Characterization of Arecoline-Induced Effects on Cytotoxicity in Normal Human Gingival Fibroblasts by Global Gene Expression Profiling

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Areca nut is the most widely used psychoactive substance and an important environmental risk factor for development of oral premalignant lesions and cancer. Arecoline, the major alkaloid of areca nut, has been known to cause cytotoxicity and genotoxicity in mammalian cells in vivo and in vitro and even contributes to carcinogenicity. However, the susceptible genes accounting for arecoline-induced damage in normal human oral cells are still lacking, which possibly involves in initial molecular damage via alternation of gene expression level on biological pathways. The present study was undertaken to characterize the toxic effects of arecoline in gene expression profiling on normal human gingival fibroblasts (HGF) using cDNA microarray and quantitative real-time reverse transcription PCR. The cytotoxicity of arecoline on HGF-1 cell line was elevated in a dose-dependent manner (p < 0.05) accompanied with distinct morphological change and formation of intracellular vacuoles were observed. At optimum concentration of arecoline determined from dose-response curve of the cytotoxicity, a large number of genes were significantly repressed than induced by arecoline in global gene expression profiling. Five induced- and seven repressed genes including glutathione synthetase were further validated, and their gene expression changes were increased in a dose-dependent manner in a concentration range of 50–150 μg/ml. In conclusion, we proposed a tentative model to explain arecoline-induced effects on contribution of oral pathogenesis. The findings identified that 12 susceptible genes can potentially serve as biomarkers of arecoline-induced damage in betel chewers.

Key Words: arecoline; cytotoxicity; gene expression; human gingival fibroblasts; betel chewing.

Betel chewing is prevalent in many areas of Asia, Pacific region, and migrant communities in United Kingdom, United States and South Africa, with an estimated 600 million chewers worldwide (Gupta and Warnakulasuriya, 2002; IARC, 1985).

There are approximately 2 million habitual chewers in Taiwan, and various epidemiology studies have consistently indicated betel quid chewing to be an independent risk factor in the development of oral premalignant lesions (OPLs) and oral cancers (Ko et al., 1995; Lee et al., 2003; Tilakaratne et al., 2006; Yang et al., 2001). Among OPLs, oral submucous fibrosis (OSF) develops in lamina propria and connective tissues of oral cavity, but its definite pathogenesis and multifactorial mechanisms are still not fully understood. However, betel quid chewing has strong contribution to formation of OSF, and particularly in Taiwan (population-attributed proportion = 85.4%) (Lee et al., 2003). According to the International Agency for Research on Cancer, Areca nut (AN) is the main component in various form of betel chewing and is constituted by alkaloids, polyphenols, carbohydrates, fats, proteins, crude fiber, and mineral matter. Also, in 2004 it has been evaluated to be a group 1 carcinogen to human based on the potential carcinogenicity of betel quid, yet unfortunately the annual global production of AN is continuously increasing (IARC, 2004).

Arecoline is the main alkaloid of AN with a soluble feature in water and alcohol. During betel chewing, the concentration of arecoline was reported to reach around 140 μg/ml (Nair et al., 1985). It is absorbed in the buccal cavity and is detected in fasting blood in dose- and time-dependent manner (Strickland et al., 2003). The documented in vitro cytotoxicity of arecoline included firstly inhibition of cellular growth, attachment, collagen synthesis, and the activity of the mitochondrial cytochromeoxidase; secondly depletion of cellular glutathione (GSH); and thirdly arrest of cell cycle at G2/M phase (Chang et al., 2001b; Jeng et al., 1996). Furthermore, in vivo and in vitro studies both indicated that arecoline has potential tumorigenicity in mouse tissues and cells, and the genotoxicity included induction of chromosome aberrations, sister chromatid exchange, micronucleus formation, and DNA strand breaks (Dave et al., 1992; Deb and Chatterjee, 1998; Shirname et al., 1984). Additionally, metabolites derived form N-nitrosation of
arecoline in saliva during betel chewing have potentially tumorigenic properties (Prokopczyk et al., 1987). To date, it is reasonable to suspect that arecoline should play an important role in formation of OSC; for instance, the increased levels of inflammatory cytokines (interleukin [IL]-1α, IL-1β, and IL-6) and growth factors (transforming growth factor-beta [TGF-β], platelet-derived growth factor, basic fibroblast growth factor, and keratinocyte growth factor-1) in OSC patients are considered to be involved in initial pathogenesis, and some of these could be attributed to arecoline (Haque et al., 1998; Jeng et al., 2003; Tsai et al., 2005). The characterization of these cytotoxicity and genotoxicity of arecoline indeed has highlighted the potential risk of arecoline exposure in betel chewers, nevertheless, molecular-based interpretation of how arecoline toxicity may inflict normal oral fibroblast cells to lesion is still lacking.

