Original Article

Expression of CLLD7 and CHC1L Proteins in Oral Potentially Malignant Disorders in A Group of Thais: A Preliminary Study

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Abstract

Chronic lymphocytic leukemia deletion 7 (CLLD7) and chromosome condensation 1-like (CHC1L) proteins are putative tumor suppressor proteins that have never been studied in oral potentially malignant disorders (OPMDs). This study aimed to evaluate the expression of these two proteins in OPMDs which encompassed oral leukoplakia, oral lichen planus (OLP), and oral lichenoid lesions (OLL). The histopathological features of oral leukoplakia were acanthosis with or without hyperkeratosis and mild to severe epithelial dysplasia. Therefore, five groups including acanthosis with or without hyperkeratosis, mild, moderate, and severe dysplasia and the last group OLP/OLL were subjected to immunohistochemistry using normal oral mucosa as a control. In each case, random areas were selected and photographed after immunohistochemistry, then at least 1000 cells were counted. For CLLD7 and CHC1L, nuclear, cytoplasmic, and/or membrane staining were considered positive. Positive cells at different locations were evaluated. SPSS version 18 was used to compare the variation of protein expression among groups with a statistical significance at $p<0.05$. CLLD7 and CHC1L proteins were expressed in all cases of NOM and OPMDs. Compared to the acanthosis group, nuclear staining of CLLD7 was significantly lower in the severe epithelial dysplasia and the OLP/OLL groups. Although increased cytoplasmic staining of CLLD7 was observed in all OPMDs groups compared to the NOM group, a statistically significant difference was observed between the mild and moderate epithelial dysplasia groups compared to the NOM group. Regarding CHC1L staining, the percentage of nuclear staining was reduced, whereas membrane staining was increased in all OPMD groups compared to the NOM group. However, a statistically significant difference was observed between the severe epithelial dysplasia and OPL/OLL groups compared to the NOM group. In conclusion, the altered subcellular localization of CLLD7 and CHC1L in OPMDs suggests that the expression of these putative tumor suppressor proteins might be dysregulated during the early malignant transformation processes of oral cancer.

Keywords: CLLD7, CHC1L, Normal oral mucosa, Oral epithelial dysplasia

Received date: **Revised date:** **Accepted date:**
Doi:

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Introduction

The International Agency for Research on Cancer (IARC) reported that lip and oral cavity cancers accounted for 377,713 cases and 177,757 deaths worldwide in 2020.[1] In the same year, in Thailand, lip and oral cavity cancers ranked as the tenth most common cancer, 2.5 % of all new cancer cases.[2] The most common malignant tumor of the oral cavity was oral squamous cell carcinoma (OSCC).[3,4] Northeastern Thailand is the region where the incidence of oral cancer is relatively high[5,6] and strongly associated with tobacco smoking, alcohol use, and betel nut chewing habits.[7] These risk factors have some ingredients and metabolites which can gradually cause normal tissues to transform into oral potentially malignant disorders (OPMDs) and malignant tumors.[8]

As suggested by the WHO, OPMDs are any clinical presentations or conditions of tissue alteration that are hazardous to becoming cancers.[9] Therefore, early detection of OPMDs leads to confirmatory investigations and timely appropriate treatments. OPMDs consist of oral leukoplakia, oral erythroplakia, palatal lesions in reverse smokers, oral submucous fibrosis, oral lichen planus (OLP), oral lichenoid lesion (OLL), discoid lupus erythematosus, actinic cheilitis, and inherited cancer syndromes.[9] Regarding leukoplakia, it could be histopathologically diagnosed as acanthosis with or without hyperkeratosis, mild or moderate or severe epithelial dysplasia. Therefore, leukoplakia specimens were selected for this study. In addition, OLP/OLL were also included since it is one of the OPMDs.

Clinical and/or histopathological examinations are not always enough to prove the malignant potentials of OPMDs; investigations at a molecular level will complement these methods to confirm occurrences of genetic alterations more precisely.[10] This comprehensive integration is based on the biological fact that cumulative alteration of subcellular structures such as DNAs, RNAs, and proteins influences the replication and differentiation of normal cells to progress to OPMDs and invasive cancer.[11]

RCBTB proteins (Regulator of chromatin condensation 1 and Broad complex, Tramtrack and Bric à brac domain-containing proteins) belong to one of the subgroups of proteins under RCC1 (Regulator of Chromatin Condensation 1) superfamily proteins, which function as a GEF (guanine nucleotide-exchange factor) of Ran (Ras-related nuclear protein). Ran acts as a biological switch cycling between GTP-bound "on" and GDP-bound "off" states. Thus, RCC1 operating through Ran plays an essential role in cell cycle regulation, chromatin condensation, nucleocytoplasmic transport, mitotic spindle formation, and nuclear envelope assembly.[12] There are three members of RCBTB proteins, namely, CLLD7 (RCBTB1), CHC1L (RCBTB2), and IBtk (inhibitor of Bruton's tyrosine kinase), but in this study, only CLLD7 and CHC1L will be investigated.

