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Human Calmodulin-Like Protein (CLP) Expression in Oral Squamous Mucosa and in Malignant Transformation

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Abstract

Purpose: The purpose of this study was to test whether calmodulin-like protein (CLP) is expressed in normal human oral mucosal cells and if downregulation of CLP occurs in malignant transformation.

Materials and Methods: Oral mucosal tissue was taken from three individuals in a double-blind manner. The samples were cut, measured, and homogenized. Total RNA was extracted and reverse transcribed. Each cDNA sample was subjected to polymerase chain reaction (PCR). PCR fragments were purified, cloned, and sequenced to verify the presence of CLP. Three oral mucosal tissue samples with biopsy-confirmed squamous cell carcinoma were obtained. These samples demonstrated regions of normal epithelial cells as well as invasive squamous cell carcinoma. One normal breast epithelial sample was also obtained for positive control. Sections were stained with an affinity-purified CLP antibody and counterstained with a diluted hematoxylin. Two observers evaluated the specimens for expression of CLP. Staining patterns and intensity were noted in normal oral mucosa, comparing them to the normal breast epithelium sample. Staining patterns and intensity were then observed in squamous tumor cells, comparing them to the patterns of benign squamous mucosa.

Results: CLP coding sequences were positively identified from the normal oral mucosal tissue samples by reverse transcription and polymerase chain reaction (RT-PCR) with 100% identity to the published CLP sequence (accession #M58026). In the three oral mucosa tissue samples with known squamous cell carcinoma, expression of CLP was readily detected in areas of normal oral mucosa, while a notable downregulation of CLP expression occurred in areas of malignant transformation. The staining intensity was equivalent to the staining seen in the benign breast epithelium used as a control. In the areas of squamous cell carcinoma, a decrease in CLP immunoreactivity occurred. There was a sharp contrast in staining quality and clarity between benign and malignant tissue. In the majority of the carcinoma regions, a complete lack of immunoreactivity was noted.

Conclusions: The RNA for human CLP is found in normal oral mucosa. CLP expression is seen in normal oral mucosa with a downregulation of CLP expression in malignant transformation.

Human calmodulin-like protein (CLP) is a calcium binding protein found in many epithelial cell types including breast, thyroid, prostate, kidney, and skin.[1] CLP closely resembles calmodulin, an intracellular Ca^{2+} sensor that participates in signaling pathways that regulate many cell activities such as growth, proliferation, and movement.[2] CLP shares 85% identity with calmodulin's 148 amino acid sequence, but appears to significantly differ in its protein-binding activity.[3-6] Furthermore, CLP appears to have a highly epithelial-specific expression.[1]

A study by Yaswen et al in 1992 suggested that CLP may have a tumor suppressor function in normal breast tissue.[7] In histologically normal tissue, the immunohistochemical distribution of the protein suggests that expression of the gene is regulated during epithelial differentiation. In comparison, malignant tissue showed a marked reduction of the protein. In a more recent study, Rogers et al analyzed 80 archival breast cancer specimens.[8] The study found a significant reduction in CLP expression in 79 to 88% of invasive ductal carcinoma and lobular carcinoma specimens compared to normal breast specimens. The authors concluded that CLP downregulation is common in breast tumorigenesis and that expression of CLP can serve as a marker for the nonmalignant state.[8]

Oral cancer is visibly detectable because of the accessibility of the oral cavity; however, because oral cancer is typically late in development when it is finally clinically visible, it has high morbidity and mortality rates. Gaining an understanding about the expression of CLP in oral epithelial cells and its possible downregulation in cancer may be a potentially valuable marker in early detection of oral cancer.

