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ORIGINAL ARTICLE

Hypoxia inducible factor-1α expression in areca quid chewing-associated oral squamous cell carcinomas

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OBJECTIVES: Hypoxia inducible factor (HIF)-1α gene expression is mainly induced by tissue hypoxia. Overexpression of HIF-1α has been demonstrated in a variety of cancers. The aim of this study was to compare HIF-1α expression in normal human oral epithelium and areca quid chewing-associated oral squamous cell carcinoma (OSCC) and further to explore the potential mechanisms that may lead to induce HIF-1α expression.

METHODS: Twenty-five OSCC from areca quid chewing-associated OSCC and 10 normal oral tissue biopsy samples without areca quid chewing were analyzed by immunohistochemistry. The oral epithelial cell line GNM cells were challenged with arecoline, a major areca nut alkaloid, by using Western blot analysis. Furthermore, glutathione precursor *N*-acetyl-L-cysteine (NAC), AP-1 inhibitor curcumin, extracellular signal-regulated protein kinase inhibitor PD98059, and protein kinase C inhibitor staurosporine were added to find the possible regulatory mechanisms.

RESULTS: Hypoxia inducible factor-1α expression was significantly higher in OSCC specimens than normal specimen ($P < 0.05$). Arecoline was found to elevate HIF-1α expression in a dose- and time-dependent manner ($P < 0.05$). The addition of NAC, curcumin, PD98059, and staurosporine markedly inhibited the arecoline-induced HIF-1α expression ($P < 0.05$).

CONCLUSIONS: Hypoxia inducible factor-1α expression is significantly upregulated in areca quid chewing-associated OSCC and HIF-1α expression induced by arecoline is downregulated by NAC, curcumin, PD98059, and staurosporine.

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Introduction

It is estimated that about 10% of the human population have the areca quid chewing habit (De Miranda *et al*, 1996). Areca quid chewing is a popular habit in Taiwan, India, and many southeastern Asian countries (Gupta and Warnakulasuriya, 2002; IARC, 2004). Reports have consistently indicated that areca quid chewing to be an independent risk factor in the development of oral squamous cell carcinoma (OSCC) (Ko *et al*, 1995; Jeng *et al*, 2001). However, the pathogenesis of areca quid chewing-associated OSCC still remains to be elucidated.

Hypoxia (poor oxygenation), owing to an inadequate blood supply, is a common feature of most solid human tumors and is associated with increased malignancy, resistance to therapy and distant metastasis (Harrison and Blackwell, 2004). Hypoxia inducible factor (HIF)-1α, a member of basic helixloop-helix-PAS protein family (Hockel *et al*, 1996; Bottaro and Liotta, 2003), is usually increased under hypoxic conditions, and can activate transcription of many genes that are critical for cellular function under hypoxic conditions (Hockel *et al*, 1996). HIF-1α is overexpressed in the majority of human cancers, including gastric adenocarcinoma (Urano *et al*, 2006), breast cancer (Dales *et al*, 2005), cervical cancer (Burri *et al*, 2003), non-small cell lung cancer (Chen *et al*, 2009), and oral cancer (Mohamed *et al*, 2004; Fillies *et al*, 2005; Lin *et al*, 2008). Moreover, it may be possible to use HIF-1α as a marker for malignant transformation of oral submucous fibrosis (Tilakaratne *et al*, 2008).

Areca quid chewing, cigarette smoking, and alcohol drinking are the main oral habits and etiologies of oral cancer. Previous studies have shown that tobacco and alcohol can enhance the expression of HIF-1α (Szabo

et al, 2001; Michaud *et al*, 2003; Jeong *et al*, 2005; Li *et al*, 2006). Recently, HIF-1α expression in OSCC was demonstrated significantly related with the consumption of drinking as well as smoking in Taiwan (Lin *et al*, 2008). However, there is limited information on the regulation of HIF-1α expression in areca quid chewing-associated OSCCs both *in vitro* and *in vivo*. The purpose of this study was to test whether HIF-1α expression regulated within areca quid chewing-associated OSCC specimens and to further explore possible pathogenic mechanisms that may lead to the induction of HIF-1α *in vivo*. The oral epithelial cell line GNM cells were also challenged with arecoline, the major areca nut alkaloid, *in vitro*. Furthermore, cell-permeable glutathione precursor *N*-acetyl-L-cysteine (NAC), AP-1 inhibitor curcumin, extracellular signal-regulated protein kinase (ERK) inhibitor PD98059, and protein kinase C (PKC) inhibitor staurosporine were added to investigate the possible mechanisms and their protective effects.

