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CDK4, CDK6, Cyclin D1 and Notch1 immunocytochemical expression of oral brush liquid-based cytology for the diagnosis of oral leukoplakia and oral cancer

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Abstract

Objectives: This study aimed to investigate the feasibility of using oral liquid-based brush cytology (OLBC) coupled with immunocytochemistry as a minimally-invasive approach to stratify the cancer risk in patients with oral leukoplakia.

Methods: 55 patients diagnosed with either oral leukoplakia (OLK) or oral squamous cell carcinoma (OSCC) were recruited. All patients underwent oral brush biopsy followed by surgical biopsy. 275 liquid-based cytology preparations were made. Pap stained OLBC slides were assessed using the modified 2014 Bethesda Cytology system. The expression of CDK4, CDK6, Cyclin D1, and Notch 1 was immunocytochemically analysed and compared against the histopathological diagnosis. A combined index score of OLBC grading and protein expression was calculated.

Results: A significant association was found between the definitive histopathological diagnosis and the cytological interpretation (p = 0.0005). The index scores of CDK4, CDK6, and Cyclin D1 were significantly associated with the development of disease from non-dysplastic epithelium to OSCC. No significant association was observed between the Notch 1 index score and disease stage. The diagnostic accuracy of OLBC showed the highest values of sensitivity, specificity, positive predictive value, negative predictive value, and accuracy; 84.6%, 70.4%, 73.3%, 82.6%, and 78.8%, respectively, compared to the cumulative protein index, CDK4/6 index, and the combined OLBC grading and CDK4/6 index.

Conclusion: This study has also demonstrated the efficacy of the use of OLBC in the detection of OED and OSCC, and showed that the use of CDK4, CDK6, Cyclin D1 and Notch 1 immunocytochemistry failed to improve the diagnostic accuracy of OLBC suggesting they are not useful in the early detection of OSCC.

Introduction

Oral cancer is a global oral health problem on the rise with more than of 350,000 newly diagnosed cases worldwide, and an associated mortality exceeding 175,000 cases.[1] Remarkably, nearly half of the newly diagnosed cases exhibit regional or distant metastases at the time of diagnosis, which significantly contributes to high mortality rates.[2] Early detection has the promise to reduce oral cancer-related burden of disease and improve overall patient survival rates.[3]

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Oral squamous cell carcinoma (OSCC) is the predominant type of oral cancer that is mostly preceded by a group of lesions and disorders with an increased risk of developing cancer termed oral potentially malignant disorders (OPMDs).[4] Nonetheless, predicting the malignant transformation of OPMDs is still challenging. Histopathological grading of oral epithelial dysplasia (OED) is the current standard for malignant transformation prediction, however its prognostic value is hampered by subjectivity and lack of confident reproducibility.[5] Further, the current scalpel method of surgical biopsy is a sensitive technique associated with high morbidity and warrants specialised training.[6] Oral brush cytology is emerging as a promising diagnostic adjunct which provides reliable minimally-invasive intra-epithelial sampling for the early detection of oral cancer.[7-10]

Recent molecular advances have revealed the multistep heterogenous nature of oral carcinogenesis that involves specific genetic and epigenetic events,[11] however the precise understanding of the underlying molecular mechanisms of oral carcinogenesis are still incomplete and warrant further investigations.[12] The pursuit of valid and reproducible biomarkers to predict the malignant transformation of OPMDs is still underway.[13] nevertheless there are several promising biomarkers that have been identified to have a role including CDK4, CDK6, Cyclin D1, and Notch 1.

