Gene expression profiling of oral cancer cells chronic exposed to areca nut extract

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Abstract

Oral cancer is the 6th most frequent cancer in Taiwan. The habit of areca nut chewing is the main etiological factor of oral cancer. To shed light on molecular basis of areca nut associated oral carcinogenesis, we established two oral cell sublines chronically treated with areca nut extract (ANE) at IC70 dose for 2 months. Affymatrix microarray was used in transcriptome profiling between parental and ANE sublines of oral cancer cells. Algorithmic analysis was applied to analyze the network regulatory pathways. RT-PCR was used to validate the genes altered expressions in ANE-sublines. Total of 35 genes was differentially expressed in both sublines. Several functional pathways were apparently altered, with lipid metabolism \((P=1.95\times10^{-10})\), oxidative phosphorylation \((P=1.02\times10^{-6})\), and cell adhesion \((P=9.66\times10^{-6})\) most significant. Seven genes were confirmed over 2-fold of changes, including HMGCS1, KRT-17 up-regulation and SMC4, CENPF, ID-1, IL1-alpha and Ches1 down-regulation. Further study revealed Ches1 was down regulation upon arecoline treatment. Consistently, this gene was reduced expression in 52\% of oral cancer tissues, which was significantly correlated with areca nut chewing habit of patients \((p=0.04)\). Thus, these results provide information regarding the molecular mechanisms of areca nut-induced oral carcinogenesis.
Background

Oral Cancer/ oral cavity cancer

Oral cavity includes lips, inside lining of the lips, cheeks (buccal mucosa), teeth, gums, the front two-thirds of the tongue, the floor of the mouth below the tongue, the bony roof of the mouth (hard palate), and the area behind the wisdom teeth.

Oral cancer (ORC) is one of the ten most frequent cancers worldwide. According to the Oral Cancer Foundation report, an estimated 481,000 new cases were diagnosed annually on 2007. The global estimates from the WHO in the year 2000 have reported an incidence rate for ORC of 14.27 per 100,000. In Taiwan, the mortality rate of ORC is significantly and most increased from 1999 to 2008. The incidence of oral cancer is also sixth leading cancer and still increasing in the recent years. Especially, oral cancer is forth leading cancer in male, appearing that males have high risk suffering oral cancer. Previous study indicates 90% of male oral cancer patient with betel quid chewing and smoking⁴.

Risk factor of oral cancer- betel quid

The habits of alcohol drinking, betel quid chewing, and cigarette smoking have been documented as risk factors for oral precancerous lesions and oral cancer². Patient combining alcohol drinking, betel quid chewing, and cigarette smoking have 123-fold increase of suffering oral cancer. Moreover, betel quid chewer has 28-fold to suffer oral cancer compared with control, and it is highest risk than other risk factors in Taiwan³.

Betel quid (BQ) is generally known as the complex of areca nut, lime, catechu, piper betel inflorescence, piper betel leaf and so on. It is an old oral habit, and
extensively chews in many Asian countries, especially in South and Southeast Asian. There are about 600 million betel quid chewers, more than 15% of the total population, the 4th most popular oral habit in the world. In the South Asian and Southeast Asian, more than 200 million, including more than 2 million, around 10% of total population in Taiwan, are addicted to betel quid. Unlike most countries in Southeast Asia which mostly used mature betel fruit, the Taiwan chewer commonly use fresh unripe betel fruit and with slaked lime as an essential ingredient.

**Areca nut extract**

Among the betel quid, areca nut extracts have been considered to be the major etiologic factors in the pathogenesis of oral cancer and other oral diseases. From NHRI report in 2000, areca nut extract is composed of 0.15-0.67% alkaloids, 11.4-26% polyphenols, 1.3-17% fats, 47.2-26% saccharide, unclear percentage of crude fiber and rare tannins. Considerable evidence suggests that alkaloids of areca nut are the major factors for genotoxicity. Arecoline is the most abundant component of alkaloids. In saliva, arecoline can transfer to arecadine by enzyme of saliva. The alkaloids in areca nut extract can undergo nitrosation reactions and the nitroso-derivatives in the oral cavity have been suggested as constituting the primary cause of oral mucosal lesions.