Mabuchi *et al.* created a high-resolution physical map of chromosome 13q14 covering the critically deleted region in B-cell chronic lymphocytic leukemia[13] and identified three novel genes that were *CLLD6, CLLD7,* and *CLLD8.* The *CLLD7* gene encodes the CLLD7 protein containing the RCC1 domain at the NH2 terminus and the broad complex, tramtrack, and bric-a-brac (BTB) domain at the COOH terminus.[13] These structural components give the CLLD7 protein the official name RCBTB1 (RCC1 and BTB domain-containing protein 1).[13] In addition, Zhou and Munger investigated the biological roles of CLLD7 in different human cancer cell lines. They reported a decreasing expression of CLLD7 in human cancer cell lines, which subsided in cell apoptosis.[14] Thus, it is possible that CLLD7, as a novel protein involved in cell cycle mechanism, and transcriptional repression of several proteins, can be a tumor suppressor protein.[15]

Another novel gene, *CHC1L*, was discovered by Devilder *et al.* The gene was later designated as RCBTB2 since it contains RCC1 and BTB domains similar to CLLD7.[16] The evaluation of the mapped clones determined the *CHC1L* gene to be located on chromosome 13q14.3. Protein analysis revealed a significant resemblance between the CHC1L N-terminal amino acid sequence and the seven intradomain repeats of the RCC1, so CHC1L is

qualified to be a new member of the RCC1-related GEF family.[12] CHC1L may function in the cell cycle, nucleocytoplasmic transport, and human cell growth, possibly in the transfer of an anti-oncogenic signal.[16] Even though there is no distinct evidence to date, *CHC1L* was still considered a candidate tumor suppressor gene. Latil *et al.* in 2002, reported the loss of heterozygosity (LOH) and decreasing expression of CHC1L in human prostate cancer.[17] According to a study by Spillane *et al.,* CHC1L-deficient mice succumbed to multiple cancers, including histiocyte-rich neoplasms suggesting that CHC1L plays a role in preventing tumorigenesis.[18]

Detection of changes or abnormalities of cells from the beginning stage by the molecular technologies accompanied by conventional examination promotes effective prevention, early diagnosis, and possible treatment.[10] Our previous study analyzed the expression of CLLD7 and CHC1L proteins in OSCC specimens and compared them to the normal oral mucosa (NOM).[19] Mislocalization of CLLD7 and CHC1L proteins were found.[19] Therefore, this study aimed to assess and compare the expression of these two proteins in NOM and OPMDs, including acanthosis with or without hyperkeratosis, various degrees of epithelial dysplasia, and OLP/OLL using immunohistochemistry in biopsy specimens obtained from a group of participants in the lower part of Northeastern Thailand.

Materials and methods

This is a laboratory-based and observational cross-sectional study. Biopsy specimens were obtained from the "Development of Disease Management Model for Oral Cancer with an Integration Network of Screening, Surveillance, and Treatment in Northeast Health District" project during 2018-2019 or the Faculty of Dentistry, Mahidol University to assess the expression of CLLD7 and CHC1L in OPMDs and NOM using immunohistochemistry. The research was approved by the Committee on Human Rights Related to Human Experimentation, Faculty of Dentistry/Faculty of Pharmacy, Mahidol University (MU-DT/PY-IRB 2020/059.2909, MU-DT/PY-IRB 2019/041.0307 and MU-DT/PY-IRB 2018/025.1106). Demographic data of the participants were retrieved from the questionnaires or pathological report forms without recording the name of the participants. Only age, gender, risk factors that contributed to OPMDs and oral cancers, site, and characteristics of the lesions were recorded.

Tissue specimens

The OPMDs identified as white and/or red patches were clinically diagnosed as oral leukoplakia, OLP, or OLL by board-certified oral medicine specialists or oral surgeons who performed biopsies at the screening clinics for the OPMDs and oral cancer project. Board-certified oral and maxillofacial pathologists at the Faculty of Dentistry, Mahidol University, made histopathological diagnoses of the biopsied specimens as acanthosis with or without hyperkeratosis, various degrees of epithelial dysplasia, OLP, or OLL. Since there was no data regarding the use of the drug(s) that can induce OLL, history of dental restoration in the oral cavity, or history of liver disease, the term OLP/OLL is used in this study. The inclusion criteria of each group were as follows. 1) NOM group; male or female patients at the ages older than 15 years old who attended the Faculty of Dentistry, Mahidol University for impacted third molar removal were asked to participate. NOM specimens that served as normal controls were obtained from the flap of tissue left over from the removal of impacted third molars. These tissues were histopathologically free from inflammation and dysplasia. 2) Oral leukoplakia; male or female participants at ages equal to or older than 40 years old attended the screening for OPMDs and oral cancer in the northeastern areas of Thailand were requested to participate. The clinical diagnosis of oral leukoplakia was made according to Warnakulasuriya and colleagues, 2007.[20] Specimens included acanthosis with or without hyperkeratosis, mild, moderate or severe epithelial dysplasia. 3) OLP/OLL; male or female participants at ages equal to or older than 40 years old attended the screening for OPMDs and oral cancer in the northeastern areas were recruited. The clinical and histopathological diagnosis of OLL/OLP was according to the WHO criteria for the diagnosis of OLP/OLL, 2016.[21] Complete demographic

data and risk factors for oral cancer were also received. The specimens with incomplete data on demography and risk factors were not included in the study. Biopsy specimens with poor orientation and superficial ulceration were excluded from the study. Due to the limitation of tissue samples and immunohistochemical technique, only six samples were used in each group of NOM and OPMDs in this preliminary study. In addition, some tissue samples were too small for the staining of both proteins. Therefore, a substitution of tissue samples was made leading to different groups of tissue specimens from different participants for CLLD7 and CHC1L immunohistochemistry.

