RESEARCH ARTICLE

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Expression of CLLD7 and CHC1L Proteins in Oral Epithelial Dysplasia in a Group of Thai Patients

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Abstract

Objectives: Previous study showed aberrant CLLD7 and CHC1L protein expression in oral squamous cell carcinoma (OSCC) compared to normal oral mucosa (NOM). This study aimed to evaluate the expression of these proteins in oral epithelial dysplasia (OED). **Materials and Methods:** Forty specimens of OED and 11 NOM were used. The expression of CLLD7 and CHC1L was determined by immunohistochemistry. In each case, at least 1000 cells were counted. Presence of nuclear, cytoplasmic, and/or membrane staining of CLLD7 and CHC1L were considered positive. Percentages of total positive cells and positive cells at different locations were recorded. SPSS version 18 was used to compare variation between groups with statistical significance at $p<0.05$. **Results:** No significant differences in the percentages of total positive cells of CLLD7 and CHC1L were found between NOM and all grades of OED. Nevertheless, there were significant differences in subcellular staining of these two proteins. In CLLD7, the nuclear staining of the moderate and the severe OED groups was significantly lower than that of the NOM group ($p<0.05$). The percentages of membrane staining of CHC1L in moderate and severe OED were significantly higher than that of NOM ($p<0.001$). In addition, the nuclear staining of CHC1L in each grade of OED was significantly lower than that of NOM ($p<0.05$). **Conclusion:** The subcellular mislocalization of CLLD7 and CHC1L in OED suggests that the expression of these potential tumor suppressor proteins might be dysregulated during the dysplastic process. The distinct membrane staining of CHC1L observed in OED but not in NOM is a useful characteristic that can be used to separate OED from NOM. Thus, CHC1L may be a good marker to assist in the diagnosis of OED.

Keywords: Normal oral mucosa- oral epithelial dysplasia- CLLD7- CHC1L- cancer

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Introduction

Oral epithelial dysplasia (OED) has been defined as "a spectrum of architectural and cytological epithelial changes caused by an accumulation of genetic change, associated with an increased risk for progression to squamous cell carcinoma" [1]. In the oral epithelial tissues, accumulating mutations, chromosomal damage, and loss of cellular control functions induce the transition from normal histology to dysplasia, to superficial cancer and invasive squamous cell carcinoma [2]. According to the malignant transformational potential of OED, early detection of molecular changes in OED may lead to a significant profit in disease surveillance. Markers of proliferation could potentially be good candidates for improving the prognostic evaluation of OED and oral squamous cell carcinoma (OSCC) [2]. Until now, a panel of molecular markers that allows for a prognostic prediction of OED and OSCC has not yet been determined [3].

Chronic lymphocytic leukemia deletion gene 7 or *CLLD7* or *RCBTB1* gene is a candidate tumor suppressor gene located on chromosome 13q14 close to the *pRB (retinoblastoma tumor suppressor)* locus. It was first isolated by Mabuchi et al. in 2001 [4]. *CLLD7* encodes an evolutionarily conserved protein that contains an RCC1 (regulator of chromatin condensation 1) domain at the NH2–terminus with broad complex, tramtrack, bric-a-brac (BTB) and POZ domains at the COOH-terminus [5]. A study suggested that CLLD7 expression is decreased in colon cancer, cervical cancer, and lymphoma cell lines [6]. The induction of CLLD7 inhibits cell growth, decreased

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cell viability, and indicates activation of the DNA damage/repair pathway [6]. However, the association between CLLD7 expression in OED is yet to be investigated.

CHC1L, also known as *RCBTB2* or *Chromosome Condensation 1-like* gene, was first discovered by Devilder et al. in 1998 [7]. CHC1L is also characterized as having RCC1 and BTB domains. It is located on the 13q14.2 region telomeric to the *pRB* gene, which contains deletion hotspots in prostate cancer, multiple myeloma, and chronic lymphocytic leukemia [8, 9]. CHC1L might have a regulatory function in intracellular transport and the transfer of anti-oncogenic signals, thereby affecting the growth of tumor cell [7]. However, there is still inadequate evidence that supports the role of CHC1L in the pathogenesis of OED.