Areca nut extract (ANE) is highly cytotoxic and genotoxic to cultured human oral mucosal epithelial cells and fibroblasts. ANE can cause DNA-protein cross-links, DNA strand breakage, unscheduled DNA synthesis, sister chromatid exchange, cytotoxicity, and transformation. Moreover, areca nut extract is shown to inhibit cell growth and generated of reactive oxygen species to cause apoptosis. Metastases is the cause of 90% of human cancer death. Metastases consist of migration and invasion mainly. There have been some evidences that ANE associate
migration and invasion. Matrix metalloproteinases (MMPs) on salivary may participate in tumor invasion and migration. Areca quid consumption stimulates elevation of MMP-9 and induces cell invasion. Similarly, the regulation of MMP-8 by betel quid affect on cell migration. In addition, LOX exerts oncogenic roles in areca-associated OSCC and regulates cell invasion and migration.

In vivo study, ANE identified as being carcinogenic for humans have typically been demonstrated to be carcinogenic for various animal models. Consequently, areca nut should be highly suspected as a human carcinogen.

CHES1

Using differentiation display technique, we have previously identified several oral cancer-associated genes, including CHES1 (checkpoint suppressor 1) (Fig. 2). Compared to tumor and normal mucosa tissue, CHES1 was found at least 2-fold under-expressed in 46% of the tumor tissue samples. Therefore, CHES1 may lose its function during oral cancer development.

CHES1 was first isolated through suppression of the mec1-1 checkpoint mutation in S. cerevisiae following to be named checkpoint suppressor 1. CHES1 locates on chromosome 14q24.3-q31, determined by using of the CA repeat in the 3’ untranslated region (UTR) of the mRNA. This repeat was found to be highly polymorphic in human DNA. Initial mapping determined linkage of this marker (named CCC1) with 14q32, which was further refined to the region between 14q24.3 and 14q31. CHES1 encodes 2667 nucleotides. The ATG and TAA codons of the open reading frame are 1473 bp. CHES1 encodes a protein of 490-amino acid with predicted molecular mass of 54 kDa. This gene shows a small but significant region of homology to HTLF (human T-cell leukemia enhancer factor), a member of the fork head/Winged Helix family. There are 51% identical and 69% conserved residues
between HTLF and CHES1. In addition to the highly conserved DNA binding domain, there are significant regions of homology between these two proteins in both upstream and downstream of this motif. Therefore, CHES1 is a member of the fork head/Winged Helix subfamily of transcription factor, which include HTLF, rodent WHN, and FKHR.

It has been reported that CHES1 is responsible for G2 arrest of the cell cycle after DNA damage and via an MEC1-independent checkpoint pathway. In study of S. cerevisiae, CHES1 suppresses the sensitivity to DNA damage and restores damage-induced G2/M arrest in checkpoint-deficient strains. CHES1 protein interacts with Sin3, a member of the S. cerevisiae Sin3/Rad3 histone deacetylase complex (HDAC). Whereas, CHES1 can not suppress the DNA damage response in Sin3 mutant strains, and over-expression of Sin3 blocks CHES1-mediated G2/M arrest after DNA damage. Therefore, CHES1 may possess function by inhibition of Sin3 in HDAC effects. However, the function of CHES1 in mammalian cells was not yet reported.
Materials and methods

Preparations of areca nut extract (ANE)

The fresh areca nuts were smashed with sterilized water by juicer, and percolated by strainer. Then, those ANE was filtrated by Grade NO.1 qualitative filter paper (Whatman, UK). The filtrate was lyophilized by FreeZone 2.5 Liter Benchtop Freeze Dry System (Labconco, USA), and re-dissolved in sterilized water. After being filtrated by 0.22 μm filters, ANE became as working solution for training cultured cells and was stored at -20°C.

Liquid chromatography

According to previous study, we modified their condition of HPLC to detect our samples 27. Analyses were performed on Waters 2690 Separations Module HPLC system (Waters, USA) with C18 reversed-phase column (Sunfire). Buffer A consisted of 50% acetonitrile plus 50% 0.01M NaH₂PO₄ with 0.01% TEA. Buffer B consisted of 10% acetonitrile plus 90% 0.01M NaH₂PO₄ with 0.01% TEA. Mobile phase consisted of 50% buffer A and 50% buffer B (v/v). All chromatographic solvents were degassed with helium before use. Isocratic chromatography was 1 mL/min at 30°C.

Arecoline (sigma) and arecadine (sigma) were used as the standards for ANE quality control. Using 2.5 μg/ml, 5 μg/ml and 10μg/ml arecoline established standard curve to monitor quantity of arecoline of ANE. Finally, using UV detector detected those compounds on 215 nm.