In order to understand the global impact of arecoline on gene transcription in oral fibroblast cells, we performed whole-genome screening by high-density microarray analysis and quantitative real-time reverse transcription PCR (qRT-PCR) technique in normal human gingival fibroblasts (HGF) in the present study. Arecoline-induced or -repressed genes can be further categorized as those related to xenobiotic metabolism, maintenance of genomic stability, DNA damage or repair, stress response, and TGF-β signaling pathway. Moreover, the dose-dependent response in expression level of selected significantly candidate genes has been validated. A tentative model of how arecoline might induce cytotoxicity and lead to initial lesions was provided.

MATERIALS AND METHODS

Chemical. Arecoline hydrobromide, an alkylating agent, was purchased from Sigma Chemical Co. (St Louis, MO).

Cell culture. In the stage I experiments, for microarray gene expression experiments, we obtained normal HGF from American Type Culture Company (HGF-1, CRL-2144). In the stage II, for dose-response analysis of arecoline, we used primarily cultured HGF obtained from a healthy subject without the habit of alcohol drinking, betel chewing, and cigarette smoking, who received a surgical crown lengthening in the Department of Dentistry of Chung-Ho Memorial Hospital, Kaohsiung Medical University, with informed consent (KMUH-IRB-950135). The cultured cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (GIBCO/BRL) and 100 IU/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. The cells were cultured in a 37°C incubator filled with 5% CO₂ and were passaged routinely at 90% confluence.

Cytotoxicity assay. For dose-response experiments, 10^4 of HGF-1 cells were refreshed and cultured for 24 h in a 96-well plate. To assess the cell viability, cells were treated with various concentration (0, 25, 50, 100, and 200 μg/ml) of arecoline for 24 h, and the cell-free supernatants were harvested for quantification of lactate dehydrogenase (LDH) activity released from damaged cells using LDH Cytotoxicity Detection Kit (Roche). The absorbance at 490 and 630 nm were measured, and the average cell cytotoxicity (%) from triplicates for each concentration was calculated according to manufacturer’s instructions.

Arecoline exposure and total RNA extraction. The arecoline-conditioned medium (100 μg/ml) was freshly prepared from arecoline hydrobromide (Sigma) in growth medium. In the stage I experiments, cultured cells from the fourth passage (about 2.4 × 10^6 cells) were cultured in arecoline-conditioned medium and kept in incubator for 24 h. The control was performed under the same conditions except for the absence of arecoline in the medium. Alterations of cell morphology were photographed with a ×400 magnification level by inverted microscope (Leica Microsystems). Total RNA was isolated using RNeasy Midi Kits (Qiagen). The integrity and quality of RNA samples were determined by using the 2100 Bioanalyzer with the RNA6000 Nano LabChip Kit (Agilent), and only RNA samples without degradation were used for further study. In the stage II experiment, the dose effects of arecoline on specific genes screened from the stage I experiments were examined in the primary culture of HGF treated with arecoline of indicated dose (0, 50, 100, 150, and 200 μg/ml) for 24 h using qRT-PCR (see below). The RNA samples were isolated as described above and stored at −86°C until used.

cDNA microarray. Total RNA of 1 μg was amplified with Microarray cDNA Synthesis Kit and Microarray RNA Target Synthesis Kit (Roche). Amplified cRNA samples were fluorescently labeled with cyanine-3 (Cy3) and cyanine-5 (Cy5) by using CyScribe Post-Labeling Kit (Amersham Biosciences). The labeling efficiency was evaluated by an UV/visible spectrophotometer. In the following ‘two-color’ experiment, 25 pmol Cy3- and Cy5-labeled cDNA targets were mixed and hybridized on a cDNA microarray which comprises of 46,897 human cDNA’s representing approximately 25,000 unique putative human genes. The dye-swap labeling was performed in all of the experiments to correct for nonspecific bias upon dye labeling. The array chips were hybridized overnight, washed, and dried in an automated hybridization station (GeneTAC), and the fluorescence intensities was measured using a GenePix 4000B scanner (Axon Instruments) at 10 μm resolution. The intensity of fluorescent signal was collected in a 16 bit/pixel format and analyzed by using GenePix 4.0 software. Furthermore, to reduce random errors of microarray, five repeated pairs (including dye-swap labeling) of the experiment were performed.