Immunohistochemical analysis

Three-µm-thick tissue sections of NOM and OPMDs were cut and mounted over aminopropyl-triethoxysilane (APES) coated slides, then deparaffinized and rehydrated. Sections were incubated with 3% H_2O_2 to block endogenous peroxidase. Antigen retrieval was performed by heating the sections in citrate buffer pH 6.0 using a microwave oven. After washing with phosphate-buffered saline pH 7.6 with 0.1% Tween 20 (PBS-T), the sections were blocked using 5% bovine serum albumin (BSA). Next, the primary antibody diluted in commercially available diluent was applied over the tissue sections. Slides were then kept inside a humidifier and incubated overnight at 4°C. The dilution of the primary antibody used for CLLD7 (ab233533, Abcam, Cambridge, UK) and CHC1L (ab175505, Abcam, Cambridge, UK) was 1:400. On the second day, after draining off the primary antibody, slides were rinsed in PBS-T with gentle agitation. A horseradish peroxidase (HRP)-conjugated secondary antibody (Dako REAL® EnVision®/HRP, Rabbit/Mouse (ENV), Dako, Denmark) was applied over the sections and incubated for 30 minutes at room temperature in a humidified environment. After thorough washing, the color was developed by incubating with freshly-made diaminobenzidine (DAB) solution. Sections were then washed and counterstained with hematoxylin before dehydration and mounting. A diluent with no primary antibody was used as the negative control reagent. Sections of the mouse brain were used as the positive controls for CLLD7 and CHC1L staining.

Evaluation of CLLD7 and CHC1L expression

For CLLD7 and CHC1L, the presence of nuclear, cytoplasmic, and/or membrane staining was considered positive. Photographs of five random fields were taken for each case using a light microscope (X400 magnification). At least 1000 cells were counted in each case. The number of positive cells at different subcellular localizations (nuclear staining, cytoplasmic staining, and membrane staining) was counted using ImageJ software, and the percentage of these cells at various subcellular locations was reported. Cells that exhibited staining with a very faint intensity which is as intense as the background were not counted. The primary investigator (S.S.) was calibrated with a board-certified oral medicine specialist (S.P.K.) to count the positive cells and identify the localization of the proteins. The intraclass correlation coefficients (ICCs) were calculated for each location of staining and it was found that the ICCs were between 0.85-0.94. After the counting, photographs were also randomly selected and re-evaluated by S.S. and S.P.K. to see if the counting was correct. If there was any discrepancy, corrections were made immediately.

Statistical analysis

Statistical analysis was performed with SPSS Statistics version 18. Demographic, clinical, and histopathological data were reported by descriptive statistics. In addition, the normality of the percentage of positive cells was analyzed by the Shapiro-Wilks test. In the case of normal distribution, Levene's Test for Equality of Variance was used. When equal variances were assumed, and at least one pair was significantly different, one-way ANOVA and Post Hoc tests were used. However, when equal variances were not assumed and at least one pair was significantly different, the Welch and Post Hoc tests were used. In cases where normal distribution was not applicable, the Kruskal-Wallis test and pairwise comparisons were used. Significant differences were established at $p \leq 0.05$.

Results

CLLD7 immunohistochemistry

The characteristics of the participants for CLLD7 immunohistochemistry are presented in Table 1. The mean age of the participants in the NOM group was approximately 20 years and in the OPMDs groups was 63.13±7.18 years. Female participants (n=24) were more common than male participants (n=12).

Data regarding the localization of CLLD7 are presented in Table 2 and Figure 1. The representative images of CLLD7 immunostaining in each group are shown in Figure 2. Membrane staining was scarcely observed in all groups which made the median number almost 0 (Table 2). Nuclear and cytoplasmic staining of CLLD7 were observed in all groups. No significant difference was observed for nuclear staining between the NOM group and all other groups (Fig. 1). However, a significant reduction was found between 1) the severe epithelial dysplasia and OLP/OLL groups compared to the acanthosis group, 2) the severe epithelial dysplasia and OLP/OLL groups compared to the mild epithelial dysplasia group, and 3) the severe epithelial dysplasia group compared to the moderate epithelial dysplasia group.