Early diagnosis of oral and oropharyngeal squamous cell carcinoma is crucial for a more favorable prognosis. Although characterized in many epithelial cell types, CLP has never been shown to exist in the oral cavity. Moreover, if CLP does exist in oral epithelium, information regarding its downregulation in tumorigenesis may prove to be of great value. The ability to easily access the oral cavity may aid in using CLP levels in detection of disease progression. Evidence of CLP expression in oral epithelial cells and its possible downregulation in oral tumor progression may lead to a system of early detection of squamous cell carcinoma. In addition, changes in CLP expression at various phases of disease progress may aid in correlation with prognosis and patient outcome. Whether as a diagnostic tool or prognostic marker, the potential to develop a simple, less invasive method for oral cancer screening would be of great value. Therefore, the aim of this pilot study was to test whether CLP is expressed in normal human oral mucosal cells and if so, whether downregulation of CLP accompanies malignant transformation of oral mucosal cells. The presence of CLP may serve as a marker for nonmalignant tissue, or if downregulated, a predictor of malignant transformation.

Materials and methods

This study was approved by the Institutional Review Board of the Mayo Clinic (IRB protocol 754-04) in accordance with the guidelines for research involving human subjects. Three patients were randomly selected in the Division of Periodontics in the Department of Dental Specialties at the Mayo Clinic, Rochester, MN. These patients were present for routine treatment for either gingivectomy for crown lengthening or implant uncovering. Inclusion criteria included healthy, nonsmoking patients free from any periodontal disease or gingival pathology. No other determining factors were involved other than patients presenting for removal of otherwise normal, healthy mucosal tissue. The donors had no known underlying pathological abnormalities.

Oral mucosal tissue was taken from the three individuals in a double-blind manner. The samples were cut, measured, and homogenized in buffer RLT as supplied by Qiagen (Valencia, CA). Total RNA was extracted using RNeasy columns from Qiagen according to the manufacturer's instructions. RNA samples were reverse transcribed using Superscript II (Gibco/Invitrogen, Carlsbad, CA) and random hexamer primers according to the manufacturer's instructions. The primer sequences were 5′-ATG GCC GAC CAG CTG ACT GA-3′ and 5′-TCA CTT GGA CAC CAG GAC ACG-3′ and were predicted to amplify a 450 bp CLP product. Five microliters of each cDNA sample were subjected to PCR in a 50 μL reaction by using a touchdown cycling profile (30 cycles) with a 94°C denaturation (2 minutes), an annealing temperature beginning at 60°C, stepping down 2°C every cycle for a final annealing temperature of 54°C and a final elongation at 72°C for 5 minutes. Polymerase chain reaction (PCR) product bands were separated and visualized by 1% agarose gel electrophoresis and stained with ethidium bromide. The bands were excised, and the PCR fragments were purified using a Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Purified PCR products were cloned using TA-topo (Invitrogen, Carlsbad, CA) and sequenced using T7 promoter primer to verify the presence of CLP.

Seven oral mucosal tissue samples with biopsy-confirmed squamous cell carcinoma were obtained from the Mayo Clinic tissue registry under IRB-approved protocol 754-04. These samples demonstrated regions of normal epithelial cells as well as invasive squamous cell carcinoma. One normal breast epithelial sample was also obtained for positive control. Three of the oral mucosa tissue samples were selected, and the sections were stained with an affinity-purified CLP antibody and counterstained with a diluted hematoxylin.

A previously described[8] affinity-purified rabbit polyclonal antibody against human CLP was used for immunohistochemical staining. The paraffin-embedded tissue specimens were mounted on positively charged slides, deparaffinized and treated with H_2O_2/methanol to block endogenous peroxidase activity. The specimens received heat-induced epitope retrieval in 1 mmol/L EDTA at a pH of 8.0 for 20 minutes. A 5% normal goat serum in a phosphate-buffered solution was used to block nonspecific protein-binding sites. The sections were immunostained by sequential incubations in an affinity-purified CLP antibody (1:2500 dilution), biotinylated goat anti-rabbit IgG, and peroxidase-conjugated streptavidin. The sections were then incubated in 3-amino-9-ethylcarbazole in the presence of H_2O_2 and counterstained with hematoxylin.

