Stimulation of MMP-9 of oral epithelial cells by areca nut extract is related to TGF-β/Smad2-dependent and –independent pathways and prevented by betel leaf extract, hydroxychavicol and melatonin

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ABSTRACT

Background: There are 200-600 million betel quid (BQ) chewers in the world. BQ increases oral cancer risk. Matrix metalloproteinase-9 (MMP-9) is responsible for matrix degradation, cancer invasion and metastasis. Whether areca nut extract (ANE), a BQ component, stimulates MMP-9 secretion, and the related signaling pathways awaits investigation.

Results: ANE (but not arecoline) stimulated MMP-9 production of gingival keratinocytes and SAS cancer epithelial cells. ANE stimulated TGF-β1, p-Smad2, and p-TAK1 protein expression. ANE-induced MMP-9 production/expression in SAS cells can be attenuated by SB431542 (ALK5/Smad2 inhibitor), 5Z-7-oxozeaenol (TAK1 inhibitor), catalase, PD153035 (EGFR tyrosine kinase inhibitor), AG490 (JAK inhibitor), U0126 (MEK/ERK inhibitor), LY294002 (PI3K/Akt inhibitor), betel leaf (PBL) extract, and hydroxychavicol (HC, a PBL component), and melatonin, but not by aspirin.

Conclusions: AN components contribute to oral carcinogenesis by stimulating MMP-9 secretion, thus enhancing tumor invasion/metastasis. These events are related to reactive oxygen species, TGF-β1, Smad2-dependent and –independent signaling, but not COX. These signaling molecules can be biomarkers of BQ carcinogenesis. PBL, HC and melatonin and other targeting therapy can be used for oral cancer treatment.

Methods: ANE-induced MMP-9 expression/secretion of oral epithelial cells and related TGF-β1, Smad-dependent and –independent signaling were studied by MTT assay, RT-PCR, western blotting, immunofluorescent staining, and ELISA.

INTRODUCTION

Betel quid (BQ) chewing is popular in Taiwan and many other countries. There are about 2.8 million BQ chewers in Taiwan and 200-600 million BQ chewers in the world [1, 2]. In Taiwan, BQ contains mainly areca nut (AN), lime, \textit{piper betle} inflorescence with/without betel leaf (\textit{piper betle} leaf). The major chemical components of AN are alkaloids (arecoline, arecaidine, guvacoline, guvacine etc.), catechol, catechin,
polyphenols (flavonol, tannin), minerals (Cu, Fe etc.), carbohydrate, fat, protein, crude fibers etc. [1, 2]. ANE, arecoline, reactive oxygen species generated during oxidation of ANE, and the AN-derived nitrosamines are considered to be the possibly carcinogens. They exhibit genotoxicity, mutagenicity and cell transformation capacities in different assay systems [1, 2]. Clinically, BQ chewing increases the risk of oral leukoplakia, oral lichenoid lesions, oral submucous fibrosis (OSF) and oral squamous cell carcinoma (OSCC) [1, 2]. BQ ingredients are involved in the initiation and promotion of oral cancer by induction of DNA damage, chromosomal aberration, tissue inflammation, fibrosis and malignant transformation [1, 3]. However, limited information is known about the BQ components in tumor invasion, metastasis and progression.

Matrix metalloproteinases (MMPs) play important roles in tissue inflammation, tumor invasion and metastasis, by degradation of extracellular matrix [4, 5]. OSCC expresses higher level of MMP-2 and MMP-9 [6]. It is intriguing to know whether BQ components may affect MMPs expression/production and contribute to oral carcinogenesis. Recently, areca nut extract (ANE) activates MMP-9, but not MMP-2 expression in gingival epithelial cells, that can be inhibited by NF-kB inhibitor and curcumin [7]. ANE also stimulates MMP-9, but decreases tissue inhibitor metalloproteinase-1 (TIMP-1) and TIMP-2 secretion of SAS tongue cancer epithelial cells [8]. Salivary MMP-9 levels and MMP-2 and MMP-9 mRNA expression in OSCC are markedly increased and related to lymph node metastasis [9]. All the above reveal the importance of MMPs in oral carcinogenesis.