CDK4 and CDK6 are cyclin-dependent kinases which interact with CyclinD1 to form a complex for the progression of G1 to S phases of the cell cycle. There is strong evidence showing that dysregulation of the cyclinD1:CDK4/6 axis plays a role in the development of many human cancers such as breast cancer.[14] Moreover, overexpression of CDK4 and CDK6 has been detected in both OSCC tissues and cell lines.[15,16] Additionally, Niwa et al. in 2010 revealed that the labelling index of Cyclin D1 and CDK4 increased immediately after administration of a carcinogen (4-nitroquinoline 1-oxide), and then increased gradually from hyperplasia to a maximum level at the dysplastic stage.[17]

Notch1 is a single-pass transmembrane receptor, in which binding of specific ligands to the extracellular domain of the receptor initiates intracellular signalling pathways.[18] These pathways are highly conserved, and it is believed that they play a significant role in malignant transformation,[18] however studies reveal that Notch 1 might have diverse functions as both an oncogene and a tumour suppressor gene.[19] In this regard, several studies have demonstrated upregulation of Notch 1 in OSCCs,[20,21] while others have shown that Notch signalling has a tumour suppressive role in oral cancer.[22,23] Interestingly, Das et al. in 2010 demonstrated that activation of Notch signalling significantly enhanced expression of Cyclin D1 and promoted cell proliferation.[24]

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To the best of our knowledge, there is no single published report analysing the expression of CDK4, CDK6, Cyclin D1, and Notch 1 in OPMDs or OSCCs, neither by means of surgical biopsy or oral brush cytology. The objective of the present study was to identify the immunohistochemical expression of CDK4, CDK6, Cyclin D1, and Notch 1 in oral leukoplakia (OLK) and OSCC using oral brush liquid based cytology (OLBC), and to estimate the relative accuracy of OLBC grading in combination with index scores of the specific proteins in the diagnosis of OLK and oral cancer.

Materials and methods

Study subjects

This cross-sectional study was approved by the University of Western Australia Human Ethics Committee (Ref. RA/4/1/9313) and conducted in accordance with the ethical and scientific principles of the Declaration of Helsinki, and the STROBE statement. Cases were selected from an ongoing project evaluating OLBC in diagnosis of OPMD and OSCC.[10] Adult patients aged more than 18 years who were diagnosed with OLK or OSCC were included. Any patient with a history of chemotherapy/radiotherapy was excluded.

Oral brush cytology and surgical biopsy

Participants underwent oral brush cytology (Orcellex, Rovers Medical Devices B.V., The Netherlands) under local anaesthesia before the surgical biopsy. The brush biopsies were taken by firmly pressing the brush head against the lesion and rotating it 20 times to induce pinpoint bleeding to ensure collection of an adequate transepithelial specimen. The brush heads were then transferred directly into vials with a methanol-based preservative (ThinPrep®PreservCyt, Hologic Inc., Bedford, MA, USA) and transported to the laboratory after appropriate labelling. A standard protocol for preparing liquid-based cytology slides using the ThinPrep™ 2000 processor (Hologic Inc., Bedford, MA, USA) was used for all specimens.

Standard scalpel surgical biopsy was performed on all lesions for histopathological evaluation. Specimens were fixed in 10% neutral buffered formalin and sent to the laboratory for routine haematoxylin and eosin staining. Histopathological diagnosis was used as the gold standard for definitive diagnosis. All samples with oral epithelial dysplasia (OED) were graded as low-grade OED or high-grade OED using the binary system of grading OED.[25]

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Cytological assessment

All slides were assessed cytologically using the modified 2014 Bethesda Cervical Cytology grading system as previously described by us.[10] Briefly, following the preparation of Pap stained liquid-based cytology slides, cellularity, quality of preparation, types of cells present, data on microbiota, presence of leucocytes/inflammatory cells, artifacts present and, when applicable, dyskaryotic epithelial changes or features suggestive of OSCC using the modified 2014 Bethesda Cervical Cytology grading system were reported.

Evaluation of CDK4, CDK6, Cyclin D1, and Notch 1 immunocytochemical expression

Four unstained ThinPrep® poly-L-lysine (PLL) coated glass slides were prepared from each specimen. The primary antibodies used in this study were mouse-derived antibodies against CDK4, CDK6 (ThermoFisher Scientific, Massachusetts, USA), and CyclinD1 and Notch 1 (Abcam, CA, USA). MCF-7 cells (a breast adenocarcinoma cell line which displays positive immunocytochemical staining for Notch 1, CDK4 and CyclinD1) were used as a positive control. No antigen retrieval was undertaken. Primary antibodies were used diluted at 1:400 with 0.1% BSA/TBS and incubated overnight at 4°C in a humidified chamber. Sections were incubated with One-Step Polymer-HPR reagent for 30 minutes and visualised using fresh diaminobenzidine tetrahydyrochloride. Slides were finally counterstained with hematoxylin, dehydrated, cleared and mounted with DPX. Negative controls were carried out by omitting the relevant primary antibody.

