MUC21. Original magnification, 100x. Scale bars, 100 μm.

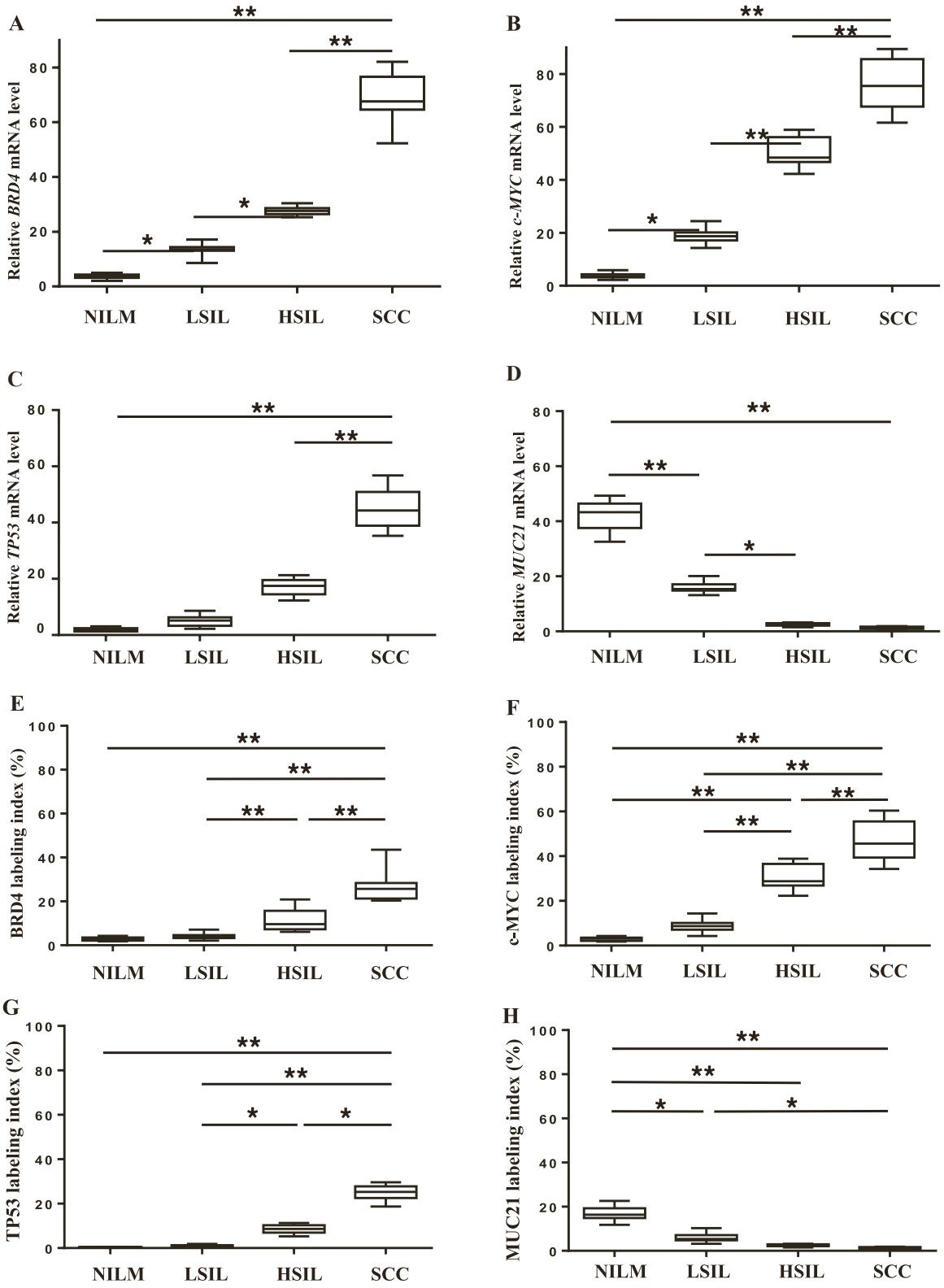

Fig. 3. The box-and-whisker diagrams show the normalized expression of (A) *BRD4*, (B) *c-MYC*, (C) *TP53*, and (D) *MUC21* in NILM, LSIL, HSIL, and SCC samples. The box shows the interquartile range (>25% and <75%), and the solid line within each box is the median candidate gene expression value. Analysis of variance (ANOVA) followed by Tukey's multiple comparisons test and the Kruskal-Wallis test followed by Dunn's post hoc test were used to determine statistical significance. Boxplots for the immunocytochemical labeling indices of (E) BRD4, (F) c-MYC, (G) TP53, and (H) MUC21 in NILM, LSIL, HSIL, and SCC samples. ANOVA followed by Tukey's multiple comparisons test was used to determine statistical significance. *$p < 0.05$, * *$p < 0.01$, as indicated.

