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Raised keratinocyte growth factor-1 expression in oral submucous fibrosis *in vivo* and upregulated by arecoline in human buccal mucosal fibroblasts *in vitro*

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BACKGROUND: Keratinocyte growth factor-1 (KGF-1) is the seventh member of the fibroblast growth factor family. KGF-1 is produced by mesenchymal cells such as fibroblasts and upregulated in a variety of hyperplastic tissues. Currently, there is limited information about the regulation of KGF-1 expression in areca quid-associated oral submucous fibrosis (OSF). The aim of the study was to compare KGF-1 expression in normal human buccal mucosa and OSF specimens and further to explore the potential mechanism that may lead to induce KGF-1 expression.

METHODS: The expression of KGF-1 from fibroblasts cultured from OSF and normal buccal mucosa were using reverse-transcriptase polymerase chain reaction and enzyme-linked immunosorbent assay. In addition, arecoline, a major areca nut alkaloid, was challenged to normal buccal mucosa fibroblasts (BMFs) to elucidate whether KGF-1 expression could affect by arecoline. Furthermore, 25 OSF specimens and six normal buccal mucosa specimens were examined by immunohistochemistry.

RESULTS: Fibroblasts derived from OSF were found to exhibit higher KGF-1 expression than BMFs both in mRNA and protein levels ($P < 0.05$). In addition, upregulation of KGF-1 mRNA gene and protein expression were found in BMFs stimulated by arecoline ($P < 0.05$). From the results of immunohistochemistry, KGF-1 expression was significantly higher in OSF specimens and expressed mainly by fibroblasts, endothelial cells, inflammatory cells, and epithelial cells.

CONCLUSIONS: Taken together, these results suggest that KGF-1 expression is significantly upregulated in OSF tissues from areca quid chewers and arecoline may be responsible for the enhanced KGF-1 expression *in vivo*.

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Introduction

Areca quid chewing is one of the most worldwide addictive oral habits (1). Epidemiological studies have clearly associated that areca quid chewing is strongly implicated in oral leukoplasia, oral submucous fibrosis (OSF), and oral cancer (2, 3). OSF is characterized by the submucosal accumulation of dense fibrous connective tissue with inflammatory cell infiltration and epithelial atrophy (4).

Our previous studies have demonstrated that upregulation of tissue inhibitor of metalloproteinase-1 (5), vimentin (6), cyclooxygenase-2 (7), and plasminogen activator inhibitor-1 (8) may play important roles in the pathogenesis in OSF. However, the precise mechanism underlying OSF is not well known. Studies of fibrotic disorder in other tissues suggest that a number of growth factors may play an important part in the molecular pathogenesis of these diseases (9).

Keratinocyte growth factor-1 (KGF-1, FGF-7) is a stromally derived factor which has an unusual specificity of targeting to epithelial cells, suggesting that it may have involved for the purpose of mediating interactions between mesenchyme and epithelium (10). KGF-1 is a unique member of the 23-member fibroblast growth factor family because they are specific paracrine mediators of epithelial cell growth (11). That is, stromal cells express KGF-1 but only epithelial cells express the KGF receptor (KGFR). Despite its importance in epithelial tissue homeostasis, however, little is known about the expression and the role of KGF-1 in oral mucosa (12). Nevertheless, the apparent fundamental importance of KGF-1 in benign prostate hyperplasia (13) and drug-induced gingival hyperplasia (14, 15), suggests that it might also have a central role in the pathogenesis of areca quid chewing-associated OSF.

In this study, we have therefore measured the relative levels of KGF-1 in OSF compared with normal buccal mucosa and the effects of arecoline, a major areca nut alkaloid, on KGF-1 in normal human buccal mucosa fibroblasts (BMFs) *in vitro*. Furthermore, immunohistochemical localization of KGF-1 and KGFR were examined in normal buccal mucosa and OSF specimens.