Ten random fields on each slide having greater than 500 cells were scored at ×100 magnification. Regarding the staining intensity, epithelial cells that showed golden brown staining in the nucleus were considered positive. The intensity of staining was scored and graded for statistical analysis as follows; no staining = 0, weak intensity = 1, moderate intensity = 2, and strong intensity = 3. The number of positive cells and the number of all cells were counted within 10 × 10 grids to avoid repetition. The ratio of positive cells was calculated and scored as follows; 0-24% = 1, 25-50% = 2, 51-75% = 3, and 76-100% = 4. Positive cases were those with at least five positive cells, and at least 500 counted cells. The final index score for each protein in each slide was calculated by multiplying the stain intensity score by the score of positively stained cells. In order to ensure reproducibility, two investigators independently examined each slide. Any slide with more than 5% variation among the examiners was reevaluated until consensus was achieved.

Index score

An index score of a total 12-points was proposed using one-way ANOVA analysis to diagnose lesions per the criteria described in **Table 1**.

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Statistical analysis

Statistical analysis was performed using SPSS Statistics, version 23.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $p < 0.05$. Chi-square test was used to study the association between different categorical variables. One-way ANOVA was used to evaluate the relation between the index score of each protein and other variables. A Pearson product-moment correlation was conducted to examine the association between continuous variables. Samples with inadequate cellularity were excluded. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of OLBC grading, cumulative biomarker index, CDK4/6 staining index and the index score of combined OLBC grading and CDK4/6 staining were calculated.

Results

A total of 55 adult patients were recruited; 30 males (54.5%) and 25 females (45.5%). Their age ranged from 31 to 92 years with a mean of 65.13 (±13.2) years. The mean age for males and females was comparable; 65.3 (±13.7) and 64.92 (±12.8) years, respectively (p = 0.917).

Table 2 illustrates the clinical characteristics of lesions included in the study. Leukoplakia was the main clinical diagnosis with 42 (76.4%) lesions. Lateral and dorsal surfaces of the tongue were the most commonly affected sites (29.1%), followed by the floor of mouth and ventral surface of the tongue (18.2%).

Cytological diagnosis using OLBC evaluation showed that 40% of preparations were associated with undetermined significance. Only 1 preparation was inadequate for assessment and was excluded (**Table 2**). Final histopathological diagnosis revealed that nearly half of the lesions (45.5%) showed either ortho or parakeratosis without OED (**Table 2**). Gender was not associated with either cytological diagnosis or histopathological diagnosis (**Table 2**). The age of participants was significantly associated with histopathological diagnosis and cytological diagnosis; p = 0.049 and p= 0.036, respectively. There was no association between the age of participants and the provisional clinical diagnosis (p = 0.848).

CDK4, CDK6, Cyclin D1, and Notch 1 index scores were assessed based on both the intensity of staining and the percentage of positive cells. Out of 220 preparations that were used for protein expression, 18 slides (8.2%) were excluded because of inadequate cellularity.

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Nuclear and cytoplasmic staining of the studied proteins was observed in 80% of the stained slides, and the intensity of staining varied from weak to strong (**Figure 1**). There were significant positive correlations between the index scores of CDK4, CDK6, and Cyclin D1 and both the cytological and histopathological diagnoses in which the expression of these proteins increased with disease severity (**Figure 2**). In contrast, there were negative, non-significant associations between the Notch 1 index score and both the cytological and histopathological diagnoses (**Figure 2**). Further, the cumulative index score of the four proteins was associated weakly with the cytological diagnosis (p = 0.049), however there was no association between this index score and histopathological diagnosis (p = 0.298). When the index scores of CDK4 and CDK6 were combined, a significant association was found between the index score of CDK4/6 and both the histopathological and cytological diagnoses; p= 0.0001 and p= 0.0001 respectively.

