Areca nut extract causes the phenotypic disruption of oral keratinocytes through PI3K/AKT activation

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Abstract:
Areca (betel) is an important etiological factor linked to the high prevalence of oral squamous cell carcinoma (OSCC) and other oral diseases in South Asians. In this study, we identified that non-toxic areca nut extract (ANE) treatment resulted in the down-regulation of involucrin, a differentiation marker of keratinocyte, and disruption in involucrin distribution in normal human oral keratinocyte (NOK). Progressive down-regulation of involucrin during oral carcinogenesis process was noted. Further, we also identified ANE treatment induced the epithelial to mesenchymal transition (EMT)-associated phenotypes in OSCC cells. ANE treatment resulted in the appearance of fibroblastoid morphology, the disappearance of E-cadherin from cell membrane, the overexpression of vimentin and the assembly of fibronectin. Activation of AKT was elicited following ANE treatment. Treatment with PI3K/AKT blockers reverted the down-regulation of involucrin in NOK and the expression of EMT-associated markers in OSCC cells. The results indicate that ANE-associated down-regulation of involucrin and genesis of EMT through AKT signaling could underlie the areca-associated epithelial pathogenesis.

ANE treatment causes morphological change and disruption of involucrin.

(Figure 1) A. NOK following ANE treatment exhibited an increase in cell population with spindle-cell morphology (a, b). A trend of dynamic involucrin in both cytoplasm and nucleus was noted (c). In comparison with cytokeratin (c, d, g) and involucrin (c, h) expression, ANE treatment at 48 h also exhibited such phenomenon, with a cytoplasm and nuclear localization of involucrin in a spindle-shaped NOK. B. A representative RT-qPCR analysis displays a decrease in involucrin mRNA expression. C. Quantitation of four individual experiments. The expression of COX-2 has not altered by each treatment. D. A representative western blotting displays a decrease in involucrin protein. E. Quantitation of D.

Involucrin mRNA expression in LCM-retrieved tissues.

(Figure 2) A. A representative RT-qPCR analysis in tissue pairs. B. Quantitation of involucrin mRNA expression in NOK, OSCC and LNM in comparison to paired NMMT.

ANE treatment activates AKT and ERK, and up-regulates COX-2.

(Figure 3) A. A representative western blotting displays an increase of p-AKT. B. Quantitation of three independent samples. C. A representative western blotting displays an increase of p-ERK. D. Quantitation of two of four independent NOKs. E. A representative RT-qPCR and western blotting analysis of COX-2 level, respectively. F. Quantitation of five NOKs.

PI3K/AKT blockers revert ANE-associated involucrin down-regulation.

(Figure 4) A. RT-qPCR analysis of a representative NOK exhibits the blocking of ANE-associated down-regulation on involucrin mRNA expression by wortmannin and LY294002. U0126 does not exert such beneficial effect. B. Quantitation of A.

ANE treatment disrupts the subcortical localization and involucrin expression in Ca++-induced differentiated NOKs.

(Figure 5) A. Fluorescence microscopy reveals that Ca++ induces NOK aggregation and subcortical localization of involucrin (b, d) relative to control NOK (a, c). Concomitant ANE treatment eliminates the intense NOK cortical and continuous subcortical involucrin localization. Cytoskeletal reorganization of involucrin is also seen. B. RT-qPCR analysis reveals that Ca++ treatment induces involucrin down-regulation, while ANE treatment further down-regulated the involucrin level. C. Upper panel: a representative western blotting displays a decrease in involucrin protein. D. Quantitation of C.

ANE-associated COX-2 activation is not essential for involucrin down-regulation in Ca++-induced differentiated NOKs.

(Figure 6) A. A representative western blotting displays a slight increase of p-AKT following Ca++ treatment. Concomitant ANE treatment for 24 h did cause further increase in p-AKT, but both p-AKT and p-ERK were increased for 24 h. B. Quantitation of A. C. A representative western blotting displays an increase of p-ERK in a Ca++-induced NOK following concomitant ANE treatment for 24 h. C. Upper panel: a representative RT-qPCR analysis of COX-2 mRNA expression on Ca++-induced NOKs. D. A representative western blotting displays an increase of COX-2 protein expression following Ca++-induced control NOKs. E. A representative western blotting displays an increase in COX-2 protein expression following Ca++-induced ANE treatment. F. Quantitation of E. G. RT-PCR analysis of a representative NOK exhibits no change in ANE-associated involucrin down-regulation when cells are treated with LY294002. H. Quantitation of H.

ANE treatment induces EMT-associated phenotypes through PI3K/AKT pathway.

(Figure 7) A. A representative western blotting displays an increased p-AKT following ANE treatment can be down-regulated by 10nM wortmannin, 10nM LY294002, and 10μM wortmannin. B. A representative western blotting displays an increase of p-ERK following ANE treatment in a Ca++-induced differentiated NOK. C. The blockage of ANE-associated up-regulation on involucrin protein expression by wortmannin, LY294002, and AKT inhibitor V. D. Quantitation of C.