Table 1 *Characteristics of participants for CLLD7 immunohistochemistry*

Groups	Sex (M/F)	Age (Mean±SD) Range (years)	Site (n)	Associated risk factors (n)
NOM (n=6)	3/3	19.83±2.71 (15-23)	Pericoronal tissue of 3rd molar from mandible (5) Pericoronal tissue of 3rd molar from maxilla (1)	None
Acanthosis (n=6)	2/4	67.00±9.25 (50-76)	Alveolar ridge (1) Buccal mucosa (4) Tongue (1)	Smoking (3) Alcohol consumption (1) Betel nut chewing (1) Working in sunlight (4)
Mild dysplasia (n=6)	3/3	64.50±8.09 (49-71)	Buccal mucosa (1) Gingiva (2) Tongue (2) Lower lip (1)	Smoking (5) Tobacco (2) Betel nut chewing (1) Working in sunlight (1) History of cancer (1)
Moderate dysplasia (n=6)	0/6	65.67±6.62 (55-72)	Buccal mucosa (3) Edentulous area (1) Labial mucosa (1) Lower lip (1)	Smoking (3) Smokeless tobacco (3) Alcohol consumption (1) Betel nut chewing (4) Working in sunlight (2)
Severe dysplasia (n=6)	1/5	65.33±6.80 (54-72)	Buccal mucosa (3) Lower lip (1) Labial mucosa (1) Tongue (1)	Smoking (4) Smokeless tobacco (1) Alcohol consumption (1) Betel nut chewing (4) Working in sunlight (3)
OLP/OLL (n=6)	3/3	63.17±9.30 (51-73)	Buccal mucosa (6)	Smoking (4) Alcohol consumption (2) Working in sunlight (4)

NOM: normal oral mucosa; OLP/OLL: oral lichen planus/oral lichenoid lesion

Table 2 *Percentage of subcellular localization of CLLD7 and CHC1L*

Protein and localization		Category						*P*-Value
		NOM	Acanthosis	Mild Dysplasia	Moderate Dysplasia	Severe Dysplasia	OLP/OLL	
CLLD7	Nucleus	56.10±3.00 [a]	64.31±6.38 [a]	60.23±1.83 [a]	59.90±5.02 [a]	45.64±5.27 [a]	46.97±4.35 [a]	0.035[#]
	Cytoplasm	0 (0.00, 0.29) [b]	1.96 (0.23, 57.46) [b]	48.44 (29.27, 61.63) [b]	58.74 (35.08, 72.88) [b]	25.17 (18.16, 51.34) [b]	10.07 (1.91, 19.92) [b]	0.005[b]
	Membrane	0 (0.00, 0.01) [b]	0 (0.00, 0.00) [b]	0 (0.00, 0.00) [b]	0 (0.00, 0.03) [b]	0 (0.00, 0.03) [b]	0 (0.00, 0.00) [b]	0.671[†]
CHC1L	Nucleus	45.52 (36.62, 64.22) [b]	16.92 (6.31, 24.33) [b]	5.75 (2.10, 11.43) [b]	6.50 (4.80, 8.57) [b]	5.60 (0.35, 11.21) [b]	4.12 (3.75,8.89) [b]	0.003[†]
	Cytoplasm	0 (0.00, 0.33) [b]	3.27 (0.00, 12.63) [b]	2.28 (0.71, 3.44) [b]	5.18 (0.00, 9.51) [b]	2.65 (0.00, 5.83) [b]	0.80 (0.38, 1.97) [b]	0.178[†]
	Membrane	1.13 (0.00, 11.29) [b]	9.88 (2.32, 15.68) [b]	32.11 (14.64, 49.70) [b]	26.34 (21.55, 52.64) [b]	50.55 (26.27, 58.05) [b]	46.19 (32.27, 61.91) [b]	0.002[†]

[a]*Mean+SEM; #One-way ANOVA test;* [b]*median (Q1, Q3);* [†]*Kruskal-Wallis test; NOM: normal oral mucosa, OLP/OLL: oral lichen planus/oral lichenoid lesions*