Materials and methods

Materials and chemicals

Arecoline, NAC, curcumin, and staurosporine were purchased from Sigma (St. Louis, MO, USA). PD098059 was obtained from Promega (Madison, WI, USA). HIF-1α (sc-10790) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All culture materials were obtained from Gibco (Grand Island, NY, USA). The final concentrations of NAC, curcumin, PD98059, and staurosporine used in this study were 1 mM, 20, 10, and 1 μM, respectively. The concentrations used are without cytotoxicity to cells.

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of 10 normal oral epithelium specimens from non-areca quid chewers, and 25 OSCC 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. Institutional Review Board permission at the Chung Shan Medical University Hospital was obtained for the use of discarded human tissues. Five micron sections from formalin-fixed, paraffin-embedded specimens were stained with the monoclonal anti-HIF-1α antibody (1:50 dilution) using a standard avidin–biotin–peroxidase complex method as described previously (Tsai *et al*, 2009). Diaminobenzidine (Zymed, South San Francisco, CA, 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. Processed immunohistochemically for HIF-1α expression, sections graded as 'low' were represented by positive stained cells $<50\%$; sections graded 'high' exhibited positive stained cells over 50% on three sections/tissue at 400× magnification (Lee *et al*, 2008a).

HIF-1α expression analysis

The oral epithelial cell line GNM cells, derived from a patient with $T_2N_{2a}M_0$ gingival carcinoma and metastasis to the cervical lymph node, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U ml^{-1} of penicillin, 100 μg ml^{-1} of streptomycin and 0.25 μg ml^{-1} of fungizone). Cells arrested in G_0 by serum deprivation (0.5% FCS; 48 h) were used in the experiments (Lee *et al*, 2008b). Nearly confluent monolayers of GNM cells were washed with serum-free medium and immediately thereafter exposed to 0, 20, 40, 80, and 160 μg ml^{-1} arecoline. Cell lysates were collected after 8 h for Western blot analysis. Cultures without FCS were used as negative control. Subsequently, various pharmacological agents were added to test their regulation effects during 8 h coincubation period.

Western blot

For Western blot analysis, cell lyates were collected as described previously (Lee *et al*, 2008c). Briefly, cells were solubilized with sodium dodecyl sulfate-solubilization buffer (5 mM EDTA, 1 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.5 and 0.5% Trition X-100, 2 mM phenylmethylsulfonyl fluoride, and 1 mM *N*-ethylmaleimide) for 30 min on ice. Then, cell lysates were centrifuged at 12 000 *g* at 4°C and the protein concentrations determined with Bradford reagent using bovine serum albumin as standards. Equivalent amounts of total protein per sample of cell extracts were run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immediately transferred to nitrocellulose membranes. The membranes were blocked with phosphate-buffered saline containing 3% bovine serum albumin for 2 h, rinsed, and then incubated with primary antibodies anti-HIF-1α (1:500) in phosphate-buffered saline containing 0.05% Tween 20 for 2 h. After three washes with Tween 20 for 10 min, the membranes were incubated for 1 h with biotinylated secondary antibody diluted 1:1000 in the same buffer, washed again as described above and treated with 1:1000 streptavidin–peroxidase solution for 30 min. After a series of washing steps, protein expression was detected by chemiluminescence using an ECL detection kit (Amersham Biosciences UK Limited, Buckinghamshire, England), and relative photographic density was quantitated by scanning the photographic negatives on a gel documentation and analysis system (AlphaImager 2000; Alpha Innotech Corp., San Leandro, CA, USA). Each densitometric value was expressed as the mean $\pm$ s.d.

Statistical analysis

The statistical significance of differences between HIF-1α staining grade and cell differentiation of OSCC were assessed by the Fisher's exact test for independence. For Western blot assay, triplicate separate experiments were performed throughout this study. The significance of the results obtained from control and treated groups was statistically analyzed by the paired Student's *t*-test. A *P*-value of <0.05 was considered to be statistically significant.