In our previous study, CLLD7 and CHC1L proteins were investigated in OSCC compared to normal oral mucosa (NOM) [10]. The expression of CLLD7 and CHC1L proteins was reduced in OSCC. Analysis of CLLD7 localization revealed predominant nuclear staining at basal and parabasal areas in NOM, whereas more cytoplasmic staining was observed in OSCC. For CHC1L, nuclear staining was prominent in NOM. In contrast, significantly increased plasma membrane and decreased nuclear staining was detected in OSCC. Since these proteins might play some roles in oral malignant transformation, studies regarding the expression of these proteins in OED seem to be of benefit and have never been reported elsewhere. Therefore, this study aimed to evaluate the expression of CLLD7 and CHC1L in OED and to assess the roles of these proteins in the diagnostic and prognostic value of OED. This may indicate whether CLLD7 and CHC1L have tumor suppressor roles in the pathogenesis of OSCC.

Materials and Methods

Ethical approval

This laboratory-based experimental study was performed at the Oral and Maxillofacial Pathology laboratory. Ethical approval was obtained from the committee on Human Rights Related to Human Experimentation (MU-DT/PY-IRB 2020/059.2909 and MU-DT/PY-IRB 2019/041.0307). The ethical guidelines of the Declaration of Helsinki were followed and informed consent was obtained from all subjects involved in the study.

Tissue samples

Forty specimens from oral cancer and oral potentially malignant disorders (OPMDs) screening for participants who reside in Northeastern Thailand, were assessed. All specimens were stored at the Department of Oral and Maxillofacial Pathology, Faculty of Dentistry. The clinical diagnosis of OPMDs during the screening was based on a consensus report from an international seminar on nomenclature and classification, convened by WHO Collaborating Center for Oral Cancer [11] including oral leukoplakia, oral erythroplakia, oral submucous fibrosis, oral lichen planus, oral lichenoid reaction, oral lupus erythematosus, and actinic cheilitis. However, in this study, merely specimens with the clinical diagnosis of leukoplakia and erythroplakia were taken for further analysis. In addition, only the specimens with histopathological diagnosis of epithelial dysplasia (with all different grading including mild, moderate, and severe) by a board-certified oral pathologist were included. The specimens which had been diagnosed histopathologically as epithelial dysplasia combined with any other conditions, such as lichen planus, lichenoid lesion, and hyperplastic candidiasis, were excluded from this experiment. An authorized officer at the Department of Oral and Maxillofacial Pathology under the permission of the Head of the Department, retrieved the information. Eleven normal control tissues were obtained from the flap or tissue left over from the removal of impacted 3rd molars from patients who attended the Faculty of Dentistry, Mahidol University. Only the samples of normal oral mucosa (NOM) that did not show any signs of inflammation on both clinical and histological examinations were included. The biopsy tissue with a layer of epithelium and/or underneath the submucosa that was completely lost or tissue that was too small to be processed was also excluded. In conclusion, 51 specimens in total were used.

Immunohistochemistry (IHC)

The IHC staining procedure was done over a period of two days. Approximately 3 μm thick tissue sections were made using a tissue microtome and mounted on APES (amino-propyl-tri-ethoxy-silane) coated slides. Tissue sections were rehydrated by washing in serial dilutions of ethanol and incubated at room temperature using 3% hydrogen peroxide (H_2O_2). Heat-induced epitope retrieval with household microwave (850 Watts) and Target Retrieval Solution 10X concentration (Dako North America, Inc. Carpinteria, California, USA.) diluted 10 times was used for antigen retrieval. The slides were blocked in 5% bovine serum albumin (BSA) inside a humidifier at room temperature. Primary antibody diluted in commercially available diluent (Dako Antibody Diluent, Dako North America, Inc. Carpinteria, California, USA.) was applied over the tissue sections. The primary antibody to the diluent ratio for CLLD7 (ab233533, Abcam, Cambridge, UK) and CHC1L (ab175505, Abcam, Cambridge, UK) was 1:800. Horseradish peroxidase (HRP)-conjugated secondary antibody (Dako REALTM EnVisionTM/HRP, Rabbit/Mouse (ENV), Dako, Denmark) was applied over the slides for 30 mins at room temperature. The color was developed with 3,3'-Diaminobenzidine (DAB) chromogen at room temperature for 30 seconds for CLLD7 and CHC1L. After counterstaining with hematoxylin, the tissue sections were dehydrated. Then slides were allowed to dry. After applying the mounting medium, the sections were observed under a light microscope.