A Pearson product-moment correlation revealed that there were no associations between the protein index scores and age of participants. Similarly, the same test was used to identify the associations between the index scores of all proteins. Accordingly, there was a strong, positive correlation between the index score of CDK4 and CDK6 (r=0.784, p=0.0005), and a moderate, positive correlation between CDK4 and Notch 1 (r=0.445, p= 0.004). Likewise, a strong posistive correlation was present between CDK4 and Cyclin D1 (r=0.534, p= 0.0001).

Cytological interpretation of OLBC using the Orcellex® brush demonstrated a significant association with the final histopathological diagnosis (p = 0.0005). **Table 3** demonstartes the sensitivity, specificity, PPV, NPV and accuracy of OLBC grading, cumulative protein index, CDK4/6 index, and combined OLBC grading and CDK4/6 index. Cytological interpretation using the Orcellex® brush achieved the highest sensitivity, specificity, PPV, NPV, and accuracy scores; 84.6%, 70.4%, 73.3%, 82.6%, and 78.8 %, respectively. The use of protein immunocytochemistry failed to improve the diagnostic accuracy of OLBC.

Discussion

The results of this study point to a disruption of the cell cycle during the development of oral leukoplakic lesions from non-dysplastic keratosis to OSCC. The frequency of CDK4, CDK6, Cyclin D1, and Notch 1 expression was found in nearly 80% of samples. A significant immuncytochemical overexpression of CDK4, CDK6, and Cyclin D1 was noted between non-dysplastic OLK to OSCC, indicating that these molecules may play a role in the development of OSCC where the level of expression is more critical than the presence or absence of expression. This finding is consistent with previous studies which have demonstrated an overexpression of CDK4 and/or CDK6 in OSCC.[15,16] The possible

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justification for this observation is that the increase of both CDK4 and CDK6 are attributable to a downregulation of the tumour suppressor genes that code for INK protein inhibitors, which bind directly to the CDK4/6-Cyclin D1 complex.[26] As the site is made available for substrates CDK4 and CDK6 to bind, aberrant phosphorylation of Rb and continuous cell cycle progression take place as a result. Therefore, dysplastic and cancerous tissues demonstrate increased CDK4 and CDK6 expression.[26]

In our cytological preparations, we demonstrated a positive nuclear and cytoplasmic staining pattern that is consistent with other studies that have used formalin-fixed paraffin-embedded tissue specimens.[17,27,28] Our study is the first to investigate the immunocytochemical expression of four proteins (CDK4, CDK6, Cyclin D1, and Notch 1) using OLBC preparations. This and previous studies support the notion that the use of immunocytochemistry with OLBC is feasible and can serve several applications.[8,9]

Poomsawat et al. showed that both CDK4 and CDK6 were overexpressed in OSCC, but no association was found between the two markers.[27] Furthermore, they found that CDK6 was overexpressed in OSCC alone whilst the overexpression of CDK4 was found in both mild dysplasia and OSCC indicating that CDK4 may play an early role in cancer development but CDK6 may have a late-stage role.[27] In contrary, current findings show that both CDK4 and CDK6 were significantly associated with each other. Moreover, both CDK4 and CDK6 were involved in a stepwise-progression model from early stages, which may indicate that these two proteins work in a synergistic pattern.