Previously we have found that AN components stimulates cytochrome P450, reactive oxygen species (ROS), check point kinase-1/2 (Chk1/Chk2), a disintegrin and metalloproteinases (ADAMs), epidermal growth factor/epidermal growth factor receptor (EGF/EGFR), Ras, Src, Janus kinase (JAK), mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling, cell cycle arrest, apoptosis and release of various inflammatory mediators such as 8-isoprostan, interleukin-1α (IL-1α), prostaglandin E2 (PGE2), IL-6, IL-8, etc. in different kind of cells [3, 10–14]. BQ components, ANE, and arecoline, are able to stimulate TGF-β signaling, and both OSCC and OSF tissues expressed higher level of TGF-β [15, 16]. ROS, TGF-β, tumor necrosis factor-α (TNF-α), IL-1α and IL-1β have been shown to induce Smad-dependent (ALK5/Smad) and -independent (transforming growth factor β-activated kinase-1, TAK1) signaling [17, 18]. TAK1 further induces downstream signaling pathways such as ROS, EGFR, mitogen-activated protein kinases (MAPKs), Akt, and nuclear factor kappa-B (NF-κB) etc. to regulate a number of cellular and clinical events, e.g., tissue inflammation-inflammatory diseases, cell death/tissue homeostasis, rheumatoid arthritis and carcinogenesis/cancer etc. [18–20].

To know whether BQ chewing and AN components can promote cancer progression, invasion and metastasis, it is interesting to know whether AN components may stimulate MMP-9 expression in oral epithelial cells and the role of TGF-β1/Smad2-dependent and Smad-independent (TAK1 and other related signal transduction) pathways. Moreover, one clinically critical question is whether including of PBL into BQ may enhance or decrease its carcinogenicity that is important for development of health policy and disease prevention for the country. PBL contains chemicals mainly hydroxycavicol (HC), eugenol, chavicol and carotene [2], and are shown to exhibit potential anti-mutagenic and anti-carcinogenic effect [2]. PBL extract and HC are found to have anti-oxidant, anti-inflammatory and anti-platelet effect possibly via scavenging ROS and inhibition of cyclooxygenase (COX) [21, 22]. Moreover, melatonin has been shown to have anti-cancer effects by mitigating the initiation, progression and metastasis of cancer development and growth possibly via receptor-dependent and –independent manners [23, 24]. Melatonin is shown to scavenge reactive oxygen species (ROS)/redox regulation, inhibition of signaling molecules, increase sensitivity of cancer cells to chemotherapeutic drugs, modulating of non-coding RNA, control of angiogenesis and apoptosis etc. [23, 24]. Since melatonin is present as natural hormone in human body and used clinically for insomnia/sleep improvement in different conditions [24], its preventive effect on ANE-induced toxicity is useful.

Since ROS and COX are associated with ANE toxicity and tissue inflammation is consistently found in tissues of OSF and OSCC [1–3, 10, 11], clarifying the preventive efficacy of betel leaf (piper betle leaf [PBL], hydroxycavicol (HC), aspirin and melatonin, on the ANE-induced alterations in oral mucosal cells is valuable for cancer prevention and treatment. Results of this study can be helpful for targeting therapy of OSCC in the future.

RESULTS

Effect of ANE and arecoline on MMP-9 mRNA expression of SAS cells

We recently found the stimulation of MMP-9 production of oral epithelial cells by ANE at non-cytotoxic concentrations as analyzed by enzyme-linked immunosorbent assay (ELISA). On the other hand, arecoline showed no marked stimulatory effect on MMP-9 production of oral epithelial cells, even decrease MMP-9
production possibly due to cytotoxicity at concentrations of 0.4 and 0.8 mM [8]. Accordingly, ANE also stimulated MMP-9 secretion of gingival keratinocytes (GK) (data not shown). ANE also induced MMP-9 mRNA expression of oral epithelial cells as analyzed by reverse transcription-polymerase chain reaction (RT-PCR) (Figure 1A). But arecoline showed no marked stimulatory effect on MMP-9 expression (Figure 1B). Accordingly, ANE stimulated the MMP-9 secretion of SAS cells after 3 days of exposure (Figure 1C), whereas arecoline showed slight decrease of MMP-9 secretion (Figure 1D). At tested concentrations, ANE showed no evident changes on viability of SAS cells (Figure 1E). Arecoline suppressed the viability of SAS cells at concentrations higher than 0.4 mM (Figure 1F).