Results

Hypoxia inducible factor-1α staining was mainly expressed in the OSCC specimens and the intensity was significantly higher than that in normal epithelial specimens ($P < 0.05$), especially in the nuclear of poorly differentiation tissues. Weak HIF-1α expression was observed in the specimens of normal epithelium (Figure 1a). The HIF-1α staining in normal oral epithelium was found predominantly in the spinous cells and rarely in the basal and superficial cells. However, high HIF-1α expression was observed in cancer cells within the specimens of OSCC. In well-differentiated OSCC, only cells located at the periphery of the epithelial pearl were labeled, while centrally situated cells remained negative (Figure 1b). In moderate differentiated OSCC, homogeneous and intensive staining for HIF-1α was labeled in the nest (Figure 1c). A mosaic pattern was observed for HIF-1α labeling in poorly differentiated OSCC and also had a strong nuclear HIF-1α staining (Figure 1d). In addition, HIF-1α expression was observed in the cytoplasm of fibroblasts, and endothelial and inflammatory cells. Immunostaining of HIF-1α in OSCC could be classified into two groups: one was low grade, 44% (11/25), and the other was high grade, 56% (14/25). There was no significant difference in HIF-1α expression in the differentiation of OSCC ($P = 0.227$).

Expression of HIF-1α in GNM cells challenged with arecoline was elevated by Western blot. As shown in Figure 2(a), arecoline was found to elevate HIF-1α expression in a dose-dependent manner ($P < 0.05$). From the AlphaImager 2000 (Figure 2b), the amount of HIF-1α was elevated about 3.5-, 4.5-, 5.4-, and 5.2-fold after exposure to 20, 40, 80, and 160 μg ml^{-1} arecoline ($P < 0.05$), respectively. Moreover, the peak of HIF-1α protein level induced by arecoline was 80 μg ml^{-1}. On the basis of these results, experiments described below were performed at a concentration of 80 μg ml^{-1} arecoline.

Investigations of the time-dependence of HIF-1α protein expression in 80 μg ml^{-1} arecoline-treated GNM cells were found to be significantly enhanced ($P < 0.05$). The kinetics of this response showed that HIF-1α protein was first detectable in cell lysates at 1-h postarecoline challenge and remained elevated throughout the 24-h incubation period (Figure 3a). From the AlphaImager 2000 (Figure 3b), the amount of HIF-1α protein increased about 2.0-, 2.1-, 3.4-, 5.4-, and 2.3-fold after exposure to arecoline for 1, 2, 4, 8, and 24 h ($P < 0.05$), respectively.

N-acetyl-L-cysteine, curcumin, PD98059, and staurosporine without cytotoxic concentrations were added to search the possible regulatory mechanisms on arecoline-induced HIF-1α expression. These pharmacological agents were found to inhibit the arecoline-induced HIF-1α expression ($P < 0.05$) (Figure 4a). From the AlphaImager 2000 (Figure 4a), NAC, curcumin, PD98059, and staurosporine were found to reduce the arecoline-induced HIF-1α expression by lowering 1.0-, 1.2-, 1.23-, and 1.5-fold ($P < 0.05$), respectively.

Discussion

The present study demonstrated for the first time to evaluate the expression of HIF-1α in areca quid chewing-associated OSCC both *in vitro* and *in vivo*. Overexpression of HIF-1α has been demonstrated in various cancers (Burri *et al*, 2003; Dales *et al*, 2005; Urano *et al*, 2006; Lin *et al*, 2008; Chen *et al*, 2009; Mohamed *et al*, 2004; Fillies *et al*, 2005). To the best of our knowledge, HIF-1α expression was first found to highly expressed in areca quid chewing-associated OSCC as compared with normal epithelium tissue. Similar results were found in previous studies that HIF-1α was highly expressed in oral cancer specimens (Mohamed *et al*, 2004; Fillies *et al*, 2005; Lin *et al*, 2008).