Establishment of ANE-trained sublines

OECM1 and SAS were used and maintained, as previously described.11,12 For
each cell line, the IC30 dose of areca nut extract was determined by calculating the concentration at which there was 70% cell viability after 24 hours of treatment. The areca nut extract–trained sublines were established by chronically treating cells with areca nut extract at IC70 doses for 30 passages.

**Affymetrix Microarray and Functional Network Analysis**

The RNA extraction and microarray analyses were similar, as previously described. Briefly, the Affymetrix U133A microarray and scanning systems were used. The functional network analyses of differentially expressed genes were performed using the MetaCore Analytical suite (GeneGo, St Joseph, MI). MetaCore is a webbased computational platform that provides cluster analysis of gene expression data in the context of regulatory networks and signaling pathways. MetaCore was used to calculate the statistical significance (p-value) based on the probability of assembly from a random set of nodes (genes) of the same size as the input gene list.

**Cloning of Ches1 Full-Length Plasmid and Cellular Transfection**

Full-length open reading frame of Ches1 was produced by RT-PCR. Polymerase chain reactions (PCRs) were carried out with 35 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, and extension at 68°C for 2 minutes. The primers for fulllength Ches1 were 5’-GGTACCAATGGTCAGTCATG-3’ and 5’-TCTAGATTAATTTTTTGTGGTT-TCTTTTT-3’. The full-length Ches1 gene was cloned in-frame into pGEM-T easy vector (Promega, Fitchburg, WI) to obtain pGEMChes1 plasmid and verified by direct sequencing. The full-length Ches1 fragments were subcloned into pFlag-CMV-2 vector (Sigma, St Louis, MO) using KpnI/XbaI restriction enzyme site to obtain pFlag-Ches1 plasmids. The oral cancer cells, SCC25, were transfected in
100-mm dishes with the mixture 6 lg plasmid DNA and 6 IL Lipofectamin 2000 (Invitrogen, Carlsbad, CA) in 3 mL OPTI-MEM medium (Gibco, Langley, OK) and incubated in 37°C, in a 5% CO2 incubator for 10 hours. After this, the medium was replaced with fresh complete medium and continuously cultured. Cell numbers were determined daily.

**Flow Cytometry Analysis**

Cells transfected with pFlag-Ches1 or the vector plasmids were harvested at a density of 8 *10^5/mL and washed with phosphate-buffered saline (PBS). The cell pellets were fixed with ice-cold 70% ethyl alcohol in PBS at 20°C for 1 hour and then centrifuged at 1500 revolutions per minute for 5 minutes. The pellets were suspended and incubated with 0.5% Triton X-100 (Sigma Chemical Co) and 0.05% RNase (Sigma) in 1 mL PBS at 37°C for 30 minutes and then centrifuged at 1500 revolutions per minute for 5 minutes. These cell pellets were resuspended and incubated with 40 mg/mL propidium iodide in 1 mL PBS at room temperature for 30 minutes. Samples were analyzed by FACScan flow cytometry (Becton Dickinson, San Jose, CA). The distribution of cell cycle phases was determined using Cell Quest Pro and ModiFit software.

**Patients and clinical tissues**

Fifty- two consecutive patients seen at the Otorhinolaryngology or Head and Neck Surgery clinics at Chang Gung Memorial Hospital (Taoyuan, Taiwan) were enrolled. Written informed consent was obtained from all subjects prior to this study, and this study was approved by the Institutional Review Board at Chang Gung Memorial Hospital. The characteristics of these head and neck cancer patients were summarized in the Table 1. There were 48 (92%) males and 4 (8%) females. The
mean age was 49.8 years, ranging from 30 to 78. A total of 34 (65%) consumed alcohol, 44 (85%) smoked tobacco, and 43 (83%) chewed betel quid. Biopsies of cancer and grossly normal mucosa tissue were obtained from each subject before chemo- or radiotherapy. Part of the diseased tissue was dissected and frozen immediately in liquid nitrogen until used for molecular assays. The remaining sample was fixed in formalin and processed for routine histopathologic examination. All patients had undergone a series of clinical evaluations, including assessment of tumor extension before treatment and the tumor response to therapy. All cancers were histologically graded as well differentiated, moderately differentiated, poorly differentiated, or undifferentiated according to the World Health Organization (WHO) classification. The diagnosis, clinical staging, and identification of the anatomic site of the HNC were based on the American Joint Committee on Cancer (AJCC) TNM Classification of Malignant Tumors.