Two observers (a staff surgical pathologist, TJS, and a maxillofacial prosthodontist, MDB) evaluated the specimens for expression of CLP. The dilution of the antibody was carried out until loss of qualitative staining occurred in both benign oral mucosa and benign breast epithelium. This was accomplished to find the optimal dilution (1:2500) for staining. First, staining patterns and relative intensity (strong, weak, or absent) were noted as previously described[8] in normal oral mucosa comparing them to normal breast epithelium used as a control. Next, the same parameters were observed in squamous tumor cells comparing them to the patterns of benign squamous mucosa.

Results

From the three normal oral mucosal tissue samples, CLP sequences were positively identified by RT-PCR with 100% identity to the published CLP sequence (Accession #M58026). The PCR product bands were separated and visualized alongside appropriate positive and negative controls (Fig 1). The samples were cloned and sequenced (Fig 2). The sequences were positively identified from the normal oral mucosa with 100% identity to the published CLP sequence.

From the three oral mucosal tissue samples with biopsy-confirmed squamous cell carcinoma, expression of CLP was readily detected in normal oral mucosa, while a notable downregulation of CLP expression occurred in areas of malignant transformation. Samples of normal epithelium were immunostained with an affinity-purified CLP antibody. The staining intensity and quality of the benign oral mucosal tissue matched those of the known benign breast epithelium (Figs 3 and 4). A crisp cytoplasmic membrane staining was obtained with intense staining in the cytoplasm itself. Expression of CLP was readily detected in normal oral mucosa adjacent to tumor cells. The staining intensity was equivalent to the staining seen in benign breast epithelium.

Adjacent to the benign oral mucosa were areas of invasive squamous cell carcinoma. In the areas of squamous cell carcinoma, a decrease in CLP immunoreactivity occurred (Fig 5). There was a sharp contrast in staining quality and clarity between benign and malignant tissue. In the majority of the carcinoma regions, a complete lack of immunoreactivity was noted.

Staining intensity is apparent with the more differentiated epithelial cells. Cells with a typical epithelial cell morphology exhibit intense staining in the periphery and nucleus. Tumor cells that lose their epithelial cell morphology or that are more de-differentiated show a diminished or complete loss of CLP staining.

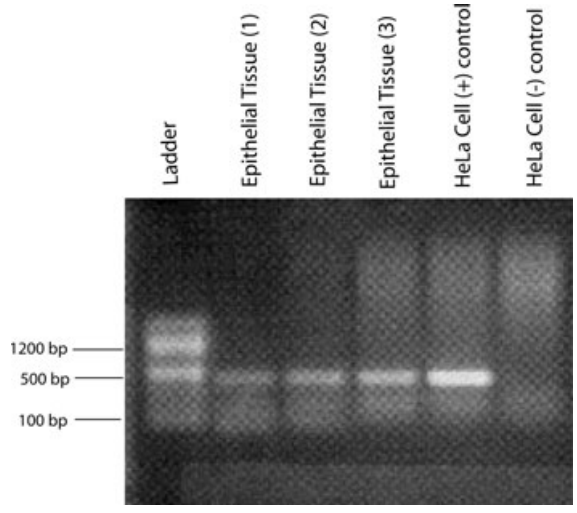

Figure 1 Results of RT-PCR gel electrophoresis. Expression of CLP in oral mucosal tissue was detected by RT-PCR. RNA was isolated from three separate oral tissue samples and from CLP-expressing HeLa cells (+control) and HeLa cells that do not express CLP (−control). CLP-specific primers were used for RT-PCR, and the products separated in a 1% agarose gel and stained with ethidium bromide. A size marker ladder is included on the left.

Discussion

Analysis of CLP expression in oral mucosa has not previously been reported. Therefore, samples of oral mucosa were collected and RNA was extracted. Reverse transcription and PCR were accomplished using known CLP-specific primers as mentioned.