Evaluation of CLLD7 and CHC1L expression

Photographs of five fields using a light microscope (X400 magnification) were randomly selected for each case. The numbers of both total positive cells and total cells in each photograph were counted using ImageJ software. At least 1,000 cells were counted in each case. In addition, the number of total positive cells and

subcellular staining pattern (nuclear staining, cytoplasmic staining, and/or membrane staining) were recorded. A board-certified oral pathologist trained the researcher to evaluate the cell counting and subcellular staining pattern.

Statistical analysis

All data were processed and analyzed using SPSS version 18.0 (SPSS Inc., Chicago, IL., USA). Descriptive statistics were used to analyze demographic, clinical, and histopathologic data. All data's normality was tested by the Kolmogorov-Smirnov test. The comparisons of the percentage of total positive cells and pattern of subcellular staining between NOM and OED (all grades) were carried out using the Independent samples t-test or Mann-Whitney U test according to the normality of the data. The comparisons among three grades of OED (mild, moderate, severe) were performed using one-way ANOVA or Kruskal-Wallis H test according to the normality of the data. Whenever the data showed normal distribution, results were expressed in mean and standard error of mean (SEM); otherwise, median and interquartile range (IQR) were used. Significant differences are established at $p < 0.05$.

Results

Demographic data of the patients

Fifty-one biopsied specimens were obtained. Eleven NOM and forty OED specimens were used for IHC analysis. The cases involved in this study were clinically diagnosed as oral leukoplakia (30 cases) and oral erythroplakia (10 cases). According to the histopathological diagnosis, there were 17 mild OED, 16 moderate OED, and seven severe OED specimens in the OED group. The demographic data are summarized in Table 1.

Pattern of CLLD7 and CHC1L staining

CLLD7

According to the immunohistochemistry, all cases in both NOM and OED were positive for CLLD7. The expression of CLLD7 was detected as golden-brown color in the nucleus and cytoplasm of cells. Generally, the membrane staining was negligible. However, the protein showed nuclear and cytoplasmic stainings in all cell layers (basal, prickle, and granular). In NOM, pale nuclear and cytoplasmic stainings were found in all cases (Figure 1A). In OED, nuclear and cytoplasmic stainings were both observed. However, nuclear staining was less frequently noted, whereas pale cytoplasmic staining was more commonly found in OED compared with NOM (Figures 1B, 1C, and 1D). Different grades of OED similarly showed nuclear and cytoplasmic staining patterns, while nuclear staining was less obvious in high-grade dysplasia compared with the low-grade.

CHC1L

All cases of NOM and OED were positive for CHC1L staining. CHC1L was observed as dark brown color in the nucleus, cytoplasm, and membrane of cells in all cell layers of NOM and OED. In NOM, all cases showed nuclear and cytoplasmic staining in all cell layers of the epithelium. Membrane staining was also detected but only in a small number of cells in each case (Figure 2A). In OED, nuclear and cytoplasmic stainings were drastically decreased and less often observed. Interestingly, membrane staining became predominant (Figures 2B, 2C, and 2D). Different grades of OED showed similar patterns of staining.