**Reverse transcription and quantitative RT-PCR**

The RNA extraction and quantitative RT-PCR analysis was performed similarly as previously describe (12, 13). Briefly, Total RNA was extracted by using TRIzol reagent (Gibco BRL, Rockville, MD). Ten micrograms of total RNA was reverse transcribed using poly-T primer. Quantitative PCR reactions were performed using an ABI Prism 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems). The primers used for each specific gene are listed in Table 2. All assays were performed in duplicates with less than 5% of variation. For an internal control, we derived a Ct using Ribosome 18S RNA expression. ΔCt was the difference in the Ct values derived from the specific gene being assayed and the 18S control, while ΔΔCt represented the difference between the paired tissue samples, as calculated by the formula ΔΔCt = ΔCt of normal tissue - ΔCt of tumor tissue. The value of the
expression of a specific gene of a tumor sample compared to the normal counterpart was expressed as $2^{\Delta \Delta Ct}$. A gene differential expression in tumor tissue over 2-fold of the normal counterpart was defined as significance difference.

**Statistical Analysis**

The Pearson chi-square test was used to examine the association of Ches1 expression and clinicopathologic features, including TNM stage and histopathologic characteristics. All P values were two-sided, and the significance level was set at a P of <0.05.
Results and discussion

Quality assurance of areca nut extract

After extraction of the areca nut with sterilized water, the high performance liquid chromatography (HPLC) was used to monitor the quality of the ANE preparations. Two major alkaloids of areca nut: arecoline and its esterase form arecadine were used as the controls for ANE quality. Results were shown in the Figure 1. As shown, the two batches of ANE preparations obtained similar results. Both have significant fractions of arecoline and arecadine, with arecoline concentrations 3.9 and 3.2 ug/ml, respectively. These results indicate that the ANE preparations are consistent and possess major active areca nut ingredients.

Establishment and Verification of ANE chronically trained sublines

For each cell line, the IC70 dose of ANE was determined by calculation the concentration at 70% cell viability for treatment of 24 hours after dose titration. The ANE doses were 900 and 600 ug/ml respectively for OECM1 and SAS cells, and which were used for long term treatment. To establish ANE chronically trained sublines, the cells were continuously treated of ANE with IC70 dose for total of 30 passages.

The ANE-sublines were subjected to ANE to determine the differential sensitivity. Various concentrations of ANE (0 ~ 1200 ug/ml) was treated to cells for 24 hours, and the viability was determined. Results were shown in the Figure 2. The ANE-subline cells showed more resistance to ANE treatment than the parental cells, and this phenomenon was more apparently with the increase of doses. At the dose of 1200 ug/ml, there was 1.39- and 1.42-fold more resistance in the ANE-trained sublines of OECM1 (Fig 2A) and SAS (Fig 2B) cells. These results indicate that the
ANE chronically trained sublines have been established.

**Differential transcriptomes and functional pathway prediction of ANE-trained subline**

The ANE-trained subline cells, along with the parental cells, were subjected to Affymetrix microarray analysis. Up-regulation or down-regulation was defined as a gene expression in the parental cells that was 1.5-fold higher or lower than that in the sublines. In comparison of OECM1 and the ANE-trained subline cells, 770 genes were found differentially expressed, with 508 genes up-regulated, and 262 genes down-regulated. In comparison of SAS and the ANE-trained subline cells, 300 genes were found differentially expressed, with 26 genes up-regulated, and 274 genes down-regulated. Total of 35 genes were found differentially expressed in both cell lines, including 10 up-regulated and 25 down-regulated. The characteristics of these proteins were listed in the Table 3. In which, 8 (23%) belongs to metabolic enzymes, 6 (17%) involve in signal transduction or functions as molecular chaperone, 6 (17%) participates in general transcription or translation process, 5 (14%) participate in cell growth control, 4 (11%) are adhesion or invasion associated molecules, 4 (11%) involve in cell immunity, and 2 (6%) are other or unknown functions (Figure 3). While most up-regulated genes are associated with metabolism, the down-regulated genes are involved in several cellular functions.

Two Raw microarray data was imported into MetaCore™ for integrate network prediction analysis. Genes differentially expressed over 1.5-fold were extrapolated using an intersection algorithm. A total of 8 regulatory pathways were found to be significantly associated with chronically ANE exposure (p<0.0001) (Table 4). Algorithm analysis showed cholesterol biosynthesis pathways critically involved in ANE chronic exposure (p = 1.95E-10), with 15 differentially expressed
genes out of 21 dataset objects from microarray experiments. Other pathways including the regulations of oxidative phosphorylation \((p = 1.02E-6)\), cell adhesion process \((p = 9.66E-6)\), cell cycle control \((p = 2.41E-5)\), and immune response \((p = 5.97E-4)\) were also involved. Altogether, these data indicate the underlined mechanisms responsible to chronic ANE treatment, which may lead to oral cancer development eventually.