Materials and methods

Cell culture

Ten healthy individuals, without areca quid chewing habits, attending the Department of Oral and Maxillofacial Surgery (Chung Shan Medical University Hospital, Taichung, Taiwan) were enrolled with informed consents for this study. Biopsy specimens were derived from histologically normal oral mucosa at the time of surgical third molar extraction. The OSF specimens were obtained from 20 male patients with areca quid chewing habits during surgical biopsy. Clinical diagnosis was confirmed by histopathological examination of the biopsy specimens. Fibroblast cultures were grown and maintained using procedures described previously (16–18). Cell cultures between the third and eighth passages were used in this study.

Expression of KGF-1 mRNA in OSF and BMF

Confluent cells were trypsinized, counted, and plated at a concentration of 1×10^5 cells in 60 mm culture dish and allowed to achieve confluence. Total RNA was prepared using TRIzol reagent (Gibco Laboratories, Grand Island, NY, USA) following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in a 15 μl reaction mixture containing 100 mg random hexamer and 200 units of Moloney murine leukemia virus reverse transcriptase (RT) (Gibco Laboratories). The reaction mixture was diluted with 20 μl of water and 3 μl of the diluted reaction mixture was used for the polymerase chain reaction (PCR). PCR reaction mixture contains 10 pmol of forward and reverse primers and 2 units of Tag DNA polymerase. Amplification was performed at 25 cycles for gleceraldehyde-3-phosphate dehydrogenase (GAPDH) and 30 cycles for KGF-1 in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 1 min of extension at 72°C. The sequences of primers used were as follows (19):

(A) GAPDH	Forward: 5′-TCCTCTGACTTCAACAGCGACACC-3′
	Reverse: 5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′
(B) KGF-1	Forward: 5′-GCAAAGTAAAAGGGACCCAAGAGA-3′
	Reverse: 5′-AGAAATCTCCCTGCTGGAACTGG-3′

The PCR products were analyzed by agarose gel electrophoresis and a 383 bp band for KGF-1 was noted. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with GAPDH mRNA was quantified by the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA).

Assessment of KGF-1 activity in OSF and BMF

Confluent cells were trypsinized, counted, and plated at a concentration of 1×10^5 cells in 60 mm culture dish and allowed to achieve confluence. The conditioned medium samples were collected after 2-day cultured period. Levels of KGF-1 antigen were determined by enzyme-linked immunosorbent assay (ELISA) [human KGF (FGF-7), Quantikine, DKG00, R&D Systems, Inc. MN, USA]. Briefly, 20 μl of conditioned media were directly transferred to the microtest strip wells of the ELISA plate. All further procedures were performed following the manufacturer's instructions. The absorbance at 495 nm was measured in a microtest plate spectrophotometer and KGF-1 levels were determined with a calibration curve using human KGF-1 as a standard.

Effect of arecoline on KGF-1 mRNA and protein in BMF

BMF derived from three strains were seeded 1×10^5 cells per well into 10 cm culture dish and incubated for 24 h. Then the medium was changed to a medium containing 10% heated activated FCS and various concentrations of arecoline (Sigma, St Louis, MO, USA) (0–80 μg/ml). Total RNA was isolated after 6-h incubation period for RT-PCR as described above. Condition medium were collected after 24-h incubation period for ELISA as described earlier.

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of six normal buccal mucosa from non-areca quid chewers, and 25 OSF specimens from areca quid chewers, were drawn from the files of the Department of Pathology, Chung Shan Medical University Hospital. Diagnosis was based on histological examination of hematoxylin- and eosin-stained sections. Sections of 5 μm were stained with the polyclonal anti-KGF-1 antibody [rabbit anti-human, FGF-7 (H-73), catalogue no. *sc*-7882, lot no. D25, Santa Cruz Biotechnology, Santa Cruz, CA, USA] (1:100 dilution) or polyclonal anti-KGFR antibody [rabbit anti-human, Bek (C-17), catalogue no. *sc*-122, lot no. E0103, Santa Cruz Biotechnology](1:100 dilution) using a standard avidin–biotin-peroxidase complex method (20). AEC (DAKO, Carpinteria, USA) was then used as the substrate for localizing the antibody binding. Negative controls included serial sections from which either the primary or secondary antibodies were excluded. The preparations were counterstained with hematoxylin, mounted with Permount (Merck, Darmstadt, Germany) and examined by light microscopy.