**Role of TGF-β1 and Smad2 signaling in ANE-induced MMP-9 secretion of GK and SAS cells**

ANE stimulated TGF-β1 protein expression and this event can be inhibited by SB431542, an ALK5/Smad2/3 signaling inhibitor (Figure 2A). ANE induced the Smad2 phosphorylation in both SAS cells and primary...
SB431542 markedly attenuated the ANE-induced Smad2 phosphorylation in SAS cells and GK (Figure 2D, 2E). Intriguingly, SB431542 also prevented the ANE-induced MMP-9 secretion of both SAS cells and GK (Figure 2F, 2G). In this experiment condition, SB431542 showed little effect on the viability of SAS cells (Figure 2H).

**Role of TAK1 in ANE-induced MMP-9 expression/secretion**

Interestingly we found the activation of TAK1 by ANE. ANE rapidly stimulated p-TAK1 protein expression of SAS cells within 30 min of exposure, as shown by increase in red fluorescence (Figure 3A). To know

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**Figure 2. Role of TGF-β and Smad2 signaling on ANE-induced MMP-9 expression/secretion of oral epithelial cells.** (A) Stimulation of TGF-β protein expression of SAS cells by ANE (800 μg/ml) and its attenuation by SB431542 (1 and 5 μM), (B) ANE (100-800 μg/ml) stimulated Smad2 phosphorylation of SAS cells after 24-hr of exposure, (C) ANE (50-800 μg/ml) stimulated Smad2 phosphorylation of GK after 24-hr of exposure, (D) SB431542 (1 and 5 μM) attenuated the ANE (800 μg/ml)-induced p-Smad2 expression of SAS cells, (E) SB431542 attenuated the ANE (800 μg/ml)-induced p-Smad2 expression of GK, (F) SB431542 prevented the ANE (800 μg/ml)-induced MMP-9 secretion of SAS cells, (G) SB431542 prevented the ANE (800 μg/ml)-induced MMP-9 secretion of GK, (H) SB431542 showed little effect on ANE (800 μg/ml)-induced cytotoxicity of SAS cells (as % of control, 100%). *denotes statistically significant difference when compared with control. #denotes statistically significant difference when compared with ANE-treated group.
Further about the role of TAK1 in ANE-induced MMP-9 expression and secretion of oral mucosal cell, we intriguingly found that 5Z-7-Oxozeaenol (a TAK1 inhibitor) prevented the ANE-induced MMP-9 mRNA expression of SAS cells (Figure 3B). Accordingly, 5Z-7-Oxozeaenol also effectively attenuated the ANE-induced MMP-9 secretion of SAS cells (Figure 3C). At this experimental condition, 5Z-7-Oxozeaenol showed no marked changes on the viability of SAS cells (Figure 3D). Similarly, 5z-7oxozeaenol also prevented the ANE-induced MMP-9 mRNA expression of SAS cells (Figure 3E) and secretion of GK (data not shown). 5Z-7-Oxozeaenol also prevented the ANE-induced Smad2 phosphorylation in SAS cells (data not shown), suggesting the presence of crosstalk between different signaling pathways.

**Effect of Catalase, PD153035 and AG490 on ANE-induced MMP-9 production**

ANE has been shown to stimulate ROS production and glutathione (GSH) depletion [10], we therefore tested and found that catalase attenuated the ANE-induced MMP-9 production of oral epithelial cells (Figure 4A). Since ROS has been shown to be important signaling molecules, we further tested the possible EGFR and JAK signaling pathways in this event. Intriguingly, we found that PD153035 (a EGFR antagonist) evidently attenuated the ANE-induced MMP-9 production of SAS cells (Figure 4B). In addition, AG490 (a JAK inhibitor) was also effective to inhibit the ANE-induced MMP-9 production of SAS cells (Figure 4C). Accordingly, PD153035 and AG490 also attenuated the ANE-induced MMP-9 mRNA expression of SAS cells (Figure 4D, 4E). At these experimental conditions, catalase, PS153035 and AG490 showed no marked effect on the viability of SAS cells (Figure 4F, 4G, 4H), as analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (a mitochondrial enzyme activity marker).

**Role of PI3K/Akt and MEK/ERK in ANE-induced MMP-9 expression/secretion**

PI3K/Akt and MEK-ERK pathways are main downstream signaling molecules of ROS, EGFR and JAK. We interestingly found that LY294002 (a PI3K/Akt inhibitor) was able to prevent the ANE-induced MMP-9 mRNA expression (Figure 5A). Accordingly, LY294002 also suppressed the ANE-induced MMP-9 production of SAS cells (Figure 5B). Similarly, U0126 (a MEK/ERK inhibitor) inhibited the ANE-induced MMP-9 production of SAS cells (Figure 5C). LY294002 and U0126 showed little effect on ANE-induced cytotoxicity (Figure 5D, 5E).