Statistical data analysis. The relative cytotoxicity (%) among the treatments compared to control (HGF-1 without arecoline treatment) was analyzed by one-way ANOVA and Bonferroni multiple comparison test. The statistically significant difference (p < 0.05) was indicated by an asterisk. For microarray data, signal values in each microarray experiment were filtered to remove data from low-quality spots and then normalized by Bayesian isotonic regression while using priors on Bernstein polynomials and Markov chain Monte Carlo methods (Jiang et al., 2006). The fold change of expression of a specific gene upon treatment, represented by the ratio of signal intensities of treatment to control for a corresponding probe, was obtained accordingly. To evaluate the reproducibility of microarray data among repeated experiments and ensure chip-to-chip consistency, the pairwise correlation coefficients (r²) of ratios from corresponding probes were calculated among all repeats (including dye-swap experiments). We further set an arbitrary cutoff value for average signal intensities, denoted by 1/2(log-treatment + log-control) of 4 to remove low-signal intensity genes. A twofold change criterion was used at first to obtain genes that were either twofold upregulated or downregulated upon arecoline treatment. Moreover, Significance Analysis of Microarrays (SAM) was also performed to further confirm the significantly differentially expressed genes (DEGs), and the number of false positive genes was estimated using the false discovery rate (Tusher et al., 2001). Only those significant genes with nonmissing log; ratio available in at least three out of five dye-swap replicates were included for further study. The biological function of each gene was classified according to Gene Ontology Annotation on NCBI-GENE database.

Quantitative real-time reverse transcription PCR. The candidate genes selected from microarray analysis were validated by qRT-PCR using the Universal ProbeLibrary Set and LightCycler 480 system (Roche). Four micrograms of total RNA of each sample was used to synthesize first-strand cDNA with oligo(dT) primers using a SuperScript III First-Strand Synthesis Kit (Invitrogen). All cDNA samples were diluted 15-fold as working template in
qRT-PCR. Unique probe and gene-specific primer pair combination for three endogenous reference genes and all target genes were designed using Roche ProbeFinder Software Version 2.32 (see Supplementary Table 1). Briefly, 1× Probes Master, 200nM of each primer, 100nM Universal ProbeLibrary probe, and 2 µl diluted cDNA template were added to each reaction in a total volume of 20 µl. The protocol consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of amplification and quantification at 95°C for 15 s, 60°C for 10 s and 72°C for 10 s, and was finally cooled at 40°C. The level of gene expression was given as relative copy numbers normalized against reference mRNA. Relative quantification applying the formula $2^{-\Delta \Delta Ct}$ was used to calculate the fold change in gene expression, and the results represented mean ± SD of triplicates. For the dose-response effects of significantly validated genes, 150 ng of target cDNA obtained from stage II experiment was used in 20 µl final volume of qRT-PCR using LightCycler Taqman Master and LightCycler 1.2 system (Roche) according to manufacturer’s guidelines. Fold change in gene expression was normalized using ACTB reference control gene and calculated by LightCycler Software version 4.05. The mean ± SD value of duplicate analysis of each condition was obtained and showed in the histograms.

