**Abstract**

Arecanut is known as a cancerogen for oral cancer in southeast Asia, however, the molecular mechanism leading to the malignancy is still unclear. To mimic the habit of arecanut chewing, our laboratory has established four oral cancer cell sublines (SAS, OECM1, K2, C9), chronically trained by arecanut extract (ANE). To elucidate the molecular basis of arecanut induced oral carcinogenesis, the differential proteomes between oral cancer cells and the ANE sublines were determined using the technique of isobaric mass tag (iTRAQ) labeling and multidimensional liquid chromatography-mass spectrometry (LC-MS/MS). Over thousand proteins were identified in four sublines, in which 194 proteins were found differential expressions in at least two ANE sublines. Bioinformatic analysis revealed that these proteins participate in several pathways, with the regulation of epithelial to mesenchymal transition (EMT) most prominent. Fourteen proteins were confirmed differential expression in the ANE sublines, including Krt17. To shed more light on the mechanism of ANE induced carcinogenesis, Krt17 was further investigated. Knockdown Krt17 significantly suppressed ANE-induced cell growth, migration, and invasion, along with the modulation of EMT process. Furthermore, in a carcinogen-induced oral cancer mice model by 4NQO/aracoline, an active compound of ANE, Krt17 was found significantly up-regulated in all hyperplasia and carcinoma (p<0.001). In conclusion, we have identified proteome associated with chronic arecanut extract in oral cancer cells. Krt17 was demonstrated contributing to arecanut induced oral malignancy. This study should attribute to risk assessment, disease prevention or other clinical applications of arecanut nut-induced oral cancer.

**Material and method**

**Fig 1.** Identification of differentially expressed proteins in arecanut extract (ANE)-trained cell sublines. (A) A schematic diagram illustrates the workflow of proteomic profiling after induction by ANE with iTRAQ-based analysis. These four ANE sublines were labeled in parallel with the corresponding iTRAQ reporter. iTRAQ labeling was performed using equal amounts of proteins from pooled parental and ANE-trained sublines followed by SCX chromatography. The fractions were subjected to LC-MS/MS analysis in an LTQ-Orbitrap Velos mass spectrometer. The data were searched and collected with the Proteome Discoverer program using Mascot software as the search engine. The numbers of identified proteins are shown as Venn diagrams. (B) The numbers of proteins identified or quantified in two iTRAQ-based experiments. and the numbers of proteins that were determined to be up-regulated or down-regulated in four ANE sublines, including SAS, OECM1, CGHNC2 and CGHNC9 cells. Venn diagrams show the overlap between proteins identified or quantified in the two experiments. The total number of proteins identified or quantified in each experiment is listed in brackets. (C) The relative expression levels of 196 significant proteins in ANE-trained sublines compared with the parental cells.

**Fig 2.** Functional classification of 196 significant proteins using Metacore analysis software.

**Fig 3.** Verification of chronic arecanut related genes expression level by RT-qPCR. Verification of 29 molecules that were differentially expressed among four paired oral parental (PT) and ANE-trained subline cells by RT-qPCR.

**Fig 4.** Krt17 protein expression is up-regulated by ANE. (A) Krt17 expression is up-regulated in response to chronic arecanut nut exposure, as observed in four pairs of parental (PT) and ANE sublines by western blot analysis. (B) Krt17 expression is up-regulated in a dose-dependent manner three oral cancer cell lines (OECM1, CGHNC9 and SAS) upon short-term (24 h) ANE treatment (0–500 µg/ml). In each sample, the relative expression of Krt17 was determined after normalization to the GAPDH expression level, as shown in the right panel.

**Fig 5.** Krt17 expression was knockdown by Krt17-specific shRNA in CGHNC9 and OECM1 cells.

**Fig 6.** Krt17 knockdown suppresses the effects of ANE-induced cell migration and invasion. Krt17 gene knockdown suppressed ANE induce cell migration ability in (A) CGHNC9 and (B) OECM1 cells and cell invasion ability in (C) CGHNC9 and (D) OECM1 cells. Right panel has shown quantitative results.

**Fig 7.** Krt17 knockdown abolished ANE-induced epithelial-mesenchymal transition (EMT). Relative expression levels of EMT-associated molecules in Krt17 knockdown cells and the control (parental) (A) CGHNC9 cells and (B) OECM1 cells.

**Fig 8.** Krt17 is elevated in the oral lesions of mice with spontaneous tumor induction.