Statistical analysis

Triplicate or more separate experiments were performed throughout this study. For testing of differences in the KGF-1 between the BMF and OSF, the Wilcoxon–Mann–Whitney rank sum test was applied. The

significance of the results obtained from control and treated groups were statistically analyzed by the Student's *t*-test. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

RT-PCR assay was used to compare KGF-1 mRNA gene expression of the fibroblasts cultured from BMF and OSF. As shown in Fig. 1, OSF specimens exhibited significantly higher KGF-1 mRNA expression than BMFs. From the AlphaImager 2000, the intensity of KGF-1 mRNA from OSF was elevated approximately 2.9-fold as compared with BMFs ($P < 0.05$).

The individual values of KGF-1 from BMF and OSF cultures from ELISA were shown in Table 1. The amount of KGF-1 protein in BMF was approximately 127.93 ± 5.41 ng/10^6 cells. The amount of KGF-1 protein in BMF was approximately 236.3 ± 44.4 ng/10^6 cells. In addition, KGF-1 was found increased approximately 1.9-fold in OSF as compared with BMF ($P < 0.05$).

To examine the effect of arecoline on the KGF-1 expression, human BMFs were treated with arecoline and the levels of mRNA and protein were measured. The effects of arecoline on the KGF-1 gene expression in three different cell strains were similar, and their intracellular variations were limited.

RT-PCR were used to verify whether arecoline could affect KGF-1 mRNA gene expression by human BMFs. Fig. 2 reveals a dose-dependent change following treatment of BMF with arecoline for 6 h. Arecoline was found to elevate KGF-1 mRNA gene expression in a dose-dependent manner ($P < 0.05$). From the AlphaImager 2000, the amount of KGF-1 was elevated approximately 1.2-, 1.5-, 2.0-, and 1.8-fold at concentrations of 10, 20, 40, and 80 μg/ml, respectively, as compared with control.

As shown in Fig. 3, arecoline was found to upregulate KGF-1 activity in BMF cultures. The effect of arecoline on KGF-1 protein in BMF during 24-h incubation period is shown in Fig. 3. Arecoline was found to elevate KGF-1 expression in a dose-dependent manner ($P < 0.05$).

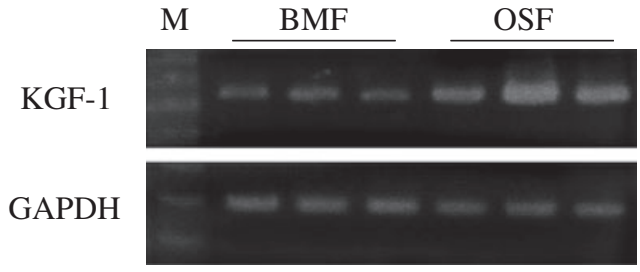

Figure 1 Comparison of the keratinocyte growth factor-1 (KGF-1) mRNA level from buccal mucosa fibroblasts (BMFs) and oral submucous fibrosis (OSF) using reverse-transcriptase polymerase chain reaction assay. Gleceraldehyde-3-phosphate dehydrogenase gene was performed in order to monitor equal RNA loading. Fibroblasts derived from OSF are significantly upregulated KGF-1 mRNA gene expression than BMFs.

Table 1 Summary of keratinocyte growth factor-1 (KGF-1) protein levels from buccal mucosa fibroblasts (BMF) and oral submucous fibrosis (OSF) by using enzyme-linked immunosorbent assay

Subject	*BMF (ng/10^6 cells) (n = 10)*	*OSF (ng/10^6 cells) (n = 20)*
KGF-1	121.13 (106.48–164.35)[a]	182.93 (103.82–856.06)*[a]
	127.93 ± 5.41[b]	236.3 ± 44.4*[b]

[a]Median (range).
[b]Mean ± SD.
*Statistically significant between BMF and OSF, $P < 0.01$.