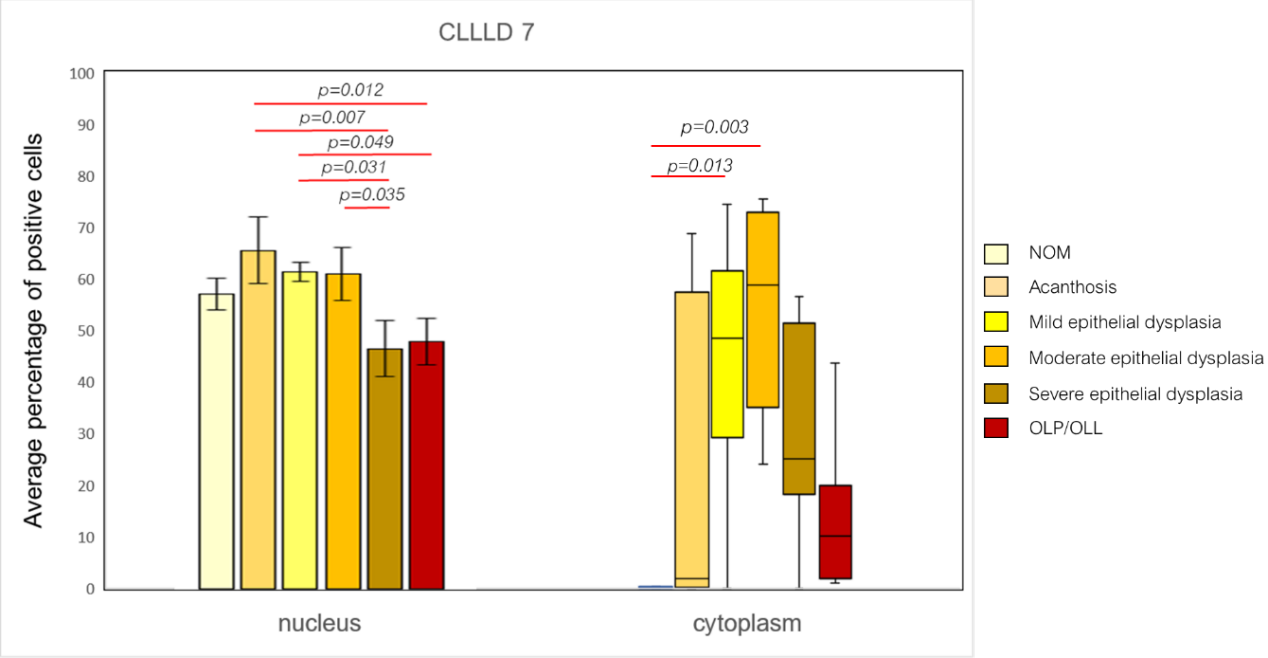

Figure 1 *Percentage of subcellular localization of CLLD 7 immunohistochemistry. No membrane staining was detected. Increased cytoplasmic staining was observed in the OPMDs groups compared to the NOM group. NOM denoted normal oral mucosa. OLP/OLL denoted oral lichen planus/oral lichenoid lesions*

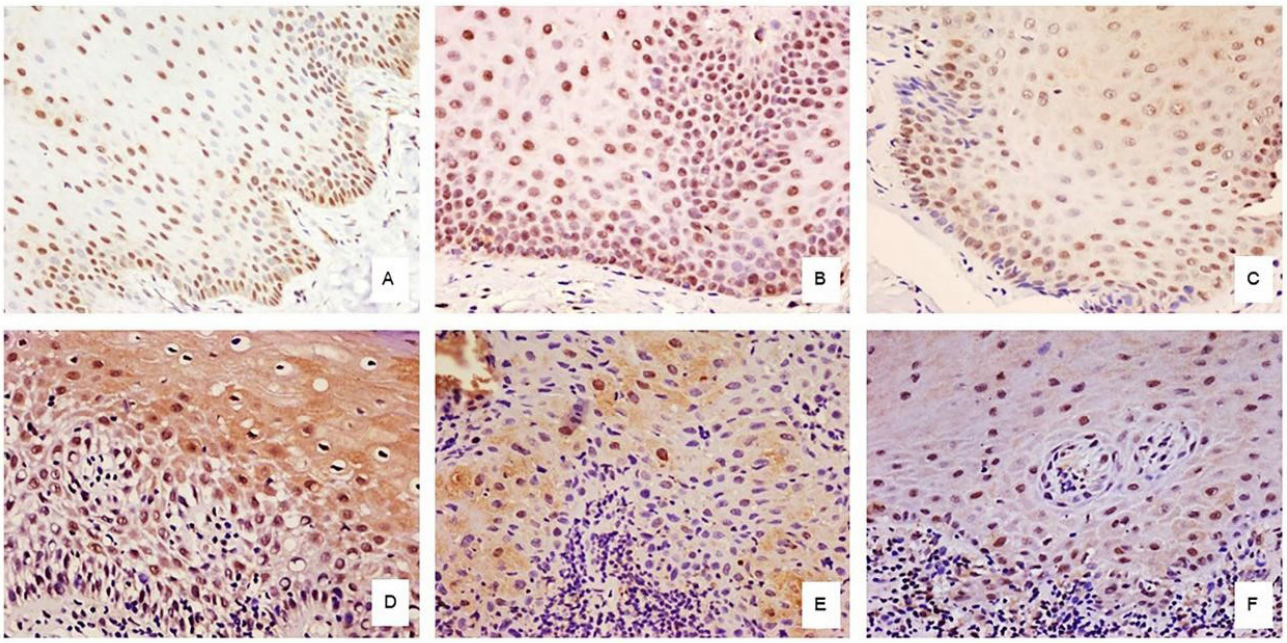

Figure 2 *Representative pictures of CLLD7 immunohistochemistry; original magnification: X400 A. Normal oral mucosa B. Acanthosis C. Mild epithelial dysplasia D. Moderate epithelial dysplasia E. Severe epithelial dysplasia F. oral lichen planus/oral lichenoid lesions. Note that cytoplasmic staining could be observed in OPMDs specimens. Positive staining was also observed in some stromal areas*

The cytoplasmic staining of CLLD7 was higher in all OPMDs compared to the NOM. However, a statistically significant increase in cytoplasmic staining was observed between the mild and moderate epithelial dysplasia groups compared to the NOM group. These data suggested some degree of changes in the localization of the CLLD7 protein in OPMDs. In addition to positive staining in the epithelial cells, positive staining was also observed in some stromal areas.

CHC1L immunohistochemistry

The biopsy specimens used for CHC1L immunostaining were not the same as those for CLLD7 due to the limited size of the biopsy specimens. Nevertheless, the mean age of each group and the sites of biopsy were similar, and this should not affect the immunohistochemical analysis.

