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Areca nut extract treatment down-regulates involucrin in normal human oral keratinocyte through P13K/AKT activation

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Summary Areca (betel) is an important etiological factor linked to the high prevalence of oral carcinoma and other oral diseases in South Asians. Involucrin is a key component of the cornified envelop and a differentiation marker of keratinocyte. In this study, we found that 5 μg/ml non-toxic areca nut extract (ANE) treatment resulted in the 0.5-fold down-regulation of involucrin and disruption in involucrin distribution in normal human oral keratinocyte (NHOK). Progressive down-regulation of involucrin during oral carcinogenesis was noted. Activation of AKT by 1.7-fold and up-regulation of COX-2 by 2-fold were elicited following ANE treatment in NHOK. Treatment with PI3K/AKT blockers reverted the down-regulation of involucrin. ANE also down-regulated involucrin by 0.6-fold and disturbed both cornified envelope and cell aggregation in calcium-induced differentiated NHOK. However, such phenomena seemed to be independent from the ANE-associated COX-2 activation. The ANE-associated down-regulation of involucrin through AKT pathway could underlie the areca-associated epithelial pathogenesis.

Introduction

Around 200–400 million people in South Asian and Southeast Asian countries are addicted to areca[1] Areca-associated oral squamous cell carcinoma (OSCC) is one of the leading cancers in these regions[2] Areca nuts contain polyphenols, arecoline, arecaidine and other alkaloids[3] Areca nut extract

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(ANE) is highly cytotoxic and genotoxic to cultured human oral epithelial cells[4] Studies have identified areca ingredients as the synergistic or promoting agents for multistep chemical carcinogenesis in hamster buccal pouch models.[5,6] Recently, we have demonstrated that chronic low dose of ANE treatment induced the senescence-associated phenotype of normal human oral keratinocyte (NHOK) and hyperdiploid chromosomal changes in low grade OSCC cells[7,8] However, the pathogenetic roles of areca for NHOK require further elucidation.

ANE can induce COX-2 expression and PGE2 production in OSCC or NHOK cells[9,10] Our previous studies have shown that ANE elicited a rapid activation of ERK and JNK1 mitogen-activated protein kinase (MAPK) as well as NF-κB in OSCC cells[11,12] Besides, ANE-modulated NF-κB activation could be the basis of COX-2 up-regulation associated with areca exposure[11] Our study also showed that the activation of Rho and Rac GTPase could underlie the areca-mediated morphological changes of OSCC cells[12] In mammals, protein kinase B or AKT (PKB/AKT) belongs to a serine/threonine kinase family consisting of PKBα (AKT1), PKBβ (AKT2), and PKBγ (AKT3). PKB/AKT is activated in cells exposed to diverse stimuli such as hormones, growth factors, oncogene and extracellular matrix components[13] AKT is the primary mediator of PI3K signaling; it has a number of downstream effectors including Bad, procaspase-9, Iκ-K, GSK-3, mTOR and so on, that can contribute to the onset of cancer.[13–15] Although AKT is highly expressed in OSCC, the activation of AKT following the stimulation of ANE have not been addressed[16] The interactive signaling cascades and phenotypic impacts induced by ANE in oral keratinocyte need extensive clarification.

The upper epidermis or oral epithelium acts as a barrier, composing mainly of terminally differentiated cells and keratinized layer, which are the endpoint of keratinocyte. During keratinocyte differentiation, cell membrane will be progressively replaced by cornified envelope, which consists of keratins that are enclosed within insoluble protein complexes cross-linked by transglutaminases (TGMs)[17] Involucrin is one of the insoluble proteins in cornified envelopes; it is abundant in the upper spinous and granular layers, and is a key marker of keratinocyte differentiation[18] A report showed that involucrin expression was reduced in intraepithelial cervical neoplasms as compared with normal epithelia[19] Moreover, involucrin expression was significantly down-regulated in highly metastatic OSCC cell lines as compared with parental cells with low metastatic potential[20] Hypoxia is an adverse status for tumor growth. Interestingly, involucrin seems to be an oxygen-regulated protein, and the majority of hypoxic cells in head and neck cancer express involucrin[21] Thereby, dysregulation or loss of involucrin expression is highly associated with neoplastic process of epithelium.

When cultured in a low-Ca^{++} (0.1 mM) medium, NHOK grows as a monolayer polygonal cell, which can be cultivated for 6 or 7 passages (for 20 population doublings)[8] In the initial passage, only scarce amount of cytosolic involucrin is present, but it is abundantly dispersed in cytosol during 3–4 passages[22] Conversely, when a higher concentration of Ca^{++} (1 mM) is used, NHOK differentiates with a squamous morphology. The regulation of keratinocyte differentiation by Ca^{++} is related to phospholipase C (PLC) and protein kinase C (PKC) activation, which might activate AKT and MAPK, and subsequently induce proteins required for differentiation[23] Activation of p38MAPK is also involved in involucrin up-regulation in keratinocytes[24] In addition, NF-κB signaling plays critical roles in regulating cell cycle withdrawal, cell survival, and differentiation in keratinocytes[25] In this study, we have identified that ANE is able to down-regulate involucrin by means of AKT activation, a signaling element lying downstream of PLC and upstream of NF-κB.

Materials and methods

Oral tissues

Sampling of tissue pairs from OSCC patients and oral precancerous lesions (OPL) for RT-PCR analysis was approved by an institutional review board. A 0.5 cm^3 tissue from OSCC, the matched metastatic OSCC lesions in cervical lymph nodes (abbreviated as LNM), OPL and a non-malignant matched tissue (NMMT) were obtained from patients. Tissue samples were embedded in OCT first, stored immediately in liquid nitrogen until use. Laser capture microdissection (LCM) was performed on frozen-sectioned tissue sections to retrieve pure epithelial components of NMMT, OPL, OSCC and LNM, representing various tumorigenic stages using a PixCell II LCM system and ARC200 v1.0.2 software (Arcturus, Mountain View, CA, USA). The clinicopathological features of the subjects are summarized in Table 1.

Table 1 Clinicopathological parameters of OPL and OSCC

Parameters	OPL	OSCC
Age (years)	38–62	41–79
Mean age ± SD	49.6 ± 10.8	53.0 ± 9.4
Sex (M/F)	5/0	20/1
Site		
Buccal mucosa	0	5
Tongue	3	7
Other sites	2	9
Diagnosis		
Epithelial dysplasia	0	
Hyperkeratosis or epithelial hyperplasia	5	
Differentiation		
Well		12
Moderate and Poor		9
Size		
T1–T3		4
T4		17
Stage		
I–III		2
IV		19
LNM		
Presence		7
Absence		14