**Effect of betel leaf, hydroxychavicol and aspirin on ANE-induced MMP-9 production**

Betel leaf (*piper betle leaf*, PBL) extract and hydroxychavicol (HC) has been shown to exhibit antiplatelet, antioxidant and even anti-inflammatory properties and inhibit cyclooxygenase (COX) enzyme activities [21, 22]. Three PBL components, hydroxychavicol (HC), catechol and catechin showed no marked stimulatory effect on MMP-9 production of oral epithelial cells (data not shown). Interestingly PBL extract and HC effectively inhibited the ANE-induced MMP-9 production of SAS epithelial cells (Figure 6A, 6B). But aspirin (a cyclooxygenase [COX] inhibitor) showed little effect on ANE-induced MMP-9 production of SAS cells (Figure 6C). Accordingly, PBL extract attenuated the ANE-induced MMP-9 mRNA expression of SAS cells (Figure 6D). In this experimental conditions, PBL and HC showed little cytotoxicity to SAS cells (Figure 6E, 6F).

**Effect of melatonin on ANE-induced MMP-9 mRNA expression and protein secretion**

Pretreatment and co-incubation by melatonin (100 and 250 μg/ml) effectively prevented the ANE-induced MMP-9 mRNA expression and MMP-9 secretion into culture medium (Figure 7A, 7B). Melatonin showed little effect on ANE-induced MMP-9 production of SAS cells (Figure 7C).

**DISCUSSION**

OSCC has been ranked as top 5 prevalent cancer in male of Taiwan. This can be due to oral habits such as BQ chewing, smoking and alcohol consumption in oral cancer patients. The 5-year survival rate of single primary OSCC patients is about 68% in Taiwan, a BQ chewing endemic area [25]. MMPs are involved in tumor invasion and metastasis and thus the prognosis/outcome of OSCC patients. Increased MMP-9 mRNA and protein expression are also reported in OSCC tissues [6, 9]. All these results suggest the contributory role of MMP-9 in oral carcinogenesis, tumor invasion and metastasis. Extracellular matrix homeostasis is important for wound healing, modulating keratinocyte behaviors, promoting keratinocyte migration, granulation tissue remodeling, and cancer [26]. Extracellular matrix turnover is tightly controlled by matrix metalloproteinases (MMP-2, MMP-9, collagenases etc.) and tissue inhibitors of metalloproteinases (TIMPs). TIMP-1 and TIMP-2 may inhibit the enzyme activity of MMP-2 and MMP-9 [27]. Degradation of basement membrane and interstitial connective tissues that contains mainly fibrillar collagens, and other ECM proteins such as...
Figure 3. Role of TAK1 activation in ANE-induced MMP-9 expression/secretion. (A) Stimulation of p-TAK1 activation of SAS cells by ANE. (upper) solvent control, and (lower) SAS cells treated by ANE (800 μg/ml) for 30 min. Pictures of p-TAK1 fluorescence (left) & merged with DAPI (right). Effects of 5Z-7-Oxozeaenol (1 and 5 μM) on (B) ANE (800 μg/ml)-induced MMP-9 mRNA expression of SAS cells as analyzed by semi-quantitative RT-PCR. One representative result was shown, and (C) ANE (800 μg/ml)-induced MMP-9 secretion in SAS cells as analyzed by ELISA. (D) Effect of ANE with/without 5Z-7-Oxozeaenol on the viability of SAS cells (as % of control, 100%), (E) ANE-induced MMP-9 mRNA expression of GK as analyzed by semi-quantitative RT-PCR.
Figure 4. Effect of catalase, PD153035 and AG490 on ANE-induced MMP-9 expression/secretion of SAS cells. Effect of (A) catalase (1000 and 2000 U/ml), (B) PD153035 (1 and 5 μM) and (C) AG490 (15 and 30 μM) on ANE (800 μg/ml)-induced MMP-9 secretion in SAS oral cancer epithelial cells. Results were expressed as Mean ± SE (pg/ml). (D) Effect of PD153035 and (E) Effect of AG490 on ANE (800 μg/ml)-induced MMP-9 mRNA expression, Effect of (F) catalase, (G) PD153035 and (H) AG490 on ANE (800 μg/ml)-induced changes of cell viability of SAS oral cancer epithelial cells. (Mean ± SE, % of control). *denotes statistically significant difference when compared with control. #denotes statistically significant difference when compared with ANE-treated group.
proteoglycans, elastin, laminin, fibronectin etc. by enzymatic proteases around the tumor cells is an essential step for cancer invasion and metastasis [27, 28]. Over-expression of MMPs is associated with tumor growth, relapse, metastasis and overall survival in breast and liver cancers [27]. Since BQ chewing is one major risk factor of chewer’s mucosa, leukoedema, oral leukoplakia, ulcer and oral carcinogenesis [1, 29], it is interesting to know whether BQ components may stimulate MMP-9 expression and secretion in oral mucosal cells. In addition to stimulation of MMP-9 secretion in SAS cancer epithelial cells [8], ANE, but not arecoline, also stimulates MMP-9 mRNA expression of SAS cells and MMP-9 secretion of