Similarly, the expression of Cyclin D1 increased with increasing grades of dysplasia towards OSCC, with the highest expression reported in OSCC samples. This result is concordant with a previous study by Patel et al.,[28] where they analysed 60 samples and found that the expression of p63 and Cyclin D1 increased as the grade of dysplasia increased.[28] Moreover, Cyclin D1 overexpression has been associated with factors that indicate a poor prognosis, such as lymph node involvement and lack of response to treatment.[29] In the present study, we were unable to check such a correlation due to the lack of clinical follow-up data. We also found that Cyclin D1 expression was significantly correlated with both CDK4 and CDK6 expression. Our findings are supportive of the notion that Cyclin D1 partners CDK4/6 in oral carcinogenesis,[29] however other reports have shown that overexpression of Cyclin D1 may contribute to tumour progression independent of CDK4.[16,30]

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Interestingly, the role of Notch 1 in oral carcinogenesis is still ambiguous. There is equivocal evidence and contradictory reports to suggest that Notch 1 may have a role as either oncogene or tumour suppressor gene.[20,21,23] Taken together, these observations indicate that the aberrant expression of Notch 1 has dissimilar roles in oral carcinogenesis, and there are other interactions within the tumour microenvironment which may influence or alter the expression of this protein. Our present study reveals that the expression level of Notch 1 was decreased from non-dysplastic tissue to OSCC, albeit the reduction was not statistically significant ($p = 0.177$). These findings may support the tumour-suppressing role of Notch 1 in oral malignant transformation. Further, our results perhaps suggest that the expression of Notch 1 is not a good indicator of OSCC development. Other studies support this notion. Sakamoto et al. demonstrated that neither Notch 1 expression nor its nuclear translocation was observed in OSCC cases.[23] Interestingly, Notch 1 down-regulation in OED and OSCC has not been well-understood and has not received much attention in the literature. Nonetheless, it is postulated that Notch 1 has two different functions. Firstly, it mediates the balance between populations of basal cells and differentiated cells in normal epithelium,[23] and secondly, it converts cells from a normal and mature state to an activated and immature state following its downregulation in pathological processes such as wound healing suggesting its essential role in maintaining epithelial integrity.[23] Further investigation is required to determine the exact role of this protein in oral carcinogenesis.

In line with previous studies,[7,10] the Orcellex® brush has been shown to be useful in harvesting transepithelial cells from oral lesions as there was significant correlation with the corresponding histopathological diagnosis. The sensitivity, specificity, PPV, NPV and accuracy of OLBC using the modified Bethesda system were satisfactory and consistent with previously published data.[7,10]

In this study, we attempted to assess the utility of using protein expression to improve the diagnostic accuracy of oral brush cytology in the diagnosis of OSCC and OED. Suprisingly, neither the cumulative protein score index nor the CDK4/6 score reached the accuracy values of OLBC grading alone. This may suggest that these proteins are not good candidates for use as biomarkers in the early detection of oral cancer, notwithstanding the limitations of the present study which include the relatively small sample size, the retrospective design, and the lack of clinical follow-up data.

In conclusion, this study has demonstrated the efficacy of the use of OLBC in the correct diagnosis of OED and OSCC, but was unable to show that CDK4, CDK6, Cyclin D1 or Notch 1 were useful surrogate biomarkers for the diagnosis of OSCC or dysplastic oral lesions.

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Further investigations are required to identify reliable biomarkers that can help in the risk stratification and malignant transformation of OPMDs.

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Conflict of interests

Authors declare no conflict of interests.

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Tables

Table 1: Criteria used to grade lesions using proposed index scores.

Index score	Criterion description
OLBC grading score	If the lesion is diagnosed as undetermined significance, a score of 3 is given
	If the lesion is diagnosed as low-grade squamous intraepithelial lesion, a score of 6 is given
	If the lesion is diagnosed as high-grade squamous intraepithelial lesion, a score of 9 is given
	If the lesion is diagnosed as OSCC, a score of 12 is given
Cumulative protein score	If the lesion has an average score between 0-3 for CDK4, CDK6, Cyclin D1, and NOTCH1 expression, it is graded as a non-OED lesion
	If the lesion has an average score between 4-6 for CDK4, CDK6, Cyclin D1, and NOTCH1 expression, it is graded as a low-grade OED lesion
	If the lesion has an average score between 7-9 for CDK4, CDK6, Cyclin D1, and NOTCH1 expression, it is graded as a high-grade OED lesion
	If the lesion has an average score between 10-12 for CDK4, CDK6, Cyclin D1, and NOTCH1 expression, it is graded as an OSCC
CDK4/6 score	If the lesion has an average score between 0-3 for CDK4 and CDK6 expression, it is graded as a non-OED lesion
	If the lesion has an average score between 4-6 for CDK4 and CDK6 expression, it is graded as a low-grade OED lesion
	If the lesion has an average score between 7-9 for CDK4 and CDK6 expression, it is graded as a high-grade OED lesion
	If the lesion has an average score between 10-12 for CDK4 and CDK6 expression, it is graded as an OSCC
Combined OLBC grading and CDK4/6 score	If the lesion has an average score between 0-3 for OLBC grading and CDK4/6 expression, it is graded as a non-OED lesion
	If the lesion has an average score between 3-6 for OLBC grading and CDK4/6 expression, it is graded as a low-grade OED lesion
	If the lesion has an average score between 7-9 for OLBC grading and CDK4/6 expression, it is graded as a high-grade OED lesion
	If the lesion has an average score of between 10-12 for OLBC grading and CDK4/6 expression, it is graded as an OSCC