The activation of HIF-1α in areca quid chewing-associated OSCCS might be explained as follows. HIF-

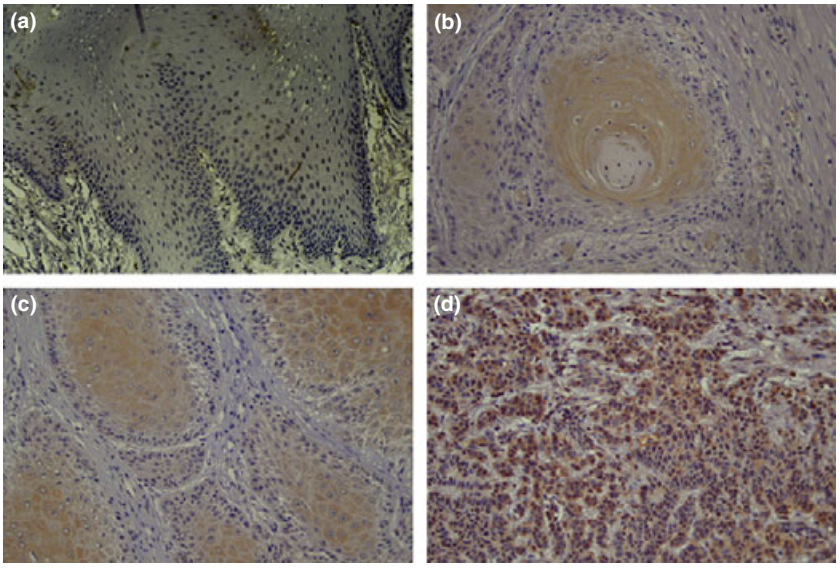

Figure 1 (**a**) Weak immunoreactivity of HIF-1α was observed in normal epithelial specimens. The HIF-1α staining was predominantly in the spinous cells and rarely in the basal and superficial cells (200×). (**b**) In well-differentiated OSCC, only cells located at the periphery of the epithelial pearl were labeled, while centrally situated cells remained negative (200×). (**c**) In moderately differentiated OSCC, homogeneous and intensive staining for HIF-1α was labeled in the nest (200×). (**d**) In poorly differentiated OSCC, a mosaic pattern was observed for HIF-1α labeling and also had a strong nuclear HIF-1α staining (200×)

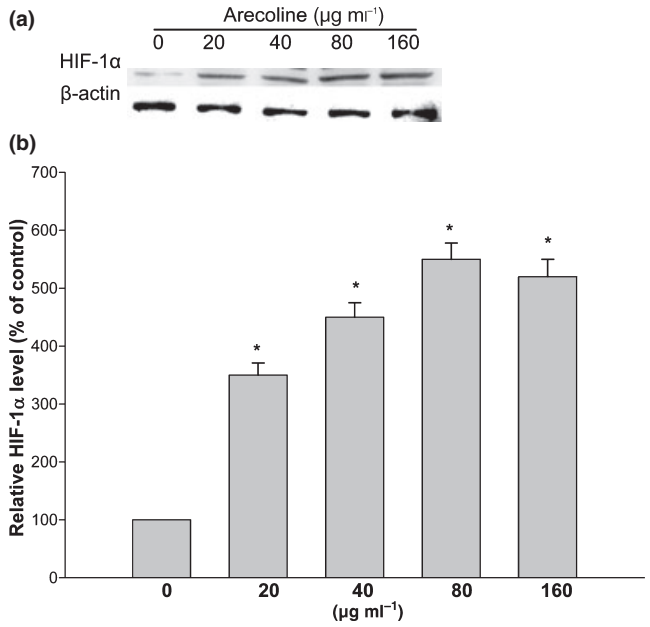

Figure 2 (**a**) Expression of HIF-1α in arecoline-treated GNM cells by Western blot. Cells were exposed for 8 h in serum-free DMEM containing various concentrations of arecoline as indicated. *β*-actin was performed in order to monitor equal protein loading. (**b**) Levels of HIF-1α protein treatment with arecoline were measured by densitometer. The relative level of HIF-1α protein expression was normalized against *β*-actin signal and the control was set as 1.0. Optical density values represent the mean ± s.d. *Significant difference from control values with $P < 0.05$