Percentage of positive CLLD7 and CHC1L cells

CLLD7

The average number of cell counts in NOM and OED were 2,607 and 2,034, respectively. There were no significant differences in the percentages of total positive cells, and the percentages of subcellular localization

Table 1. General Characteristics of the Specimens by Tissue Types

Characteristics	Sex (M/F)	Age Mean± SD; range (years)	Site	Associated risk factors
NOM (n=11)	6/5	23.67±5.96; 15-37	Pericoronal tissue of 3rd molar Right Mandible – 5 Left Mandible – 3 Right Maxilla – 2 Left Maxilla – 1	None
OED (Mild, n=17)	9/8	56.5±7.23; 48-63	Lateral tongue – 1 Ventral tongue – 1 Dorsum tongue – 2 Gingiva – 2 Hard palate – 4 Buccal mucosa – 4 Retro commissural area – 2 Lower lip – 1	Smoking – 4 (23.52%) Smoking with alcohol consumption – 1 (5.88%) Betel nut chewing – 2 (11.76%) Ill-fitting denture – 1 (5.88%) Defective restoration – 1 (5.88%) Sunlight – 1 (5.88%) None – 7 (41.18%)
OED (Moderate, n=16)	3/13	70.7±10.33; 55-90	Lateral tongue – 2 Gingiva – 2 Buccal mucosa – 8 Retro commissural area – 1 Upper lip – 1 Lower lip – 2	Betel nut chewing – 6 (37.5%) Defective restoration – 1 (6.25%) None – 9 (56.25%)
OED (Severe, n=7)	2/5	53.8±4.82; 46-59	Lateral tongue – 2 Ventral tongue – 1 Buccal mucosa – 4	Betel nut chewing – 1 (14.29%) None – 6 (85.71)

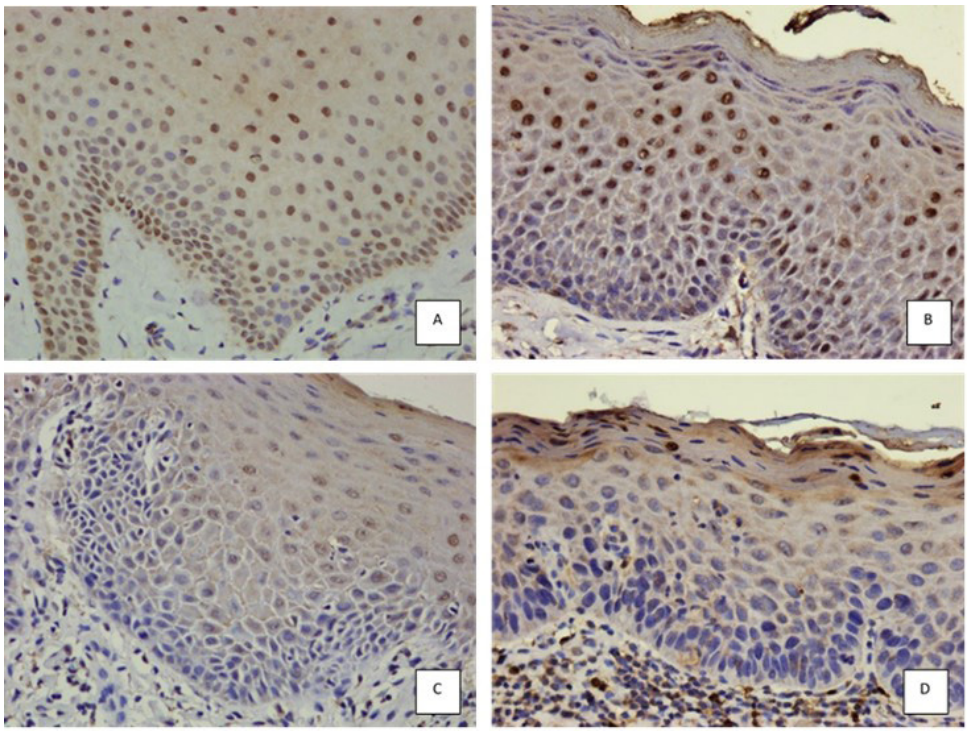

Figure 1. Representative Area of Immunostaining for CLLD7, Original Magnification: X400. A: Normal oral mucosa shows nuclear and cytoplasmic staining. B: Mild OED, C: Moderate OED, and D: Severe OED. In OED, nuclear staining is gradually decreasing.

between NOM and all grades of OED (Figure 3A and Supplementary file 1). However, when different grades of OED were compared with NOM, there were significant differences in the percentages of cells with nuclear staining between the moderate and the severe OED groups compared to the NOM group (Figure 4A and Supplementary file 2). There were no significant differences in the percentages of total positive cells and the percentages of subcellular expression when the three groups of OED were compared (Supplementary file 3).