**Six genes were validated associated with chronic ANE exposure**

Six candidate genes were selected for further validated by RT-PCR methods, and the results were shown in the Figure 4. As shown, the gene alterations were consistently found in both cell lines, indicating the common phenomenon response to chronic ANE exposure. HMGCS1 and KRT-17 were up-regulated in both ANE sublines, with averages of 3.25 and 1.87-fold increase. SMC4, CENPF, ID-1, and Ches1 were all down-regulated in both ANE sublines, with average decrease to 0.15 ~ 0.35-folds compared to the parental cells. All these results further confirmed the significance of these genes participating in the ANE induced molecular alterations.

**Ches1 is under-expressed in cancer tissues and associated with areca nut chewing habit**

To validate the effect of Ches1 induced by ANE, the OECM1 cells were treated with arecoline, a major alkaloid of areca nut, and Ches1 expression was determined. As shown in the Figure 5, Ches1 expression was significantly inhibited with a time dependent manner. This result indicated that areca nut component renders a suppression effect on CHES1 expression, especially after a longer time of exposure.

To examine the clinical significance of Ches1 in oral cancer, normal and cancerous tissues from 52 patients with oral cancer patients were obtained for study.
For each tissue sample, total RNA was extracted and subjected to quantitative RT-PCR analysis for Ches1 expression. Using a cut-off value of a 2-fold greater expression in tumor versus normal tissues, 24 patients (46%) were found to have under-expression in the tumor tissues. Pearson’s chi-square method was used for statistical analysis to determine the potential association between Ches1 mRNA expression and disease status, as summarized in the Table 5. There was no significant correlation with T stage, N stage, overall stage, differentiation status, or pathological tumor depth. However, significant correlations were found between Ches1 under-expression and the habit of areca nut chewing ($P=0.02$). This result suggests that Ches1 plays a role in the areca nut induced oral cancer development.

In conclusion, we reported differential transcriptome and predicted several pathways associated with chronically ANE exposure in oral cancer cells. Six genes were confirmed differential expression, including Ches1. These molecules may play important roles in areca nut induced oral carcinogenesis. It is conceivable that cell modulation of these genes might be effective in the correction of areca nut induced pathogenic imbalances. This study may eventually contribute to clinical investigation as risk assessment, disease prevention or other clinical applications of areca nut associated diseases as well.
References


Figure 1. Quality controls of areca nut extract (ANE) preparation as determined by high performance liquid chromatography (HPLC) method. Two major alkaloids of areca nut: arecoline (red line) and its esterase form arecadine (green line) were used as the controls for ANE quality. Two batches of ANE preparations (black and blue lines) were determined. Assay protocol of the HPLC was described in the Material and Method section.
Figure 2. Verification of ANE chronically trained sublines. Two oral cancer cell lines OECM1 and SAS were used in this study. Each cell line was treated with IC70 dose of ANE for total of 30 passages to establish ANE subline. These ANE-sublines were subjected to various concentrations of ANE (0 ~ 1200 ug/ml) for 24 hours, and their viabilities were determined.
Figure 3. Functional category and percentage of gene distribution in oral cancer cells chronically exposure to ANE. Differential transcriptomes between oral cancer cells and the ANE-sublines were determined by cDNA microarray methods. Total of 35 genes were found differentially expressed in both cell lines. Functional category of these 35 genes was displayed.
Figure 4. Differential expression of 6 genes between parental (PT) and ANE subline of OECM1 and SAS cells as revealed by RT-PCR analysis. Actin gene expression was used as an internal control for each gene. The relative density in each sample was indicated at the bottom of the band after normalization to the actin level in each individual sample. Differential expression of greater than 20% in both cell lines indicated significant difference.

<table>
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<th>Gene</th>
<th>OECM1 PT</th>
<th>OECM1 ANE</th>
<th>SAS PT</th>
<th>SAS ANE</th>
<th>(Average fold change)</th>
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<td>HMGCS1</td>
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<td>1.00</td>
<td>0.28</td>
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<td>Actin</td>
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Figure 5. Ches1 expression was inhibited by arecoline, a major alkaloid of areca nut. OECM1 cells were treated with 120 μM of arecoline for 3 days. Ches1 expression was determined by RT-PCR analysis, while using actin expression as an internal control. The relative density in each sample was indicated at the bottom of the band after normalization to the actin level in each individual sample.

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