Figure 5. Effect of LY294002, and U0126 on ANE-induced MMP-9 expression/secretion. (A) Effect of LY294002 (10 and 20 μM) on ANE (800 μg/ml)-induced MMP-9 mRNA expression as analyzed by semi-quantitative RT-PCR. One representative result was shown, (B) Effect of LY294002 on ANE (800 μg/ml)-induced MMP-9 secretion in SAS cells as analyzed by ELISA. (C) Effect of U0126 (10 and 20 μM) on ANE (800 μg/ml)-induced MMP-9 secretion in SAS cells as analyzed by ELISA. *denotes statistically significant difference when compared with control. #denotes statistically significant difference when compared with ANE-treated group.
primary oral keratinocytes. Stimulation of MMP-9 production and expression by ANE indicates that BQ chewing possibly may contribute to the pathogenesis of chewer’s mucosa, leukoedema, ulcer, oral carcinogenesis and cancer progression by induction of MMP-9 and thus promote tumor invasion and metastasis. Since arecoline does not stimulate MMP-9 production, presence of other components in ANE that may stimulate MMP-9 production should be further clarified.

Recently AN components are reported to stimulate TGF-β, IL-1α and IL-1β [12, 13, 15, 16], that may potentially induce downstream transforming growth factor β-activated kinase-1 (TAK1) signaling. Interestingly we found that ANE increased TGF-β1

Figure 6. Effect of PBL, HC and aspirin on ANE-induced MMP-9 secretion. Effect of (A) PBL (500 and 1000 μg/ml), (B) HC (50 and 100 μM) and (C) aspirin (100 and 200 μM) on ANE (800 μg/ml)-induced MMP-9 secretion in SAS oral cancer epithelial cells. Results were expressed as Mean ± SE (pg/ml). (D) Effect of PBL on ANE-induced MMP-9 expression, and effect of (E) PBL and (F) HC on ANE-induced changes of cell viability of SAS cells (as % of control, Mean ± SE).
Figure 7. Effect of melatonin on ANE-induced MMP-9 expression and secretion. (A) Effect of melatonin (100 and 250 μg/ml) on ANE (800 μg/ml)-induced MMP-9 mRNA expression (one representative result was shown) and (B) Effect of melatonin on ANE-induced MMP-9 secretion in SAS oral cancer epithelial cells. Results were expressed as Mean ± SE (pg/ml). (C) Effect of melatonin on ANE-induced changes in cell viability of SAS cells (as % of control). *denotes statistically significant difference when compared with control. #denotes statistically significant difference when compared with ANE-treated group.

Elevated expression of p-Akt in OSCC and possible induction by nicotine is reported [38]. BQ, ANE and protein expression as well as the downstream p-Smad2 and p-TAK1 protein expression and signaling. SB431542 (an ALK5/Smad2/3 signaling inhibitor) attenuated the ANE-induced p-Smad2 and TGF-β1 protein expression as well as MMP-9 secretion. Moreover, 5Z-7-Oxozaenol (a selective TAK1 inhibitor) prevented the ANE-induced MMP-9 mRNA expression and protein secretion of SAS and GK. These results suggest the involvement of both Smad-dependent and TAK1 signaling in ANE-induced MMP-9 activity of oral mucosal cells. Similarly, Yang et al. (2017) found the regulation of MMP-9 by TAK1 in gastric cancer cells [30]. Targeting and knockdown of TAK1 inhibits chemokine receptor 7, MMP-9, IL-8, COX-2, tumor growth and metastasis of breast cancer [31]. Similarly, TGF-β1 is shown to stimulate MMPs in hepatocellular carcinoma cells via ALK5/Smad and PI3K/Akt signaling [32, 33]. These results raise the possible targeting TAK1 and ALK5/Smad molecules in future prevention and treatment of oral cancer.