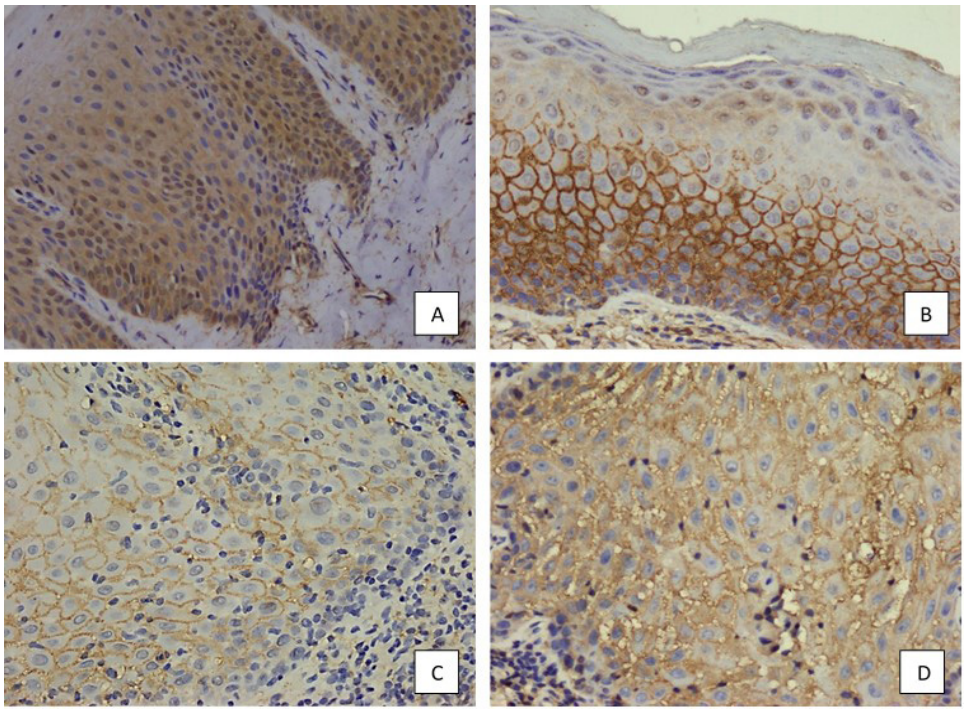

Figure 2. Representative Area of Immunostaining for CHC1L, Original Magnification: X400. A: Normal oral mucosa obviously shows nuclear and cytoplasmic staining. B: Mild OED, C: Moderate OED, and D: Severe OED. In OED, membrane staining is predominant and nuclear staining is less often observed.