Figure 4a represents normal human buccal mucosa with very faint KGF-1 expression. The strongest signal was seen in the basal epithelial cells, with almost no staining in the lamina propria. All OSF samples exhibited

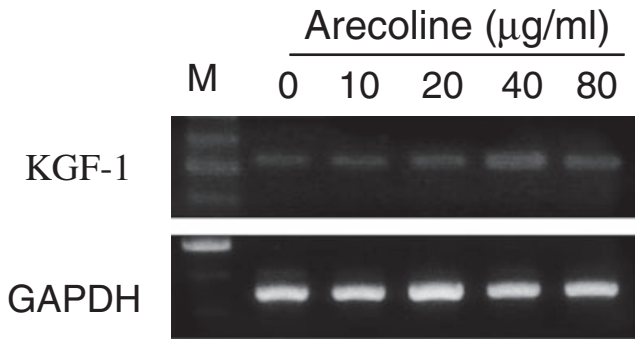

Figure 2 Expression of keratinocyte growth factor-1 mRNA gene in arecoline-treated human buccal mucosa fibroblasts by reverse-transcriptase polymerase chain reaction assays. Cells were exposed for 6 h containing arecoline concentrations as indicated. M = DNA molecular size marker. Gleceraldehyde-3-phosphate dehydrogenase gene was performed in order to monitor equal RNA loading.

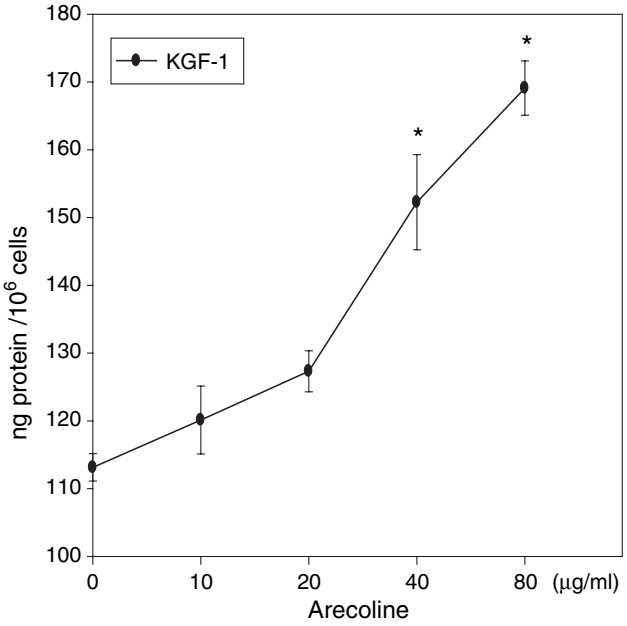

Figure 3 Expression the protein level of keratinocyte growth factor-1 in arecoline-treated human buccal mucosa fibroblasts by using ELISA. *Significant differences from control values with $P < 0.05$.