Reactive oxygen species (ROS) production shows reciprocal interaction with TAK1 activation. Whereas ROS activate TAK1/AMPK signaling, TAK1 activation also stimulates further ROS production in different kind of cells [17, 34]. Interactions and crosstalk between TGF-β/TAK1 and EGFR and JAK signaling are reported [19, 35]. Interestingly we also found the cross-talk between TAK1 and Smad2 signaling in ANE-induced events in oral epithelial cells (data not shown). BQ components may stimulate ROS production of oral mucosal cells [10, 13, 22]. Antioxidants such as N-acetylcysteine (NAC) and catalase are able to prevent the ANE- and arecoline-induced toxic events such as COX-2 expression, PGE2, IL-1α, cytotoxicity etc. [13, 22] as well as MMP-9 secretion of SAS cancer epithelial cells in this study. ANE can activate EGFR [12], and inhibition of EGFR and JAK signaling by PD153035 and AG490 also suppressed the ANE-induced MMP-9 production of SAS cancer cells. Similarly, ROS may stimulate COX-2 expression of synovial fibroblasts via TAK1 signaling [17]. Moreover, inhibition of EGFR and JAK may potentially prevent the ANE-induced ADAM17, IL-1α, PGE2 production, COX-2 expression, ADAM9 maturation, and the ANE-induced decline in keratin 5 and 14 [12, 13]. EGFR overexpression in head/neck SCC is reported and associated with tumor stages and tumor-free survival [36]. Further, alteration of JAK2/Stat3 related genes’ expression in late stage OSCC is also noted [37]. These indicate that ROS scavengers, EGFR and JAK targeting molecules can be potentially used for future therapy of OSCC.
arecoline may stimulate MEK/ERK and/or PI3K/Akt signaling, to induce heat shock protein (hsp) 47, IL-1α, ADAM17, 8-isoprostane, and COX-2, but down-regulate involucrin expression of oral mucosal cells [11–14, 39]. Similarly, PI3K/Akt and MEK/ERK signaling pathways are also important for ANE-induced MMP-9 gene expression/production in oral epithelial cells. While arecoline stimulates PI3K/Akt to mediate hsp47 expression [39, 40], it does not stimulate MMP-9 expression/secretion of SAS epithelial cells [8]. While both ANE and arecoline may stimulate PI3K/akt signaling, their downstream reactive molecules are perhaps different and should be further addressed.

PBL is a popularly-used additive in BQ. Whether addition of PBL into BQ is beneficial or harmful to oral and systemic health is an intriguing health issue. PBL components and HC are demonstrated to exhibit hepatoprotective, immunomodulatory, anti-platelet, anti-inflammatory and antioxidative effects [12, 21, 22]. In addition, PBL and HC have potential cancer treatment effects toward various cancers [41, 42]. However, the anticancer effect of PBL is not clear. In this study, PBL extract and one major PBL component - HC (at non-toxic concentrations) prevent the ANE-induced MMP-9 production in oral cancer epithelial cells, suggesting attenuation effects to cancer invasion and metastasis. This is possibly due to antioxidant or other effects, but not through inhibition of COX enzyme activity, because aspirin showed little effect on ANE-induced MMP-9 production in this study. More studies are necessary to further clarify the pharmacological effects of PBL and HC.

Melatonin is secreted from pineal gland and has physiological regulation of sleep and circadian rhythms [43]. It is suggested for use in prevention and treatment of various cancer including head and neck SCC, possibly via effects on angiogenesis, cancer cell proliferation, apoptosis, matrix turnover, matrix metalloproteinase activity and epithelial-mesenchymal transition that are important in primary tumor and cancer metastasis [43, 44]. To further clarify the anticarcinogenic effect of melatonin, we found that melatonin prevents the ANE-induced MMP-9 mRNA expression/secretion of oral cancer cells. Why melatonin inhibits ANE-induced MMP-9 awaits further investigation. The potential mechanisms are blocking of signaling transduction molecules, its antioxidant property and prevent free radicals-mediated cell damage.