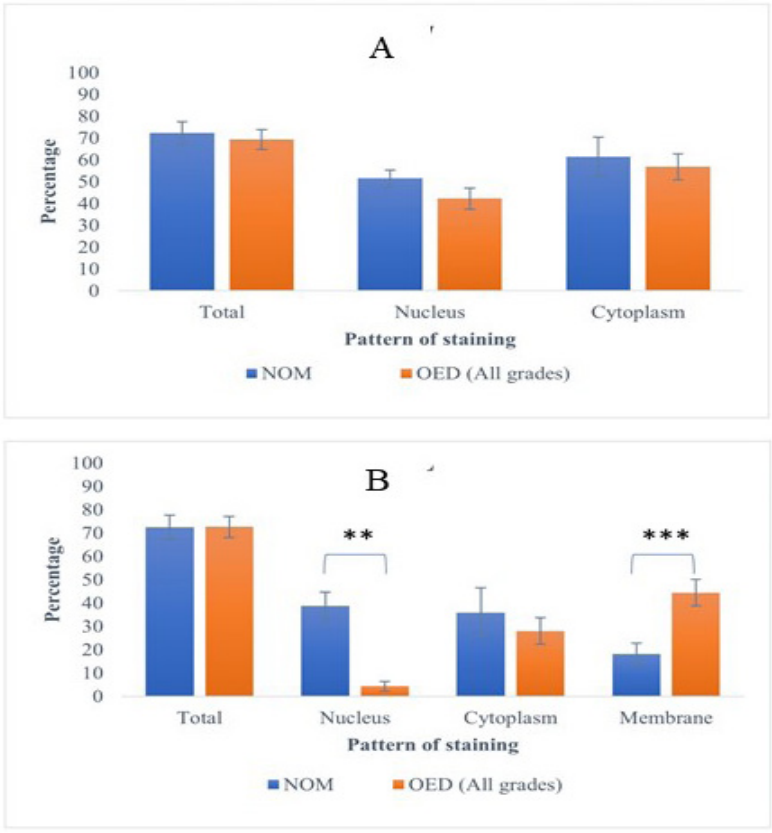

Figure 3. The Comparison of Percentages of Total Positive Cells and Subcellular Expression of CLLD7 (A) and CHC1L (B) between NOM and All Grades of OED. **p<0.001, ***p=0.001

CHC1L

The average number of cell counts in NOM and OED were 2,633 and 2,073, respectively. No significant difference in the percentages of total positive cells between NOM and all grades of OED was found (Figure 3B and Supplementary file 1). The percentage of membrane

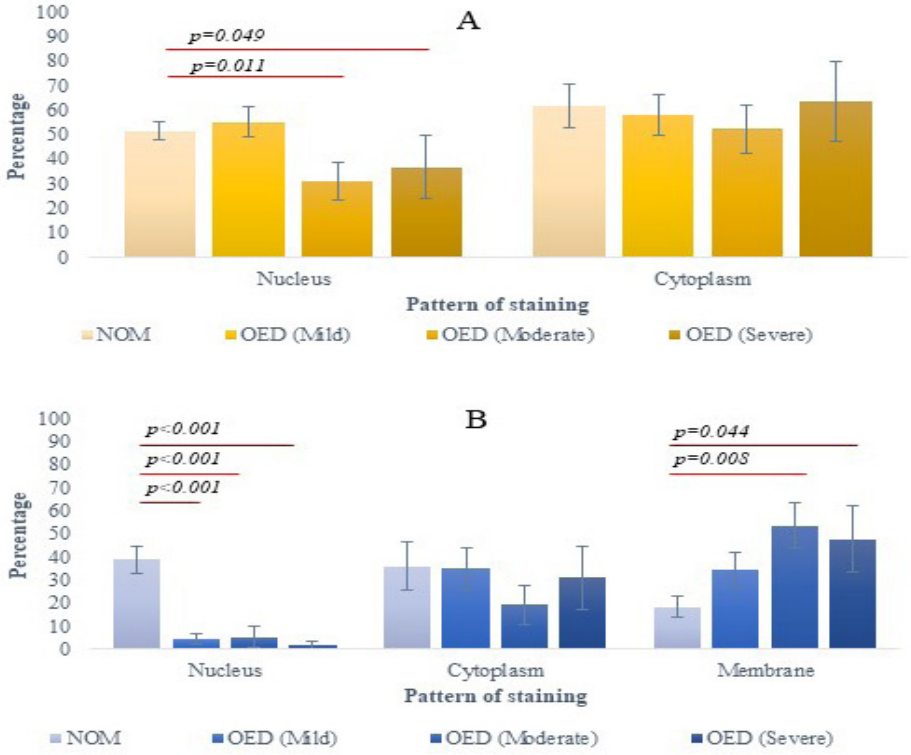

Figure 4. The Comparison of Subcellular Expression of CLLD7 (A) and CHC1L (B) between Each Grade of OED and NOM.

staining in all grades of OED was significantly higher than those in NOM (Figure 3B and Supplementary file 1). In contrast, the percentage of nuclear staining in all grades of OED was significantly lower than those in NOM (Figure 3B and Supplementary file 1). For cytoplasmic staining, there was no significant difference between the OED (all grades) and the NOM groups. When different grades of OED were compared to NOM, a significantly lower number of cells with nuclear staining was observed in the mild, the moderate, and the severe OED groups than in the NOM group (Figure 4B and Supplementary file 2). A significantly higher number of cells with membrane staining was observed between the moderate and the severe OED groups compared to the NOM group (Figure 4B and Supplementary file 2). There were no significant differences in the percentages of total positive cells and the percentages of subcellular expression when the three groups of OED were compared (Supplementary file 3).