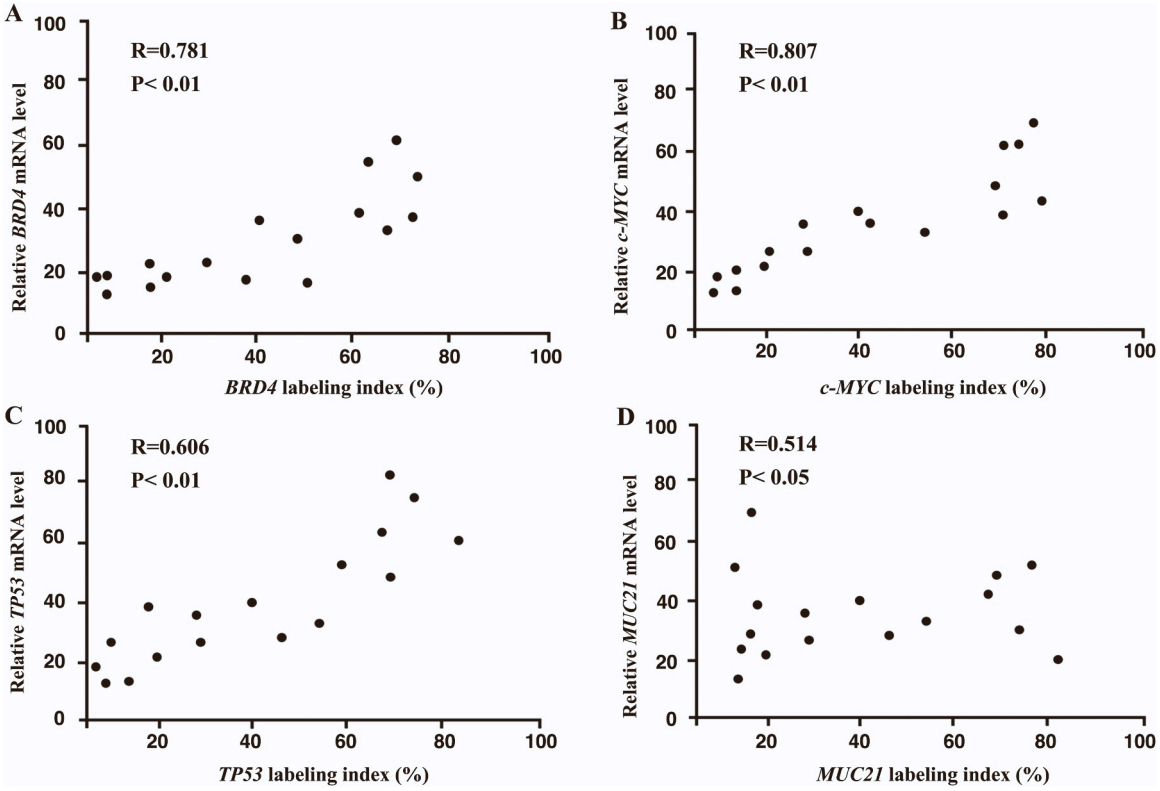

Fig. 4. The correlation between the labeling index and the relative mRNA levels of each marker (*BRD4*, *c-MYC*, *TP53*, and *MUC21*) in the oral cytological specimens. These markers displayed significant positive correlations: (A) *BRD4* (R= 0.781, $p < 0.01$), (B) *c-MYC* (R=0.807, $p < 0.01$), (C) *TP53* (R=0.606, $p < 0.01$), and (D) *MUC21* (R=0.514, $p < 0.05$).