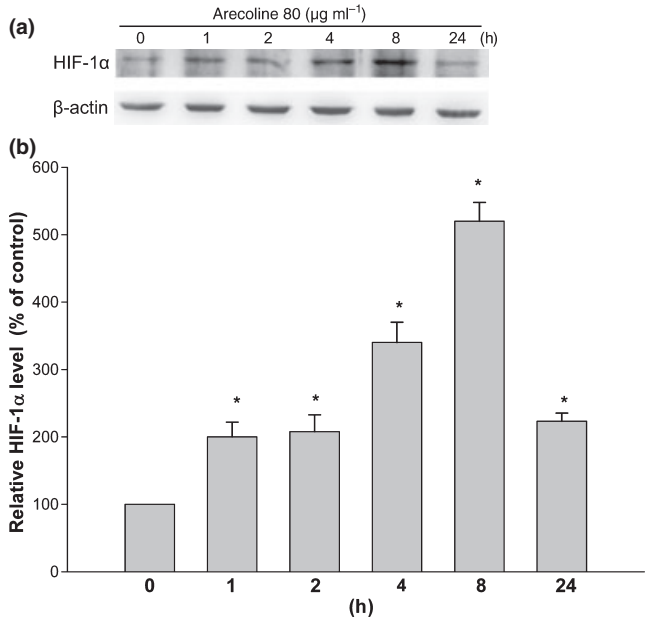

Figure 3 (**a**) Kinetics of HIF-1α expression in GNM cells exposed to 80 μg ml^{-1} arecoline for 0, 1, 2, 4, 8, and 24 h, respectively. *β*-actin was performed in order to monitor equal protein loading. (**b**) Quantization was achieved by densitometer as described in Figure 2. *Significant difference from control values with $P < 0.05$

1α is known to be induced by reactive oxygen species (ROS) (Bell and Chandel, 2007) and also activated by induced by a variety of heavy metals such as copper (van Heerden *et al*, 2004; Martin *et al*, 2005). Areca nut was found to contain a high copper content (Trivedy *et al*, 1997). The etiology of OSCC may be the generation of ROS during areca quid chewing (Nair *et al*, 1992; Chen *et al*, 2002). It is therefore feasible to suggest that high levels of copper released and ROS generated may at least in part be responsible for the upregulation of HIF-1α in areca quid chewing-associated OSCCs.

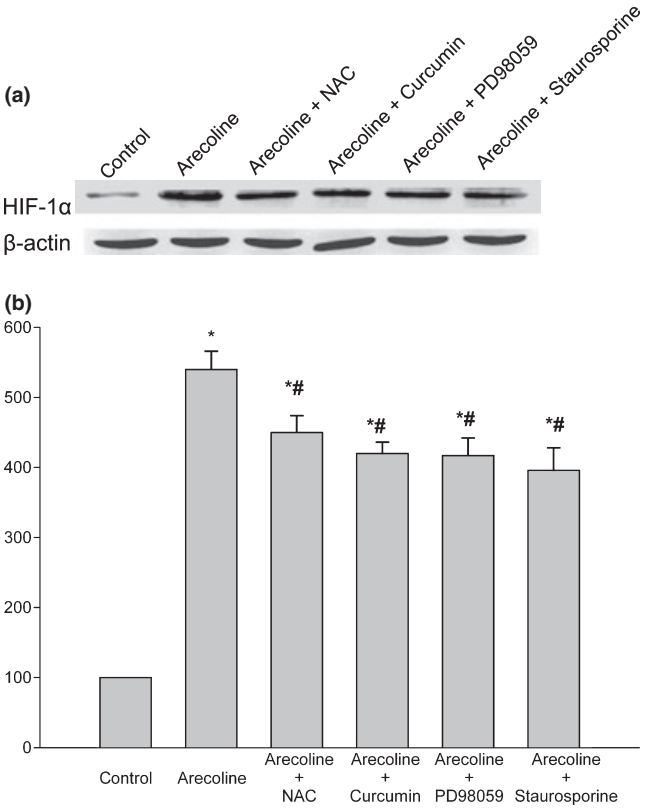

Figure 4 (**a**) The regulatory effects of NAC, curcumin, PD98059, and staurosporine on arecoline-induced HIF-1α expression in GNM cells. Cells were coincubation with various pharmacological agents in the presence of 80 μg ml^{-1} arecoline. *β*-Actin was performed in order to monitor equal protein loading. (**b**) Quantization was achieved by densitometer as described in Figure 2. *Significant difference from control values with $P < 0.05$. #Statistically significant between arecoline alone and arecoline with pharmacological agents; $P < 0.05$

In this study, we first reported the upregulation of HIF-1α protein expression in oral epithelial cell line GNM cells stimulated by arecoline. This suggests that one of the pathogentic mechanisms of OSCC may be the synthesis of HIF-1α expression by epithelial cells in response to areca quid challenge.