Discussion

In our previous study, CLLD7 and CHC1L expression was examined in OSCC. Aberrations in the expression of these proteins were observed [10]. In this study, the expression of these 2 proteins was investigated in OED to verify the role of these 2 proteins in oral malignant transformation.

CLLD7 locates on the 13q14 deletion hotspot region of the chromosome and is inferred as a potential tumor suppressor gene for various tumors [12, 13]. Our previous study in OSCC demonstrated that the expression of CLLD7 was reduced in OSCC [10]. In this study, the percentages of total positive cells in all grades of OED were slightly lower than those in NOM with no statistical difference (Figure 3A), and there was no significant difference in total positive cells among various grades of OED (Supplementary file 3). According to the outcome, we may assume that the expression of CLLD7 might not yet decrease in the NOM-OED turning process.

Regarding the pattern of subcellular staining of CLLD7, in our previous study, nuclear staining was less frequently noted, whereas cytoplasmic staining was more commonly found in OSCC compared to NOM [10]. In this present study, there was no significant difference in the percentages of subcellular expression between NOM and all grades of OED (Figure 3A). However, in further analysis, we found that the percentages of nuclear staining in moderate and severe OED were significantly lower than those in NOM (Figure 4A), which might support the concept of tumor suppressor protein mislocalization [14]. One of the examples of tumor suppressor protein mislocalization that could affect tumor development is p53. To regulate the target gene transcription, p53 has to be translocated into the nucleus when DNA damage occurs, or when cellular stress reaches a dangerous level [15]. Thus, the nucleus is the primary functional site of p53. However, increased levels of wild-type p53 in the cytoplasm and a lack of nuclear staining were reported in various types of tumors [16].

Similarly, *CLLD7* encodes a protein that contains an RCC1 domain [5], which serves as a guanine nucleotide exchange factor (GEF) for the Ras-related nuclear protein (Ran) GTPase. Therefore, the nucleus is supposed to be the primary site of CLLD7. Our previous study demonstrated the reduction of nuclear staining of CLLD7 and increase cytoplasmic staining of this protein in OSCC [10]. The lower nuclear staining in moderate and severe OED could imply that dysregulation of CLLD7 has already occurred in OED. Nevertheless, to confirm this concept, future studies at gene and protein levels are tremendously important to verify the nature of this protein, and its nucleocytoplasmic transportation, interaction, and mechanism.

CHC1L is a candidate tumor suppressor in prostate cancer [8], multiple myeloma [17], and chronic lymphocytic leukemia [9]. Our previous study demonstrated the reduction of the expression of CHC1L in OSCC [10]. In this present study, we found that there was no significant difference in total positive cells between NOM and all grades of OED (Figure 3B), and among the different grades of OEDs (Supplementary file 3). As no reduction of this protein in OED but reduced expression in OSCC, it might be postulated that the deletion of this protein occurred at the late state of malignant transformation.

Interestingly, the analysis of the subcellular staining revealed that membrane staining of CHC1L was predominant, while nuclear staining was less often observed in OED (Figures 3B and 4B). This aberration was also found in OSCC in our previous study [10]. The mislocalization of this protein can be observed as early as in mild OED although statistically significant differences were observed in moderate and severe OED compared to NOM (Figures 3B, 4B and Supplementary files 1 and 2). Our results support the concept of protein mislocalization of CHC1L during malignant transformation. Protein synthesis primarily occurs in the cytosol and is then transported to their functional sites, such as the nucleus, plasma membrane, mitochondria, and other organelles. In cancer cells, several mechanisms are responsible for the dysregulation of protein trafficking, which leads to the abnormal subcellular localization of proteins. The mechanisms include mutation of protein-targeting signals, dysregulation of transporter machinery, aberrant endocytosis and vesicular trafficking, dysregulation of signal transduction and protein post-translational modification, alteration of protein–protein interactions, and cross-regulation of cancer-related proteins [14].