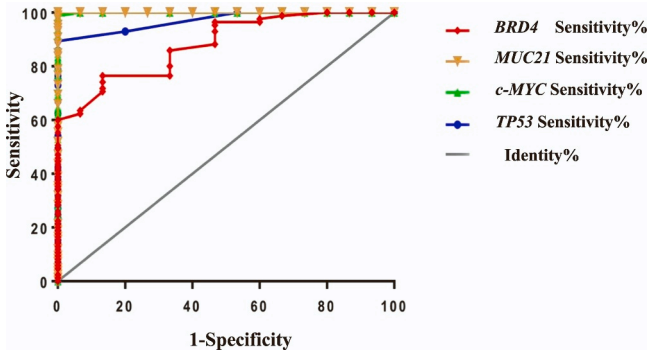

Fig. 5. Receiver operating characteristic analysis for LSIL or higher category specimens were screened using *BRD4* (red line), *c-MYC* (green line), *MUC21* (brown line), and *TP53* (blue line) as candidate markers. The optimal cut-off values of each markers were calculated using 'closest-topleft' (10, 28).

[SE]: 0.041; 95% CI: 0.738–0.919) for *BRD4*, 0.999 (SE: 0.044; 95% CI: 0.718–0.925) for *c-MYC*, 0.971 (SE: 0.052; 95% CI: 0.717–0.918) for *TP53*, and 1.00 (SE: 0.032; 95% CI: 0.647–0.902) for *MUC21*. We classified the samples into negative (NILM) and positive (LSIL, HSIL, and SCC) categories.

Table 2 shows the sensitivity, specificity, false-negative rate, and screening accuracy of the markers. Using the Oral Bethesda System [25], cut-off BRD4-LI values greater than the cut-off of 6.0% were observed in 60.0% of LSIL and 100% of HSIL samples, whereas c-MYC-LI values greater than the cut-off of 12.0% were observed in 50.0% of LSIL and 78.0% of HSIL samples. In contrast, a TP53-LI value greater than the cut-off of 3.6% was observed in only 20% of LSIL and 58% of HSIL samples.

Table 2
Cut-off values, sensitivity, specificity, and FNRs for BRD4–LI, c-MYC-LI, TP53–LI and MUC21-LI.

Markers	Cut-off value (%)	Sensitivity (%)	Specificity (%)	FNR (%)
BRD4	3.550	76.47	86.67	23.53
c-MYC	4.800	98.82	100.0	1.180
TP53	0.350	89.41	100.0	10.59
MUC21	11.05	100.0	100.0	0.000

LI, labeling index; FNR, false negative ratio; BRD4, bromodomain protein 4

4. Discussion

In the JSCC Atlas and Guideline for Oral Cytopathological Diagnosis in 2015, the new diagnostic algorithm and criteria were established by our members [25]. Recently, oral LBC has developed as a means of analysis because it is easy, reproducible, and produces fewer artifacts than the conventional method [9,10]. In our previous papers, oral LBC staining showed the carcinoma sequence from NILM, LSIL, HSIL to SCC, and the morphological changes were that observed during the progression of oral carcinogenesis [9,10]. Many oral cytological studies have shown that candidate markers expressed in the superficial and/or keratinized cell layers could be usefulness for monitoring diseases [26,27].

Our previous IHC staining studies on cytokeratin 13, cytokeratin 17, TP53 and Ki-67 demonstrated their useful for the histological diagnosis of OED and SCC [29,30]. Overexpression of the candidate marker TP53 is reportedly involved in SCC development, and TP53 mutations have been observed in OED and SCC [9,10,30,31]. In this cytological IHC study, we observed the loss of TP53 overexpression in NOE, HYP, and OED samples; therefore, we conclude that there were no TP53 expressions in NOE samples. In contrast, NOE, HYP, OED, and SCC, samples showed BRD4 expression in the basal to superficial layers. c-MYC was expressed in the basal to spinous cell layers in HYP samples and in the

basal to superficial layers in OED and SCC samples, and cytoplasmic MUC21 expression in the prickle cell layers was detected in NOE to HYP samples [9,11]. Therefore, we nominated BRD4 and c-MYC as candidate markers based on their detection by ICC in the HYP and OED samples, and examined their usefulness and reliability in this oral carcinoma sequence study.