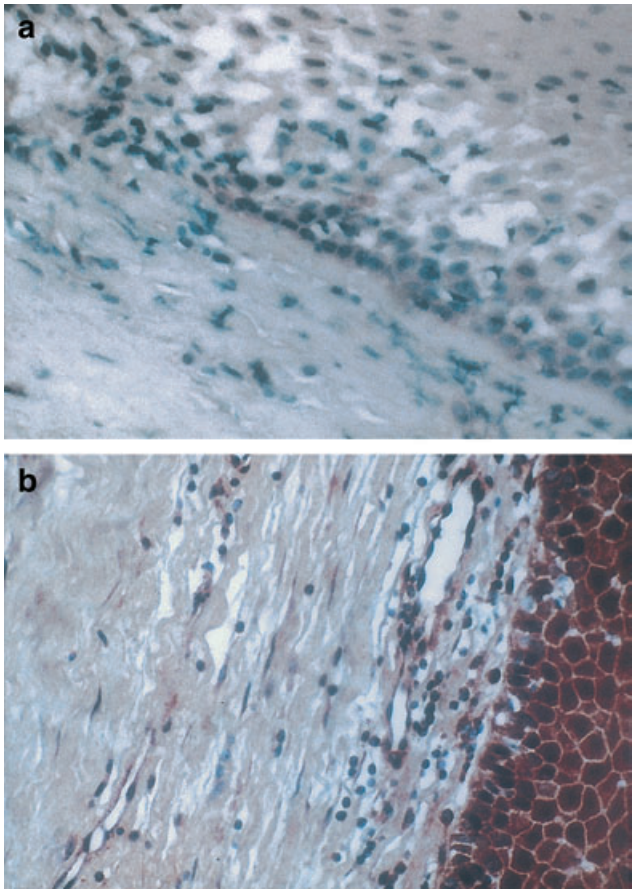

Figure 4 Immunohistochemical analysis of keratinocyte growth factor-1 (KGF-1) in normal and fibrosis buccal mucosal specimens. The brown enzyme reaction product indicates the presence of KGF-1 in buccal mucosal tissues. (a) Very faint immunoreactivity of KGF-1 was observed in normal human buccal mucosal connective tissue and the strong signal was seen in the subepithelial connective tissue (×400). (b) KGF-1 was evident as intense, diffuse brown coloring throughout the connective tissue and detected at relatively high levels in the basal and spinous layers of the epithelium (×400).

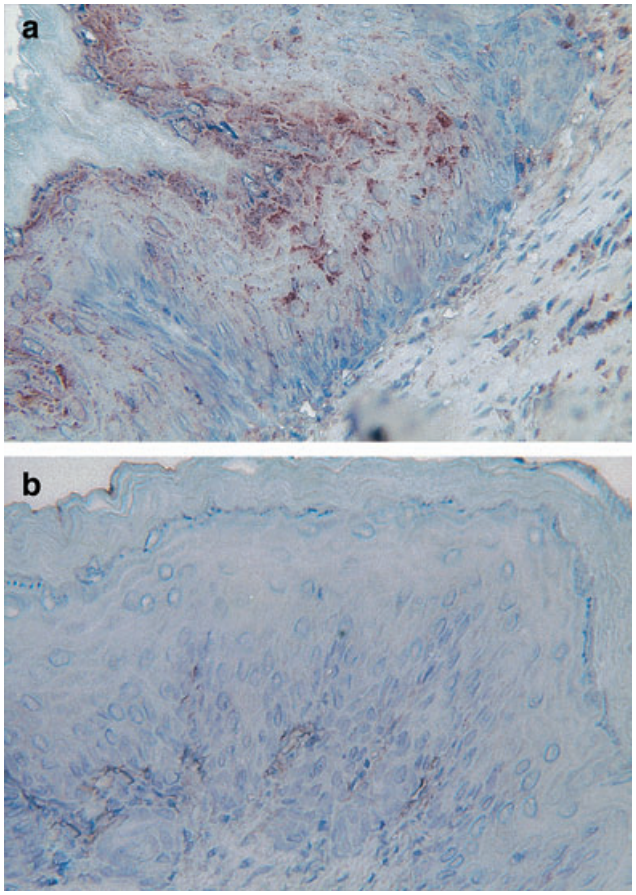

Figure 5 Immunohistochemical analysis of keratinocyte growth factor receptor (KGFR) in normal and fibrosis buccal mucosal specimens. The brown enzyme reaction product indicates the presence of KGFR in buccal mucosal tissues. (a) Section of normal buccal mucosa showing the presence of KGFR throughout the epithelium. A relatively much higher level of KGFR was found to be expressed in the granular and prickle layers of buccal mucosal epithelium (×400). (b) Very faint immunoreactivity of KGFR was observed in the epithelium of the oral submucous fibrosis specimens. A relatively much higher level of KGFR was found to be close to the basement membrane (×400).

the features of either moderately advanced or advanced submucous fibrosis. Intensive staining for KGF-1 expression was observed in the epithelial cells, with less prominent staining in the endothelial cells, infiltrating cells and fibroblasts of the connective tissue (Fig. 4b). In addition, immunoreactivity of KGF-1 was expressed in epithelium, mainly in basal and spinous layers.

Immunoreactivity of KGFR was found throughout the epithelium of normal buccal mucosa yet minimal staining is present in the basal and suprabasal layers (Fig. 5a). A relatively much higher level of KGFR was found to be expressed in the granular and prickle layers of buccal mucosal epithelium. The KGFR staining present in OSF appears to be close to the basement membrane, with no staining present in the epithelium as reported (Fig. 5b).