Table 3 summarizes the characteristics of the participants for CHC1L immunohistochemistry. The mean age of the participants in the NOM group was approximately 20 years old. The mean age of the participants in the OPMDs groups was 66.63±12.95 years old. There were more female participants (n=24) than male participants (n=12).

Table 2 and Figure 3 show the data on the localization of CHC1L. Figure 4 illustrates the representative images of CHC1L staining in each group. In addition to the nuclear and cytoplasmic expression, membrane staining was detected for CHC1L. Except for the acanthosis group, the percentage of nuclear staining was significantly reduced in all OPMDs groups compared to the NOM group. Although cytoplasmic staining appeared to be increased in all OPMDs groups compared to the NOM group, a significant difference was not observed.

Interestingly, the percentage of membrane staining was higher in all OPMD groups compared to the NOM group. However, a statistically significant difference was observed between the severe epithelial dysplasia and the OLP/OLL groups compared to the NOM group. These data implied that there were changes in the localization of CHC1IL in the OPMDs groups compared to the NOM group.

Similar to CLLD7 immunohistochemistry, positive staining was also observed in some stromal areas.

Table 3 *Characteristics of participants for CHC1L immunohistochemistry*

Groups	Sex (M/F)	Age (Mean±SD) Range (years)	Site (n)	Associated risk factors (n)
NOM (n=6)	3/3	19.83±2.71 (15-23)	Pericoronal tissue of 3rd molar from mandible (5) Pericoronal tissue of 3rd molar from maxilla (1)	None
Acanthosis (n=6)	1/5	70.83±3.06 (68-76)	Buccal mucosa (5) Tongue (1)	Smoking (4) Betel nut chewing (3) Working in sunlight (3)
Mild dysplasia (n=6)	3/3	67.83±10.01 (49-79)	Buccal mucosa (2) Gingiva (1) Lower lip (2) Labial mucosa (1)	Smoking (3) Smokeless tobacco (1) Betel nut chewing (1) Working in sunlight (2) History of cancer (1)
Moderate dysplasia (n=6)	0/6	69.33±2.07 (66-72)	Buccal mucosa (3) Labial mucosa (2) Lower lip (1)	Smoking (1) Smokeless tobacco (2) Alcohol consumption (2) Betel nut chewing (4) Working in sunlight (2)
Severe dysplasia (n=6)	1/5	65.33±6.80 (54-72)	Buccal mucosa (3) Labial mucosa (1) Tongue (1) Lower lip (1)	Smoking (4) Smokeless tobacco (1) Alcohol consumption (1) Betel nut chewing (4) Working in sunlight (3)
OLP/OLL (n=6)	4/2	59.83±7.14 (51-71)	Buccal mucosa (6)	Smoking (3) Smokeless tobacco (1) Alcohol consumption (2) Betel nut chewing (1) Working in sunlight (5)

NOM: normal oral mucosa; OLP/OLL: oral lichen planus/oral lichenoid lesions

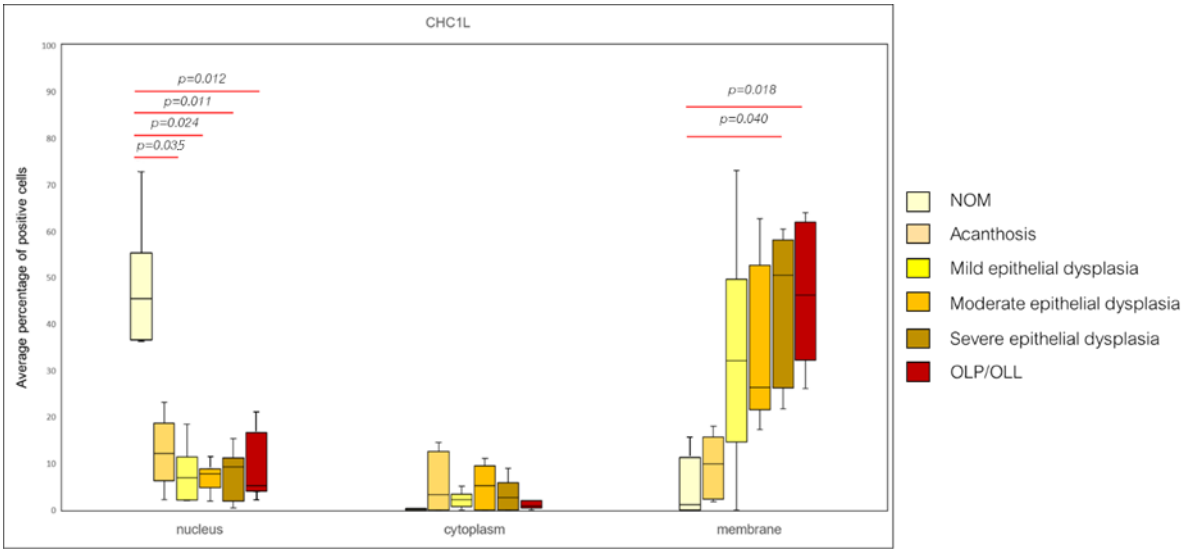

Figure 3 *Percentage of subcellular localization of CHC1L immunohistochemistry. Increase membrane staining and decrease nuclear staining were observed in the OPMDs groups compared to the NOM group. NOM denoted normal oral mucosa. OLP/OLL denoted oral lichen planus/oral lichenoid lesions*