Previously, we demonstrated that arecoline significantly depletes intracellular GSH in human buccal mucosal fibroblasts (Chang *et al*, 2000). NAC is easily deacetylated inside the cells and provides cysteine for cellular GSH synthesis and thus stimulates the cellular GSH system (Gillissen and Nowak, 1998). In this study, NAC was found to inhibit arecoline-stimulated HIF-1α expression. These indicate that arecoline-stimulated HIF-1α expression may be partially related to the GSH levels.

Curcumin, a major active component of dietary spice turmeric and the yellow pigment in curry powder, has

been widely used in traditional medicine for the treatment of inflammation and diseases (Ammon & Wahl 1991). It is also well known as an AP-1 inhibitor. In this study, curcumin was found to reduce arecoline-stimulated HIF-1α expression in GNM cells. Curcumin was reported to significantly decrease HIF-1α protein levels in HepG2 hepatocellular carcinoma cells and down-regulate HIF-1α expression in vascular endothelial cells (Bae *et al*, 2006). Taken together, these results suggest that the AP-1 signal transduction pathway may be involved in the arecoline-stimulated HIF-1α expression. Curcumin may play a pivotal role in tumor suppression via the inhibition of HIF-1α.

The ERK signaling pathway is one of the mitogen-activated protein kinase cascades and plays important roles in the regulation of cell growth and differentiation (Johnson and Lapadat, 2002). Previous studies demonstrated that HIF-1α is phosphorylated in hypoxia by an ERK-dependent pathway (Minet *et al*, 2000). PD98059 was found to inhibit HIF-1α protein induced by 17*β*-Estradiol in human mesenchymal stem cells (Yun *et al*, 2009). In this study, PD98059 was found to reduce HIF-1α protein expression by arecoline. Consistently, Chang *et al* (2004) who reported that areca nut extract treatment resulted in significantly induction of p-ERK in human oral keratinocytes. These data provide the demonstration that activation of the ERK pathway may involve in arecoline-induced HIF-1α expression in GNM cells.

Protein kinase C is a specific serine/threonine kinase regulating a variety of homeostatic processes and is important in tumor promotion (Nishizuka, 1984). PKC inhibitor staurosporine analog CGP41251 has been used in colorectal cancer, adeno-carcinomas of unknown primary, breast cancer, and lung cancer in a phase I clinical trail (Thavasu *et al*, 1999). Previously, we also reported that staurosporine could inhibit oral cancer cell lines *in vitro* (Tsai *et al*, 2003). In this study, our results demonstrated that the addition of staurosporine significantly reduces the arecoline-induced HIF-1α expression. Recently, PKC inhibitors staurosporine and bisindolylmaleimide 1 were found to inhibit HIF-1α protein induced by 17*β*-estradiol in human mesenchymal stem cells (Yun *et al*, 2009). Taken together, the activation of the PKC pathway is involved in arecoline-induced HIF-1α expression in GNM cells. Staurosporine may provide a valuable tool in the reduction of areca quid chewing-associated OSCCs.

This study represents that HIF-1α is elevated in OSCCs specimens from areca quid chewers. Arecoline was capable of stimulating HIF-1α expression in GNM cells. This suggests that areca quid chewing may contribute the pathogenesis of OSCCs via HIF-1α expression. HIF-1α inhibited by NAC, curcumin, PD98059, and staurosporine suggest that AP-1, ERK, and PKC transduction pathways may be involved in the arecoline-stimulated HIF-1α expression. Therefore, studying the signal transduction pathway involved in HIF-1α expression may prove versatile. However, more detailed studies should be undertaken to clarify the agents that can regulate HIF-1α *in vitro* and *in vivo*.