Discussion

The etiology of OSF is complex and the pathological process remains poorly understood. The reason for localization of buccal mucosa may be exposed to higher concentrations of areca quid. The underlying mechanism of OSF is not known, but the components of areca nut appear to directly or indirectly influence the growth and function of the BMF. These processes are regulated by cytokines and growth factors (21, 22) and the expression of these mediators and their corresponding receptors is thus likely to be of fundamental importance in the pathophysiology of OSF.

Fibroblasts are the principal cell type residing in connective tissue and the responsive for the formation and turnover of the extracellular matrix. Our findings demonstrated for the first time, that KGF-1 mRNA and protein were upregulated in fibroblasts derived from OSF than BMFs. KGF-1 is 100-fold more effective than bFGF at inhibiting the expression of epithelial cell specific collagenase-1 (23) thereby possibly contributing further to the excessive accumulation of extracellular matrix as occurs in OSF and other fibrotic pathologies

such as benign prostate hyperplasia (13) and drug-induced gingival hyperplasia (14, 15).

Areca quid chewing-related oral mucosal lesions are potential hazards to a large population worldwide. Many of the undesirable effects of areca nut have been attributed to arecoline. Data from our *in vitro* experiments also demonstrated that arecoline was capable of stimulating KGF-1 secretion and gene activity in human BMFs. The effects of arecoline may occur directly on KGF-1 or indirectly, mediated via other growth factors such as platelet-derived growth and transforming growth factor β which have been reported to be upregulated in areca quid chewing-associated OSF (14) and have also been shown to unregulate KGF-1 (24). However, our results differed from Ko et al. (25), who reported that KGF-1 was decreased by ripe areca nut extract in human oral fibroblasts and concluded that downregulation of KGF-1 expression in oral fibroblasts potentially impairs the proliferation of epithelial cells, which might lead to epithelial atrophy *in vivo*. The reason for this contrary result is not clear. It probably results from different origins of the cells or different experimental protocols used in each laboratory. An *in vivo* evaluation to define the association between KGF-1/KGFR and epithelial atrophy in OSF is worth further investigating.

From immunohistochemistry, our findings demonstrated, for the first time, that positive staining for KGF-1 was first found upregulated in OSF specimens compared to normal buccal mucosa. Strong immunostaining for KGF-1 was detected throughout the connective tissues, mainly in fibroblasts, endothelial cells and inflammatory cells. However, KGF-1 staining was also presented in the basal layer and spinous layer of epithelium. As the specific receptor for KGF is expressed only by epithelial cells. It is likely that KGF-1 staining observed in the OSF epithelium may be the presence of KGF–KGF receptor complex (26). The reason for this phenomenon is not known. From the results of our study, arecoline was found to upregulate KGF-1 in BMFs. This may partly explain why KGF-1 was accumulated in OSF epithelium.

It may be interesting that KGF-1 is upregulated in OSF as compared with normal buccal mucosa and suggests that KGF-1 may have important role in its enhanced epithelial proliferation *in vivo*. However, most clinical cases of OSF were demonstrated the atrophy of epithelium (27). In this study, KGFR was observed in the epithelium of normal buccal mucosa. However, KGFR was not detected in the epithelium of OSF by immunohistochemistry. These differences are not clear. It is less clear from our present studies. It could be conceivable that lack of KGFR in OSF epithelium may partly explain why epithelium atrophy in chronic areca quid chewers. In addition, the epithelial atrophy in OSF could result from cytotoxicity of areca quid. The oral mucosa microtrauma caused by the coarse fibers of areca nut, and the atrophic and more permeable OSF epithelium (28), could accelerate the diffusion of areca quid components into epithelial barrier.

This systematic attempt to evaluate the role of KGF-1 expression in areca quid associated-OSF in human at both *in vivo* and *in vitro* levels. We have demonstrated for the first time that KGF-1 is elevated in OSF than normal buccal mucosa. Data from our *in vitro* experiments showed that arecoline was capable of stimulating KGF-1 mRNA and protein expression in human BMFs. This suggests that one of the pathogenic mechanisms of OSF *in vivo* may be the synthesis of KGF-1 by resident cells in response to areca nut challenge. In addition, lack of KGFR expression in OSF epithelium might be the reason why epithelium atrophy in patients who have the habit of areca quid chewing.

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