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Table 2: Clinical, cytological and histopathological characteristics of lesions.

Lesion characteristic	Male n (%)	Female n (%)	Total n (%)	*p*-value
Lesion site				
Lateral/dorsum surface of the tongue	6 (10.9)	10 (18.2)	16 (29.1)	0.173
Floor of the mouth/ ventral surface of the tongue	5 (9.1)	5 (9.1)	10 (18.2)	
Buccal mucosa	8 (14.5)	1 (1.8)	9 (16.4)	
Hard/soft palate	4 (7.3)	3 (5.4)	7 (12.7)	
Maxillary tuberosity and retromolar pad area	2 (3.6)	2 (3.6)	4 (7.3)	
Lingual/alveolar mucosa	4 (7.3)	1 (1.8)	5 (9.1)	
Labial mucosa/ lip	1 (1.8)	3 (5.4)	4 (7.3)	
Provisional clinical diagnosis				
Leukoplakia	22 (40)	20 (36.4)	42 (76.4)	0.828
Erythroplakia	2 (3.6)	1 (1.8)	3 (5.4)	
Squamous cell carcinoma	6 (10.9)	4 (7.3)	10 (18.2)	
Conventional cytology				
Undetermined significance	13 (23.6)	9 (16.3)	22 (40)	0.272
Low grade squamous interepithelial lesion	9 (16.4)	3 (5.5)	12 (21.8)	
High grade squamous interepithelial lesion	5 (9.1)	7 (12.7)	12 (21.8)	
Squamous cell carcinoma	3 (5.5)	5 (9.1)	8 (14.5)	
Inadequate	0 (0)	1 (1.8)	1 (1.8)	
Final histopathological diagnosis				
Ortho/parakeratosis without dysplasia	14 (25.5)	11 (20)	25 (45.5)	0.483
Low-grade OED	7 (12.7)	3 (5.5)	10 (18.2)	
High-grade OED	4 (7.3)	7 (12.7)	11 (20)	
Differentiated OSCC/Carcinoma in situ	5 (9.1)	4 (7.3)	9 (16.4)	
Total	**30 (54.5)**	**25 (45.5)**	**55 (100)**	

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Table 3: Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of score indices used in the diagnosis of oral leukoplakia and OSCC.

Index	Sensitivity (%)	Specificity (%)	PPV (%)*	NPV (%)**	Accuracy (%)
OLBC grading score	84.6	70.4	73.3	82.6	78.8
Cumulative protein score	10.3	69.6	30	38.1	36.5
CDK4/6 score	11.1	95.4	75	46.7	49
Combined OLBC grading and CDK4/6 score	50	66.7	60	88.9	58.3

*PPV: positive predictive value, **NPV: negative predictive value.

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Figure legends

Figure 1: Nuclear and cytoplasmic immunocytochemical staining (x200), black arrows refer to strong positive stained cells and red arrows refer to negative stained cells. a) CDK4 staining, b) CDK6 staining, c) Cyclin D1 staining, d) Notch1 staining, e) negative staining.