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Author contributions

Chang YC and Lee SS designed the study, analyzed the data and prepared the manuscript. Tsai CH, Lee SS, Yang SF, and Ho YC performed the plan of the study and data analysis.

References

Ammon HP, Wahl MA (1991). Pharmacology of curcuma longa. *Planta Med* **57:** 1–7.

Bae MK, Kim SH, Jeong JW *et al* (2006). Curcumin inhibits hypoxia-induced angiogenesis via down-regulation of HIF-1. *Oncol Rep* **15:** 1557–1562.

Bell EL, Chandel NS (2007). Mitochondrial oxygen sensing: regulation of hypoxia-inducible factor by mitochondrial generated reactive oxygen species. *Essays Biochem* **43:** 17–27.

Bottaro DP, Liotta LA (2003). Cancer: out of air is not out of action. *Nature* **423:** 593–595.

Burri P, Djonov V, Aebersold DM *et al* (2003). Significant correlation of hypoxia-inducible factor-1α with treatment outcome in cervical cancer treated with radical radiotherapy. *Int J Radiat Oncol Biol Phys* **56:** 494–501.

Chang YC, Tai KW, Chou MY *et al* (2000). Synergistic effects of peroxynitrite on arecoline-induced cytotoxicity in human buccal mucosal fibroblasts. *Toxicol Lett* **118:** 61–68.

Chang MC, Wu HL, Lee JJ *et al* (2004). The induction of prostaglandin E2 production, interleukin-6 production, cell cycle arrest, and cytotoxicity in primary oral keratinocytes and KB cancer cells by areca nut ingredients is differentially regulated by MEK/ERK activation. *J Biol Chem* **279:** 50676–50683.

Chen CL, Chi CW, Liu TY (2002). Hydroxyl radical formation and oxidative DNA damage induced by areca quid *in vivo*. *J Toxicol Environ Health A* **65:** 327–336.

Chen YQ, Zhao CL, Li W (2009). Effect of hypoxia-inducible factor-1α on transcription of survivin in non-small cell lung cancer. *J Exp Clin Cancer Res* **28:** 29.

Dales JP, Garcia S, Meunier-Carpentier S *et al* (2005). Overexpression of hypoxia-inducible factor HIF-1alpha predicts early relapse in breast cancer: retrospective study in a series of 745 patients. *Int J Cancer* **116:** 734–739.

De Miranda CM, Van Wyk CW, Van Der Vijl P, Basson NJ (1996). The effect of areca nut on salivary and selected oral microorganisms. *Int Dent J* **46:** 350–356.

Fillies T, Werkmeister R, van Diest PJ, Brandt B, Joos V, Buerger H (2005). HIF1-alpha overexpression indicates a good prognosis in early stage squamous cell carcinomas of the oral floor. *BMC Cancer* **21:** 5–84.

Gillissen A, Nowak D (1998). Characterization of N-acetylcysteine and ambroxol in anti-oxidant therapy. *Respir Med* **92:** 609–623.

Gupta PC, Warnakulasuriya S (2002). Global epidemiology of areca nut usage. *Addict Biol* **7:** 77–83.

Harrison L, Blackwell K (2004). Hypoxia and anemia: factors in decreased sensitivity to radiation therapy and chemotherapy? *Oncologist* **9:** 31–40.

van Heerden D, Vosloo A, Nikinmaa M (2004). Effects of short-term copper exposure on gill structure, metallothionein and hypoxia-inducible factor-1alpha (HIF-1alpha) levels in rainbow trout (*Oncorhynchus mykiss*). *Aquat Toxicol* **69:** 271–280.

Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P (1996). Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* **56:** 4509–4515.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2004). Betel-quid and areca-nut chewing and some areca-nut derived nitrosamines. *IARC Monogr Eval Carcinog Risks Hum* **85:** 1–334.

Jeng JH, Chang MC, Hahn LJ (2001). Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives. *Oral Oncol* **37:** 477–492.

Jeong HJ, Hong SH, Park RK, An NH, Kim HM (2005). Ethanol induces the production of cytokines *via* the Ca2+, MAP kinase, HIF-1α, and NF-κB pathway. *Life Sci* **77:** 2179–2192.

Johnson GL, Lapadat R (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298:** 1911–1912.