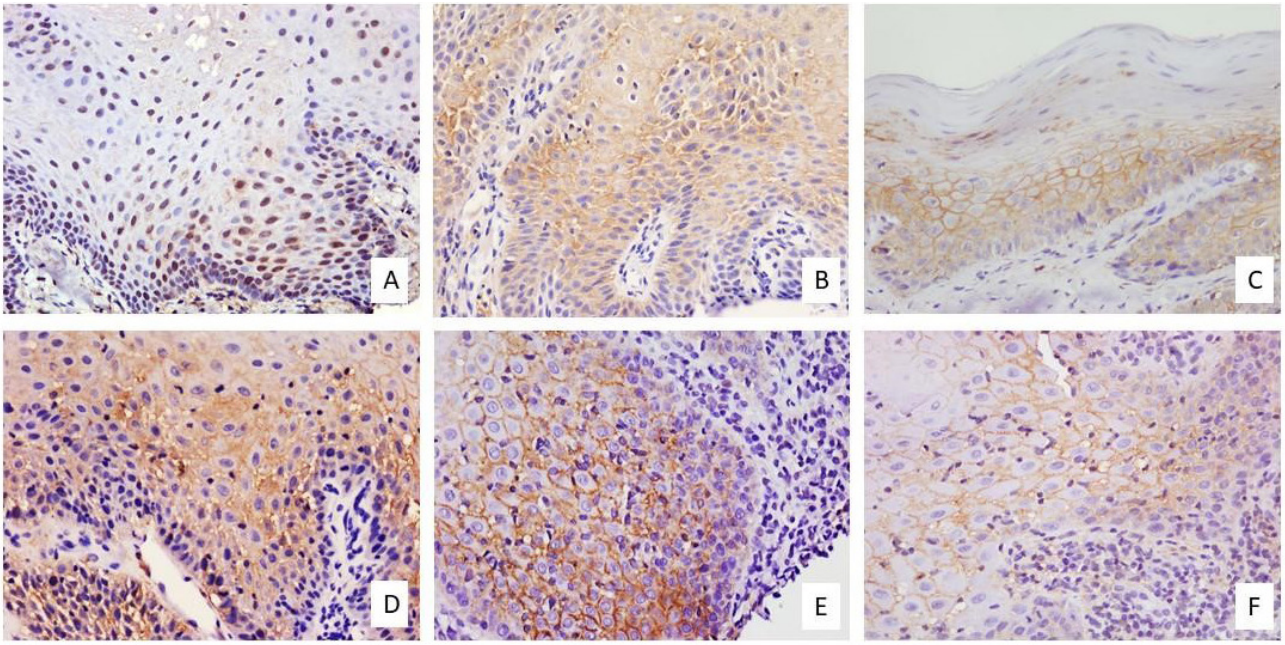

Figure 4 *Representative pictures of CHC1L immunohistochemistry, original magnification: X400 A. Normal oral mucosa B. Acanthosis C. Mild epithelial dysplasia D. Moderate epithelial dysplasia E. Severe epithelial dysplasia F. oral lichen planus. In the parabasal and prickle cell layers, membrane staining could be observed in OPMDs specimens. Positive staining was also observed in the stromal areas*

Discussion

All OPMDs participants in this research were recruited from the "Development of Disease Management Model for Oral Cancer with an Integration Network of Screening, Surveillance, and Treatment in Northeast Health District" proactive inspection project. The NOM controls were the participants who visited the Faculty of Dentistry, Mahidol University for impacted teeth removal. As presented in Tables 1 and 3, most of the participants with OPMDs lesions were from the senior population with an average age of 60. In addition, the participants exhibited the following associated risk factors; smoking, use of smokeless tobacco, alcohol consumption, prolonged exposure to sunlight during the day, and history of cancer elsewhere. Aitiwarapoj and colleagues reported that OPMDs and OSCCs occurred most frequently in the sixth and seventh decades when they surveilled 208 Thai patients with OPMDs and OSCCs at the tongue.[22] Juntanong *et al.* found that the most critical factors strongly associated with increased risk for OPMDs are smoking, alcohol consumption, and betel nut chewing.[7] Therefore, the distribution in age and characteristics of the OPMDs participants in our study are consistent with previous studies done in Thailand.