Figure 2: The association between studied proteins (mean IHC score ± SD) and the definitive histopathological (a) and cytological (b) diagnosis. *OED: oral epithelial dysplasia, **OSCC: oral squamous cell carcinoma, SIL*: Squamous intraepithelial lesion.

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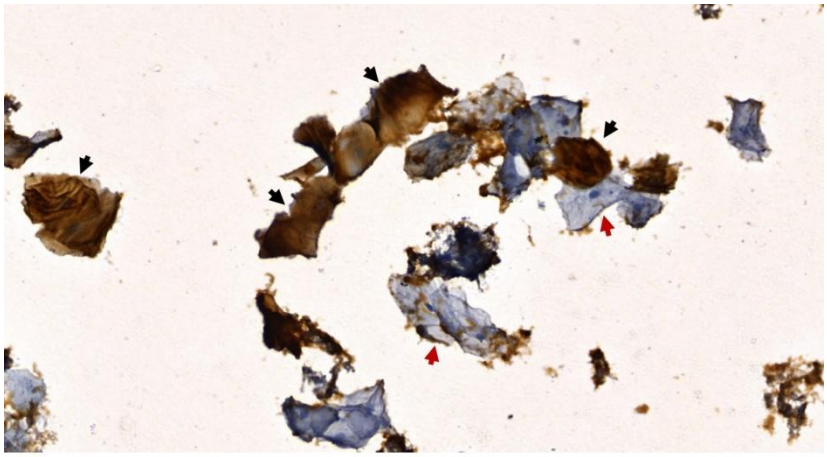

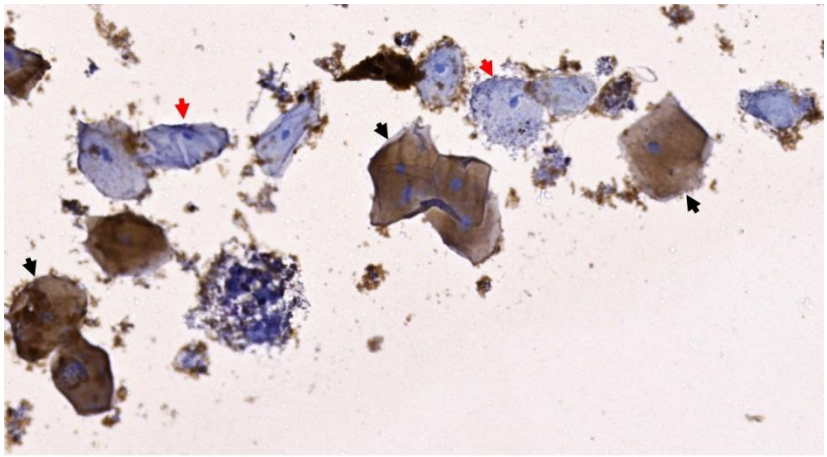

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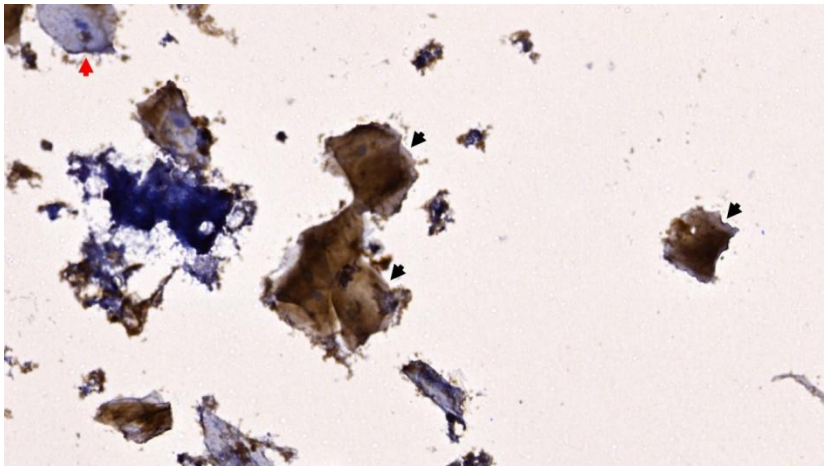