Prior to RT-PCR analysis of the oral mucosal samples, western blot analysis was performed to detect the presence of CLP; however, western analysis did not conclusively show expression of CLP in the oral tissue samples, likely due to the difficulty in obtaining a critical mass of mucosal cells from the oral tissue samples collected.

The mucosal cells in the oral cavity are the thin, outermost layer lining the structures of the cavity. Beneath the mucosal layer is a thicker layer of connective tissue. The procedures performed in procuring the oral mucosal samples excise primarily connective tissue with only a small amount of squamous cells present. The homogenization of the tissue samples likely did not create enough mucosal cell protein for detection of CLP by western blot. This might be expected given the reported specificity of CLP to epithelial tissues[1] and the absence of CLP in the connective tissue underlying the mucosa.

Although western blot was not conclusive, the more sensitive RT-PCR method readily detected the expression of CLP transcripts in the oral mucosa. The PCR product bands were cloned and sequenced and unequivocally identified the presence of CLP RNA in benign oral mucosal tissue. Although the RNA for CLP was evident from the normal oral mucosal samples, further investigation by way of immunohistochemistry was necessary to confirm its expression at the protein level.

Staining became more intense toward the outer layers of mucosa. This is consistent with previous reports that noted markedly increased staining in more differentiated layers.[1] This pattern suggests that CLP expression increases during keratinocyte differentiation. Therefore, CLP may play a role in terminal differentiation of oral keratinocytes. CLP may be involved in inducing terminal differentiation or it may be expressed to maintain a differentiated state. Downregulation of CLP in tumorigenesis may directly correlate with tumor cells turning off the normal differentiation of epithelial cells. Alternatively, CLP downregulation in tumors could merely be a secondary effect of a tumor suppressor at an earlier stage of cell differentiation manifesting at the terminal differentiation level. Regardless of why or how CLP downregulation occurs, our findings suggest that CLP is expressed in normal oral mucosal cells and that upon malignant transformation, a downregulation of CLP occurs. This information may aid in early detection of malignant transformation.

Cancer markers in general commonly upregulate in tumorigenesis, meaning that cancer has to be present or developing for the marker to manifest. CLP may serve as a marker for normalcy, with lower CLP levels indicative of potential tumorigenesis.