CLLD7 is a nuclear protein with the potential for tumor suppression. It has RCC1 and BTB domains, which are involved in key steps in cell division, nucleocytoplasmic transport, and protein-protein interaction.[13,23] Many previous studies pointed out that the genes at 13q14, where the CLLD7 gene is also located, were frequently deleted in different cancers.[24-26] However, those studies experimented with other tissues, but not with oral epithelium. Recently, there was a pioneer study comparing CLLD7 expression in OSCC and NOM.[19] Bhattarai *et al.* presented the mislocalization of this protein in OSCC compared to NOM. They reported that nuclear and cytoplasmic staining was observed in NOM and OSCCs, while OSCCs had a much higher cytoplasmic staining than normal mucosa.[19] It was suggested that the nuclear activity of CLLD7 may be compromised in OSCC, and the dislocation of CLLD7

may have a role in tumorigenesis. This study did not find a significant difference in nuclear staining between NOM control and other OPMDs. However, significantly different numbers of nuclear staining were appreciated in the acanthosis group compared to other groups. This may be due to the very high percentage of nuclear staining in the acanthosis group. Since only a limited number of cases were included in this study, a future study is mandatory to confirm this result.

For cytoplasmic staining of CLLD7, it was found that the average percentage of cytoplasmic staining in all OPMDs groups was higher than that of the NOM group. Still, a significant difference was observed between the mild and moderate epithelial dysplasia groups compared to the NOM group (Fig. 2). This result implied a potential sign of CLLD7 protein dislocation to the cytoplasm. Since CLLD7 must be localized in the nucleus to function properly, its localization in the cytoplasm may suggest an improper function of this protein in early oral carcinogenesis. Nevertheless, future studies are necessary to confirm this concept.

CHC1L is a candidate tumor suppressor located telomeric to the Retinoblastoma (Rb) gene on chromosome 13q14.3. It contains similar key domains to CLLD7 and, therefore, carries the same potential to be a tumor suppressor protein as CLLD7. CHC1L may play its functional role in the cell cycle, nucleocytoplasmic transportation, and human cell growth, a possible conveyer of an anti-oncogenic signal.[16] However, many studies reported the under-expression of CHC1L in tumors such as prostate cancer[17], histiocyte-rich neoplasms[18], and multiple myeloma.[27] Bhattarai *et al.* conducted a recent study to compare the expressions of CHC1L between OSCC and NOM.[19] It was suggested that the mislocalization of CHC1L as nuclear staining was reduced, whereas membrane staining was significantly increased in OSCC compared to NOM control.[19] The staining pattern in our study revealed reduced nuclear staining of CHC1L in almost all groups of OPMDs. However, increased membrane staining was appreciated in OPMDs. Furthermore, a significant difference was observed between the severe epithelial dysplasia and OLP/OLL groups compared to the NOM control group. Surprisingly, the membrane staining was gradually increased in accordance with the severity of epithelial dysplasia and was even more pronounced in the OLP/OLL group. These results were in line with the previous study of Bhattarai *et al.* that reported predominant nuclear staining in all cell layers of the normal epithelium with very few cytoplasmic and membrane staining.[19] While in OSCC, membrane staining was dominant, and only very few cells with nuclear staining were spotted.[19] Our results seemed to support the concept of protein dislocation in CHC1L proposed by Bhattarai *et al.*

Protein synthesis usually occurs in the cytosol, and the proteins are then transported to their functional sites, including the nucleus, plasma membrane, mitochondria, or other organelles. Several mechanisms are involved in the dysregulation of protein trafficking in cancer cells, causing abnormal subcellular localization of proteins. Mutation of protein-targeting signals, dysregulation of transporter machinery, aberrant endocytosis and vesicular trafficking, dysregulation of signal transduction and post-translational protein modification, alteration of protein-protein interactions, and cross-regulation of cancer-related proteins are some examples of such mechanisms.[28] Another interesting point is the mislocalization of these proteins in the OLP/OLL group. This implied that the potential dysregulation of this protein in OLP/OLL might support the concept of malignant transformation in OLP/OLL by protein mislocalization.

Another interesting point is the positive staining in the stroma. There might be some crosstalk between oral epithelial cells and the stromal cells as some studies indicated the interaction between the tumor microenvironment and its influence on the growth and metastasis of head and neck squamous cell carcinoma.[29] We believe that there might be an interaction between the stromal cells within the underlying connective tissue and the epithelial cells in dysplasia and OLP/OLL but this needs to be further elucidated in future studies.

Despite the exciting results, we acknowledge the limitations of this study in that only a small number of

specimens were included in each group. In addition, there was no investigation concerning the mechanism responsible for protein mislocalization. Therefore, future studies using molecular techniques are needed to declare the potential tumor suppressive function of these two proteins.

Conclusion

Increasing cytoplasmic expression of CLLD7 and subcellular mislocalization of CHC1L from the nucleus to the membrane were interesting. Both these proteins could be putative markers during oral malignant transformation; nevertheless, further study is still needed to confirm these results.

Acknowledgment

The authors would like to thank the Department of Oral and Maxillofacial Pathology and the Department of Advanced General Dentistry, Faculty of Dentistry, Mahidol University, for their support. This work is supported by a Mahidol University research grant (051/2562) to Siribang-on Piboonniyom Khovidhunkit, Boworn Klongnoi, and Vanvisa Sresumatchai under the Development of Disease Management Model for Oral Cancer with an Integration Network of Screening, Surveillance, and Treatment in Northeast Health District project and in part by the Faculty of Dentistry, Mahidol University (2018).

Conflict of interest

The authors have no relevant financial or non-financial conflict of interests to disclose.

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