**CONCLUSIONS**

In conclusion, these results indicate that ANE components (BQ chewing) may enhance tumor invasion and metastasis via stimulation of MMP-9 mRNA expression and secretion. This event is associated with ROS, TGF-β1/Smad-dependent (Smad2), TAK1 and Smad-independent (EGFR, JAK, PI3K/Akt, MEK/ERK) signaling pathways, but not COX activation. ROS scavengers and various small molecules such as PBL, HC, melatonin or antibody for target therapy of these signal transduction pathways can be used for prevention and treatment of BQ chewing-related oral cancer and other diseases (Figure 8).

**Figure 8.** Proposed signaling mechanisms of ANE-induced MMP-9 expression/secretion of oral mucosal cells, contribution to oral carcinogenesis and its prevention by PBL, HC and melatonin.
MATERIALS AND METHODS

Materials and chemicals

Dulbecco’s modified Eagles’ medium (DMEM), penicillin/streptomycin, trypsin/EDTA, fetal bovine serum (FBS), KGM-SFM medium, and phosphate-buffered saline (PBS) were from Gibco (Life Technologies, USA). Arecoline, melatonin and MTT were from Sigma (Sigma-Aldrich Chemical Company, St. Louis, MO, USA). ELISA kits for MMP-9 were from PeproTech (PeproTech, Inc., Rocky Hill, NJ, USA). Catalase, SB431542, 5Z-7-Oxozeaenol, PD153035, AG490, U0126, LY294002, and aspirin were purchased from Tocris. Phospho-TAK1 (p-TAK1) antibody was from Abcam (ab79583). Antibodies for TGF-β1, and GAPDH were from Santa Cruz. P-Smad2 antibody was from Cell Signaling Technology. PBL extract and ANE were prepared as before [8, 12, 13, 21]. HC was synthesized as previously [22].

Culture of GK and SAS cancer epithelial cells

GK were cultured as previously in KGM-SFM with supplements [3, 11], by the approval of Ethics Committee, National Taiwan University Hospital (Ethical Approval Number: 201012090RC, 201112153 RID, 201512152RINC). SAS tongue cancer epithelial cells were cultured in DMEM containing 10% FBS and penicillin/streptomycin. They were regularly subcultured before confluence for use.

Cytotoxicity of ANE and arecoline to SAS cells

Briefly 5 x 10^5 cells were inoculated onto 6-well culture wells in 2 ml medium. After 24 hours, medium was changed with fresh medium containing various concentrations of ANE (100-800 µg/ml) or arecoline (0.05-0.8 mM) for 24 hours. Culture medium was collected for ELISA analysis of MMP-9 secretion as before [8, 45, 46]. Total RNA was isolated for semi-quantitative RT-PCR.

In some experiments, cells were exposed to different concentrations of some inhibitors (PD153035, AG490, 5Z-7-Oxozeaenol, LY294002, PBL, melatonin) for 30 min before the addition of ANE (800 µg/ml). Cells were further co-incubated for 3 days. Medium was collected for ELISA analysis. Cell layers were used for MTT assay for estimation of cytotoxicity. Cell layers were used for RNA isolation.

Semi-quantitative reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was done as described before [12]. In short, 3 µg of total RNA was reverse transcribed in a total volume of 44.5 µl reaction solution comprising 8 µl of deoxyribonucleotide triphosphate (dNTP) (2.5 mM), 4 µl of random primer (500 µg/µl), 1 µl of RNase inhibitor (40 U/µl), 4.5 µl of 10x RT buffer, and 0.5 µl of RT (21 U/µl) at 42°C for 90 minutes. Thereafter, 4 µl of complementary deoxyribonucleic acid (cDNA) was taken for PCR amplification in a reaction mixture of 50 µl containing 4 µl of dNTP (2.5 mM), 5 µl of 10x Super Taq buffer, 0.2 µl of Super Taq enzyme (2 U/µl) and 1 µl of each specific primer. The PCR program was set at 94°C for 5 minutes for the first cycle, and then further amplified for 20-35 cycles at 94°C for 1 min, 55°C for 1 min, and then 72°C for 2 min by a thermal cycler (Perkin Elmer 4800, PE Applied Biosystems, Foster City, CA, USA). Finally, the reaction was completed at 72°C for an additional 10 min. The specific primer pairs for β-actin (218 bp) was described previously and used as control [12]. The MMP-9 primer sequence was GTTGTAGCCGACCAGGCG and ACCGCAACTACGA CCGGGA with amplified product of 179 bp. The PCR-generated products were loaded into 1.8% agarose gel electrophoresis with reference of molecular weight markers ranging 100-1200 bp. The gels were finally stained with ethidium bromide and photographed for presentation.