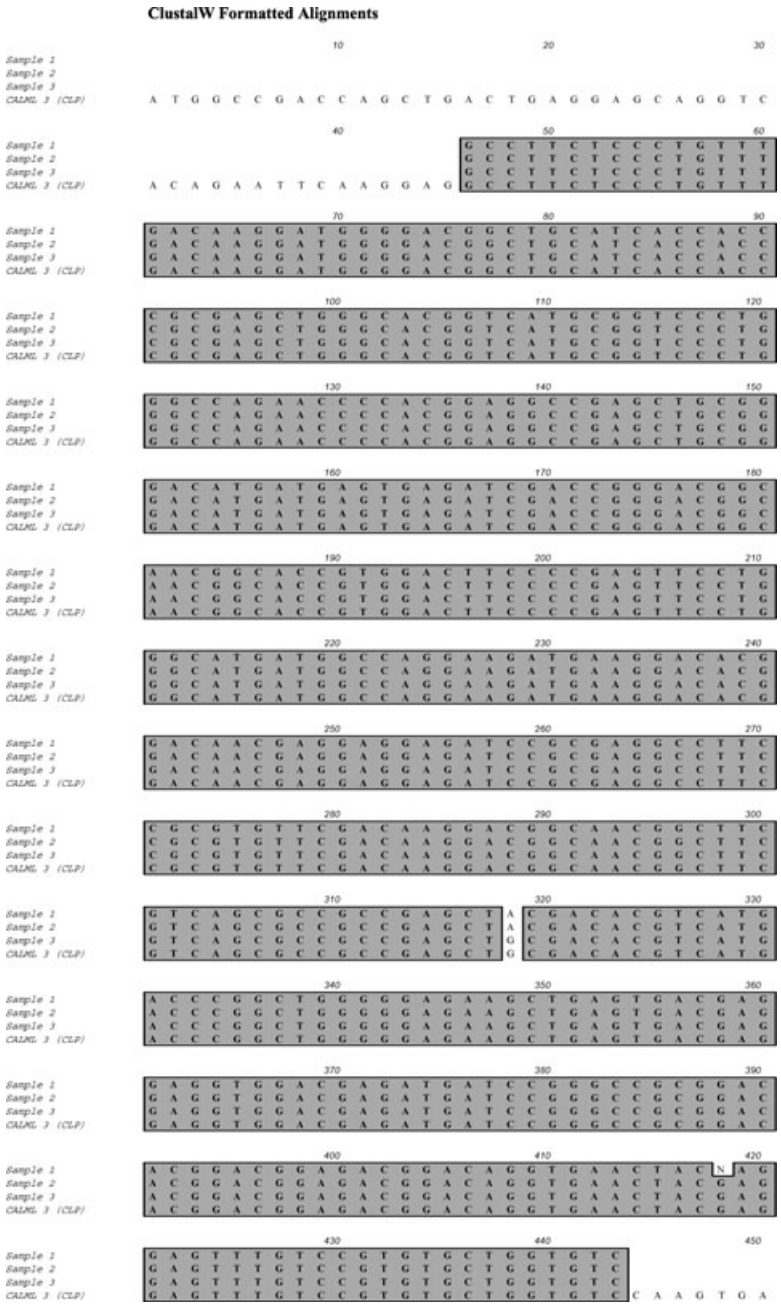

Figure 2 Oral mucosal sample sequences. The three oral mucosal sample sequences are listed alongside the known CLP sequence. Shaded areas denote matching identity of oral epithelial samples to known CLP.

In prostate cancer, prostate-specific antigen (PSA) levels are a marker for nonpalpable, clinically localized disease. Although PSA has had a positive impact on detection, diagnosis, and management of prostate cancer, there are notable problems with specificity. Significant cancers have been found even in the presence of low PSA levels. It has been suggested that since PSA is organ specific, it is not cancer specific.[9] PSA levels reflect not only changes due to cancer but also changes due to inflammation, trauma, and benign proliferation. Nonetheless, monitoring PSA has proven to be an effective, noninvasive weapon in the fight against prostate cancer.

A noninvasive means of testing for oral cancer would also be of great value. The oral cavity is so accessible for cancer screening, and yet oral cancer is typically not detected until later stages of tumor development. Like PSA levels, CLP levels are indicative of normalcy. CLP levels may merely reflect changes in the epithelial organ itself; however, unlike PSA levels, which are organ specific, CLP downregulation appears to be specific to tumorigenesis.

Currently there are few diagnostic tests for oral cancer. Techniques being implemented as diagnostic tests include transepithelial brush biopsy/exfoliative cytology, fine needle

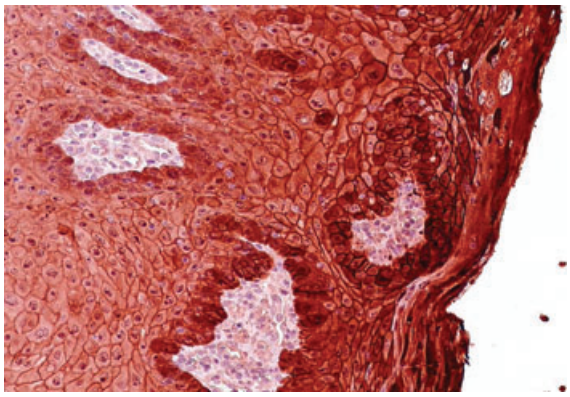

Figure 3 Benign oral epithelium immunostained with a CLP antibody. Intense staining of benign epithelial cells is apparent compared to areas of invasive squamous cell carcinoma (center). The expression of CLP in the benign tissue characterized by the immunostaining (especially to the right border) is appreciable compared to the lack of staining in the invasive squamous cell carcinoma. The staining intensity increases toward the more superficial layers. Dense cytoplasmic membrane staining and nuclear staining can be seen.