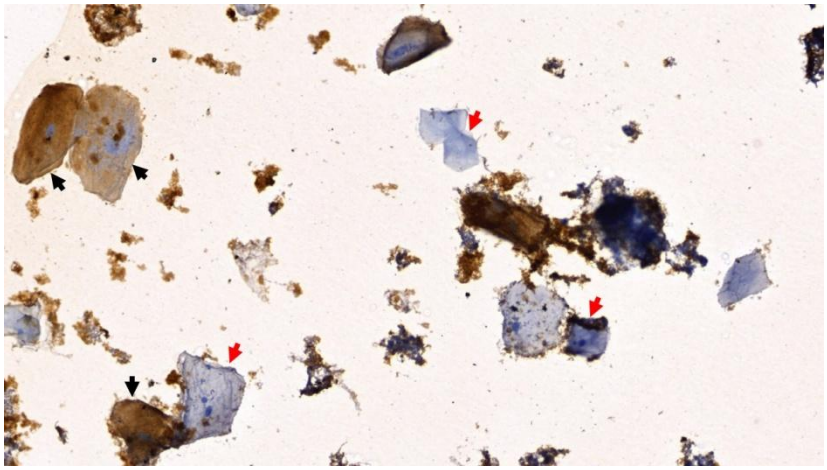

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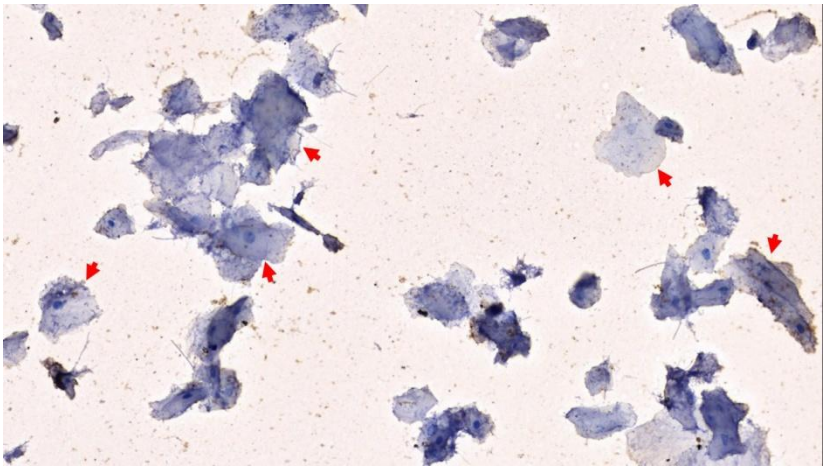

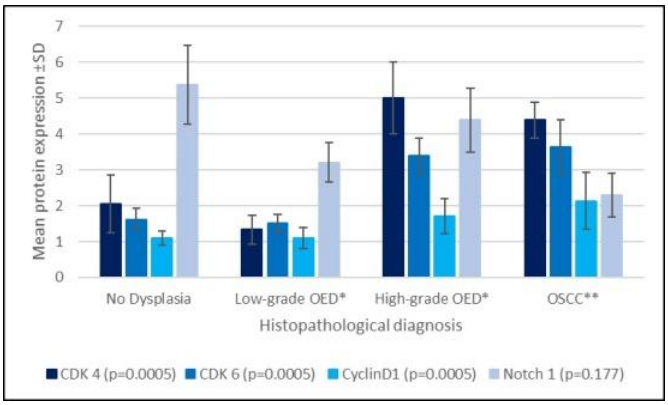

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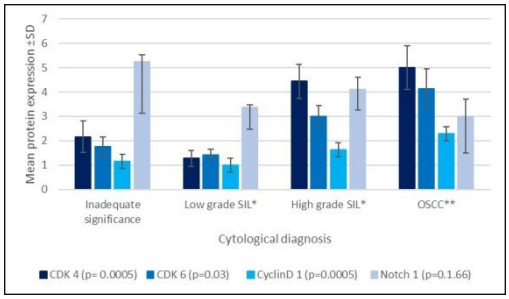

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