The qRT-PCR results for *BRD4*, *c-MYC*, and *TP53* showed gradually increasing expression with progression from NILM to SCC, and these markers were significantly overexpressed in SCC samples compared with NILM, LSIL, and HSIL samples [32]. These observations are consistent with those reported in our oral cancer model and other studies on oral SCC [18,27–31]. mRNA expression of *BRD4* was aberrantly upregulated in head and neck oral SCC samples and in the 4NQO-induced TC model [9,18]. Co-overexpression of *c-MYC* and *TP53* in oral SCC samples was linked with premalignant lesions [31,32].

The LIs for BRD4, c-MYC, and TP53 significantly increased with progression from NILM to SCC, whereas the LI for MUC21 decreased. BRD4 is highly expressed in many tumors, and cancer prognosis is tightly linked to BRD4 expression. BRD4 may serve as a potential prognostic and early detection marker [18]. c-MYC is known to shuttle between the nucleus and cytoplasm. In settings of elevated expression, c-MYC accumulates in the nuclei of some cells, consistent with saturation of a nucleolus-associated degradation system [33]. Therefore, c-MYC expression in OED and SCC remains debatable. However, our studies collectively show that c-MYC is unlikely to be localized to the cytoplasm, and it could be appropriate to assess the nuclear localization of c-MYC in OED and SCC. Moreover, the LI calculation method was validated because we observed a significant correlation between the LI and mRNA expression levels for these markers (Fig. 4). ICC was a more accurate method for detecting positive cells than qRT-PCR because ICC determined the LI for hotspots in oral LBC samples, whereas qRT-PCR required the evaluation of all samples. Consequently, we propose that ICC is more useful for the early detection of OED and SCC using oral LBC than qRT-PCR.

The LIs for BRD4 and c-MYC were significantly increased during the early stages of oral carcinogenesis over those in NOE; therefore, they could be good predictors of OED and SCC. Cytological screening based on morphological changes is frequently difficult because of the occurrence of false negatives. This applies particularity for oral cytology, in which distinguishing between reactive/regenerative or neoplastic changes occurring in NILM and LSIL samples is important [31]. Our result showed that BRD4 and/or c-MYC expression were detected more frequently in LSIL samples than TP53 expression. Therefore, evaluating the BRD4-LI and c-MYC-LI using ICC, in addition to oral cytological screening, could improve the screening accuracy of the oral cytology from NILM to SCC. c-MYC is a well-known of proto-oncogene that regulates various cellular processes including cell growth, differentiation and apoptosis, and expression of the downstream target of BRD4 [33]. c-MYC promotes TP53 mutations and reduced expression of the Cdk inhibitor $p27^{Kip1}$ are frequently observed in human oral cancer, but it is not known whether alterations in oral cancer suppressors interact to influence oral cancer progression [34–37]. BRD4 and c-MYC expression are usually upregulated in OED and SCC, and they are significantly associated with clinicopathological features and poor survival [18,38]. However, their usefulness as markers for early detection of OPMDs and oral SCC was unknown. Combining cytology and ICC to increase screening accuracy has been widely reported for other cancers; however, only a few studies have evaluated pathological conditions of the oral cavity [29,30]. MUC21, which showed 100% sensitivity and specificity (Fig. 5), is not recommended for screening because no immunostaining was observed in OED and SCC samples (Fig. 1 and Fig. 2) [11,39]. This study presents a novel oral cytological screening tool for OED and oral SCC.

In conclusion, building on the results of our previous study [9,10], in the present study, we monitored continuous morphological changes and patterns of mRNA and protein expression during carcinogenesis in human samples. Our findings indicate that combination of ICC and oral cytology for the detection of BRD4 and c-MYC expression could improve the accuracy of OED and oral SCC diagnosis.

5. Conclusions

From the results of this research, we suggest to examine mRNA and protein expression patterns of BRD4 and c-MYC analyzing by LBC using human materials and clarify whether these markers are useful for the early detection of OED and oral SCC.

Ethical approval

This study was approved by the Human Research Ethics Committee of Niigata university (2018–0228). The requirement for informed consent was waived owing to the retrospective study design and the anonymization and deidentification of the dataset. The study was conducted in accordance with the principles of the Declaration of Helsinki.

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Patient consent for publication

Not applicable.

Declaration of Competing Interest

The authors declare no conflict of interest.

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