aspiration, and toluidine blue.[10-18] These techniques all rely upon cytological markers to indicate the presence of malignant or premalignant cells. Diagnostic tests for oral cancer such as these are advantageous over conventional biopsy confirmation because they are less invasive and more tolerable for the patient. However, the reliability and efficacy of these tests have not been validated.[19,20] Like many diagnostic tests in various fields, diagnostic tests for oral cancer lack sufficient sensitivity to indicate the presence of disease and/or specificity to indicate the absence of disease.

Because the expression of CLP is an indicator for normal cell function, the potential for the development of a simple diagnostic test for oral cancer using CLP as a marker is promising and unique. Transepithelial brush biopsy and fine needle aspiration are used to detect the presence of disease. In other words, oral cancer has to be present to mark the disease. Unlike these techniques used, CLP levels could verify normalcy of the cells with a drop in its levels indicative of disease progress. A change in CLP levels could indicate the presence or even the early development of oral cancer. Perhaps this could be implemented by the use of exfoliative brushing cytology, mini-biopsy, or "swish-and-spit" procedure. This would allow a clinician who observes a suspicious area in the mouth to noninvasively test for CLP levels. If CLP levels were below an established normal level, this would indicate that cancer is present. A routine clinical test could be developed that could screen all individuals for normal CLP levels.

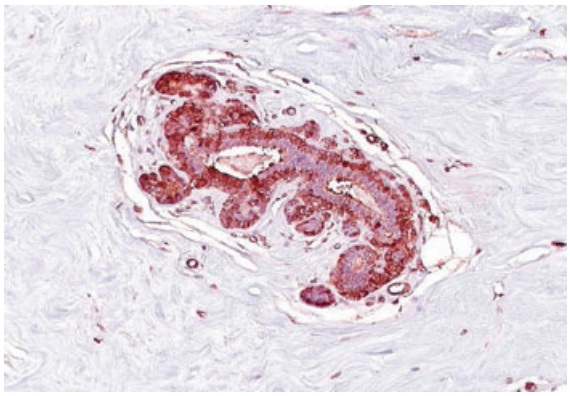

Figure 4 Benign breast epithelium immunostained with a CLP antibody. Rich immunostaining can be appreciated in this sample of benign breast epithelium. This level of immunoreactivity is evidence of CLP expression.

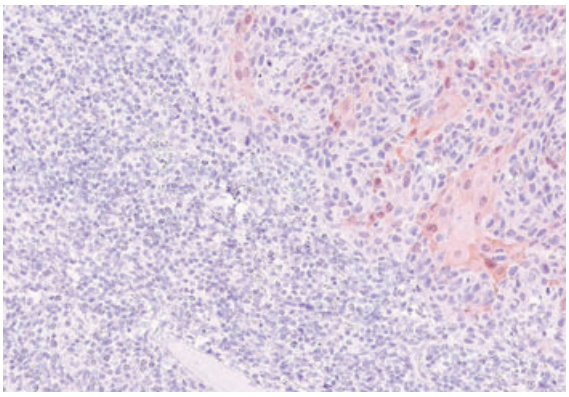

Figure 5 Invasive malignant oral epithelium immunostained with a CLP antibody. In areas of invasive squamous cell carcinoma, a marked decrease in immunoreactivity is noted. The tumor cells to the left show a complete lack of immunoreactivity denoting no expression of CLP.

Conclusion

Through RT-PCR, the RNA for human CLP is found in normal oral mucosa. By way of immunohistochemical analysis, CLP expression is seen in normal oral mucosa with a downregulation of CLP expression in malignant transformation.

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