Similar to *CLLD7*, *CHC1L* encodes a protein that contains an RCC1 domain [5], hence, the nucleus is supposed to be the primary site of CHC1L as well. The lower nuclear staining in OSCC and OED could imply that dysregulation of CHC1L has already occurred during the step of the OSCC malignant transformation. In addition to the RCC1 domain, a part of its structure, the BTB domain, which facilitates protein-protein interactions and works as an adaptor for cullin-3-based ubiquitin ligases [18-20], might take a role in these processes. The ubiquitin ligases E3 or E3s were considered the only subjects to regulate the processes of ubiquitination of membrane proteins. There are many different types of E3s, each of them specific to its substrates, which could be found in any part of the

cells, such as the nucleus, cytoplasm, and membrane. In the scope of membrane localization, the E3s were found to regulate the membrane protein trafficking and degradation of membrane proteins [21]. This finding could support the evidence of membrane localization of CHC1L. Therefore, in NOM, it might be possible to observe CHC1L localized in the membrane (from the BTB domain) along with in the nucleus (from the RCC1 domain). However, when the nuclear function was compromised in OED and OSCC, the nucleocytoplasmic transportation might be interrupted and lead to decrease nuclear expression, whereas plenty of storage of this protein may lead to increase membrane expression. As mentioned above, this might explain why the membrane expression was predominant in OED. Similar to CLLD7, future studies at gene and protein levels are needed to confirm the mechanism of aberration of this protein in OED.

According to the present study and our previous study, non-significant changes of CLLD7 and CHC1L in total positive cells were observed in OED but significant reductions of both proteins were revealed in OSCC [10]. This might demonstrate that CLLD7 and CHC1L could involve in multistep cell dysregulation before OSCC occurs. It is interesting that although CLLD7 and CHC1L proteins belong to the RCC1 superfamily of proteins and both possess RCC1 and BTB/POZ domains, the aberration of these proteins was observed in different patterns in OED and OSCC. This might be because each protein may have a different function and different types of aberration may occur in different types of tumors. Therefore, the signs of protein mislocalization in CHC1L and CLLD7 might be promising predictive factors of OED. However, due to the small amount of evidence in the mislocalization concept of these proteins, further studies such as biochemical basis and biological consequences are necessary to determine the actual functions of these proteins.

We acknowledged the limitation of this study in that the sample size of OED specimens was low. As a consequence, further investigation into more OED specimens along with OSCC is still needed. In addition, aberration of *CLLD7* and *CHC1L* genes should be investigated in future studies.

In conclusion, CLLD7 and CHC1L subcellular mislocalization in OED suggests that the expression of these potential tumor suppressor proteins might be dysregulated during the formation of OED. Reduction of nuclear staining of CLLD7 has been observed in moderate and severe OED. The distinct membrane staining of CHC1L observed in each grade of OED but not in NOM is a useful characteristic that can be utilized to separate OED from NOM. Thus, CHC1L may be a good marker to assist in the diagnosis of OED. Further studies regarding the mechanisms of CLLD7 and CHC1L mislocalization are still needed to clarify the tumor suppressor role of these proteins.

Author Contribution Statement

The study was conceptualized by SPK, who was also responsible for establishing the methodology. The immunohistochemistry was carried out by PW, RJ, SP, and OM. The data analysis was performed by PW, SP, and SPK. The resources for the study were obtained by BK, WB, and SPK. The original draft writing was done by PW and editing and revision were carried out by BPB, SP, WB, and SPK. The funding was acquired by BK and SPK. All authors read and approved the final manuscript.

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Ethical Declaration

This study was approved by the Faculty of Dentistry/ Faculty of Pharmacy, Mahidol University, institutional Review Board (COA.No.MU-DT/PY-IRB 2020/059.2909 and COA.No.MU-DT/PY-IRB 2019/041.0307) and was performed following the ethical standards of International Guidelines for Human Research Protection such as Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP). Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

The datasets used and/or analyzed during the current study are not publicly available due to the confidentiality of the participants but are available from the corresponding author at reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

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