Effect of ANE and arecoline on MMP-9 production and cytotoxicity of oral epithelial cells

Briefly 5 x 10^5 cells were inoculated onto 6-well culture wells in 2 ml medium. After 24 hours, medium was changed with fresh medium containing various concentrations of ANE (100-800 µg/ml) or arecoline (0.05-0.8 mM) for 24 hours. Culture medium was collected for ELISA analysis of MMP-9 secretion as before [8, 45, 46]. Total RNA was isolated for semi-quantitative RT-PCR.

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**Effect of ANE on TGF-β1, p-Smad2, and p-TAK1 protein expression**

**Western blotting analysis**
Briefly near confluent GK (in 6-well) or SAS cells (1 × 10^6 cells) were cultured on 10-cm culture dishes. They were then exposed to different concentrations of ANE (100–800 μg/ml) for 24 hours. Protein from cell lysates were collected and subjected to western blotting analysis as described previously [11, 12], except that TGF-β1 and p-Smad2 antibodies were used as primary antibodies. In some experiments, SB431542 was used for pretreatment and co-incubation with ANE (800 μg/ml) for 24 hours before cell harvesting.

**Immunofluorescent staining analysis**
Briefly SAS cells (1 × 10^5 cells/well) were cultured on coverslips in 24-well culture dishes. They were then exposed to ANE (800 μg/ml) for 5–120 min. Immunofluorescence staining of p-TAK1 expression was performed as before [45]. Medium was decanted, and cells were rinsed by PBS and fixed by paraformaldehyde (4%) for 20 min. Cells were then rinsed by PBS, permeabilized by Triton X-100 (2%), incubated in 0.3% v/v H_2O_2 for 20 min. After washed by PBS, bovine serum albumin (BSA, 5%) was applied for blocking of non-specific binding for 1 h, and cells were further incubated at room temperature to primary antibodies (p-TAK1) for overnight. Cells were washed with PBS and then incubated in TRITC-conjugated secondary antibodies (red fluorescence) in the dark for 1 h and counterstained by DAPI (1:1000) of nucleus staining for 30 min. Cells in coverslips were finally mounted and photographed/observed under an inverted microscope and DP Controller/Manager software (Olympus IX71, Olympus Corporation)

**Statistical analysis**
At least four or more independent experiments were conducted. If necessary, the results of cell study were analyzed by One-way ANOVA and post-hoc Turkey test.

**Ethics approval**
This study is approved by the Ethics Committee, National Taiwan University Hospital.

**Abbreviations**
ADAM17: a disintegrin and metalloprotease domain 17; ANE: areca nut extract; BQ, betel quid; Chk: checkpoint kinase; COX: cyclooxygenase; DMEM: Dulbecco’s modified eagle’s medium; DMSO: dimethylsulfoxide; EGFR: epidermal growth factor receptor; ELISA: enzyme-linked immunosorbent assay; HC: hydroxychavicol; GK: gingival keratinocyte; GM-CSF: granulocyte macrophage-colony stimulating factor; IL-1β: interleukin-1β; MAPK: mitogen-activated protein kinase; MMP-9: matrix metalloproteinase-9; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; NAC: N-acetyl-cysteine; OSCC: oral squamous cell carcinoma; OSF: oral submucous fibrosis; PBL: *piper betle* leaf; PBS: phosphate buffered saline; PGE2: prostaglandin E2; ROS: reactive oxygen species; RT-PCR: reverse transcription-polymerase chain reaction; TAK1: transforming growth factor activated protein kinase 1; TCIPA: tumor cell induced platelet aggregation; TGF-β: transforming growth factor-β; TIMP-1: tissue inhibitor metalloproteinase-1; TNF-α: tumor necrosis factor-α.

**AUTHOR CONTRIBUTIONS**
M.C.C, Y.H.P, J.J.L, and J.H.J. acquired the financial support, designed and performed the experiments, provide resources, contribute the experiments, and validated and performed the formal analysis of data, write and review the manuscript. H.L.W., E.J.L., W.C.L., C.Y.Y. performed some experiments, review manuscript draft and comments.

**CONFLICTS OF INTEREST**
All of the authors declare no conflict of interest for this submitted paper.

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