Ko YC, Huang YL, Lee CH *et al* (1995). Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. *J Oral Pathol Med* **24:** 450–453.

Lee SS, Yang SF, Ho YC, Tsai CH, Chang YC (2008a). The upregulation of metallothionein-1 expression in areca quid chewing-associated oral squamous cell carcinomas. *Oral Oncol* **44:** 180–186.

Lee SS, Yang SF, Tsai CH, Chou MC, Chou MY, Chang YC (2008b). Upregulation of heme oxygenase-1 expression in areca-quid-chewing-associated oral squamous cell carcinomas. *J Formos Med Assoc* **107:** 355–363.

Lee SS, Tsai CH, Ho YC, Chang YC (2008c). The upregulation of heat shock protein 70 expression in areca quid chewing-associated oral squamous cell carcinomas. *Oral Oncol* **44:** 884–890.

Li L, Chen SH, Zhang Y, Yu CH, Li SD, Li YM (2006). Is the hypoxia-inducible factor-1α mRNA expression activated by ethanol-induced injury, the mechanism underlying alcoholic liver disease? *Hepatobil Panc Dis Int* **5:** 560–563.

Lin PY, Yu CH, Wang JT *et al* (2008). Expression of hypoxia-inducible factor-1α is significantly associated with the progression and prognosis of oral squamous cell carcinomas in Taiwan. *J Oral Pathol Med* **37:** 18–25.

Martin F, Linden T, Katschinski DM *et al* (2005). Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. *Blood* **105:** 4613–4619.

Michaud S, Menard C, Guy LG, Gennaro G, Rivard A (2003). Inhibition of hypoxia-induced angiogenesis by cigarette smoke exposure: impairment of the HIF-1α/VEGF pathway. *FASEB J* **17:** 1150–1152.

Minet E, Arnould T, Michel G *et al* (2000). ERK activation upon hypoxia: involvement in HIF-1 activation. *FEBS Lett* **468:** 53–58.

Mohamed KM, Le A, Duong H, Wu Wu *et al* (2004). Correlation between VEGF and HIF-1alpha expression in human oral squamous cell carcinoma. *Exp Mol Pathol* **76:** 143–152.

Nair UJ, Obe G, Friesen M *et al* (1992). Role of lime in the generation of reactive oxygen species from betel-quid ingredients. *Environ Health Perspect* **198:** 203–205.

Nishizuka Y (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308:** 693–698.

Szabo IL, Kawanaka H, Jones MKm *et al* (2001). Activation of hypoxia inducible factor-1α in gastric mucosa in response to ethanol injury: a trigger for angiogenesis? *Life Sci* **69:** 3035–3044.

Thavasu T, Propper D, McDonald A *et al* (1999). The protein kinase C inhibitor CGP41251 suppresses cytokine release and extracellular signal-regulated kinase 2 expression in cancer patients. *Cancer Res* **59:** 3980–3984.

Tilakaratne WM, Iqbal Z, Teh MT *et al* (2008). Upregulation of HIF-1α in malignant transformation of oral submucous fibrosis. *J Oral Pathol Med* **37:** 372–377.

Trivedy C, Baldwin D, Warnakulasuriya S *et al* (1997). Copper content in Areca catechu (betel nut) products and oral submucous fibrosis. *Lancet* **349:** 1447.

Tsai CH, Hsieh YS, Yang SF *et al* (2003). Matrix metalloproteinase 2 and matrix metalloproteinase 9 expression in human oral squamous cell carcinoma and the effect of protein kinase C inhibitors: preliminary observations. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **95:** 710–716.

Tsai CH, Chang TY, Chang YC (2009). Upregulation of lysyl oxidase expression in cyclosporin A-induced gingival overgrowth. *J Dent Sci* **4:** 13–17.

Urano N, Fujiwara Y, Doki Y *et al* (2006). Overexpression of hypoxia-inducible factor-1 alpha in gastric adenocarcinoma. *Gastric Cancer* **9:** 44–49.

Yun SP, Lee MY, Ryu JM *et al* (2009). Role of HIF-1alpha and VEGF in human mesenchymal stem cell proliferation by 17beta-estradiol: involvement of PKC, PI3K/Akt, and MAPKs. *Am J Physiol Cell Physiol* **296:** 317–326.