RESULTS

Cell Viability and Morphological Changes

To evaluate the cytotoxic effects of arecoline on cultured HGF-1 cells, we firstly used LDH release assay to measure cell cytotoxicity following exposure to five different concentration of arecoline for 24 h. As shown in Figure 1, the arecoline-induced cytotoxicity increased remarkably in a dose-dependent manner ($p < 0.05$) and reached to about 40% at the concentration of 100 µg/ml, although there was only marginal increase in cytotoxicity when concentration was elevated to 200 µg/ml. At the concentration of 100 µg/ml, a pathological atrophy of cell morphology was also noted. Interestingly, we observed several intracellular vacuoles in moderate to severely damaged cells (Fig. 2B), a phenomenon similar to earlier reports where cultured oral mucosal fibroblasts were treated with areca nut of 800 µg/ml following 24 h (Chang et al., 2001a). Since the content of arecoline in unripe areca nut was in the range of 11–14% (Jayalakshmi and Mathew, 1982), we believed the effective concentration of arecoline in these studies was also around 100 µg/ml. These suggest arecoline of this dosage exerts extensive toxicity and induces common morphological changes in various cell types of interest.

Arecoline-Induced DEGs

We used whole-genome cDNA microarray assay in order to screen the arecoline-induced or -repressed genes in HGF-1
cells. To evaluate the intrinsic technical errors and the reliability of microarray data, we firstly examined the consistency of repeated experiments by performing a pairwise comparison of the results among repeated arrays. The average Pearson correlation coefficients ($r^2$) of all possible paired combinations of signal intensities of arecoline-treated samples labeled with Cy5 or Cy3 (in dye-swap labeling) were 0.974 and 0.969, respectively; the values for control samples were 0.978 and 0.978, respectively, indicating highly reproducible results were obtained across extensively repeated microarray experiments (also see Supplementary Figure).

The global gene expression changes reflected by 8490 probes reporting consistent gene expression pattern in five repeated and dye-swap experiments were shown in an MA plot (Fig. 3A). It shows that a great number of genes ($n = 1027$ genes) were downregulated more than twofold while a lesser population of genes ($n = 201$ genes) were upregulated to the same extent. Further analysis by SAM indicated the numbers of genes that were significantly changed for at least twofold were 162 and 708 for up- and downregulated genes, respectively. Most of arecoline-induced and -repressed genes with known biological function were related to transcription/mRNA processing, cellular metabolism, cell communication/transport, cellular metabolism, biological protein synthesis, and ubiquitin system (see Supplementary Table 2). After removing genes of low-intensity spots, replicate probes, transcribed sequences, hypothetical proteins, and unknown molecular function, we further focused on the interesting gene sets of xenobiotic biotransformation, DNA damage or repair, stimulus and immune response, and TGF-beta signaling. The average fold changes of selected genes from repeated and dye-swap microarray analysis are listed in Table 1.

Related to xenobiotic metabolism, six genes involved in phase I biotransformation (AKR1A1, CYP26B1, S100A12, ALDH9A1, MAOA, and UGCG1) and three genes involved in phase I biotransformation (GSS, LCMT1, and NAT8) were all repressed by arecoline. The only gene related to DNA damage signaling was DNA damage-inducible transcript 4 (DDIT4; also known as REDD1 or RTP801), which was induced moderately. Seventeen human DNA repair–related genes were significantly changed in gene expression level in microarray analysis, including BRCA1 and RAD50, which were strongly repressed and moderately induced by arecoline, respectively. These human DNA repair genes can be further classified into the following subgroups according to their biological activity: homologous recombination (RAD50 and BRCA1), nucleotide excision repair (GTF2H2, ERCC2, GTF2H5, and CCNH), base excision repair (APEX1), ATM/NBS1-dependent S-phase checkpoint (SMC1A), mismatch excision repair (MSH2 and MLH1), DNA damage repair function (DCLRE1B), genes whose deficiency are responsible for increased sensitivity to DNA damaging agents (FANCG), chromatin structure (CAF1A and CAF1B), DNA damage response (RAD1), ATM/ATR-mediated p53 activation (EEF1E1), and nonhomologous end joining (PRKDC).

The genes related to stress response (HSPA14 and DNAJA1) and inflammatory response (COX-2/PTGS2) were moderately induced by arecoline. The growth differentiation factor 15 (GDF15), also known as transforming growth factor-beta (PTGFB) and macrophage inhibitory cytokine-1 (MIC-1), was strongly induced on HGF-1 cell line by arecoline treatment. Totally, six significantly induced genes and 25 repressed genes were selected for further analysis.

**qRT-PCR Validation and Dose-Dependent Analysis**

To further verify the reliability of results from microarray analysis, thirty-two unique significant DEGs were validated by qRT-PCR. According to the triplicate qRT-PCR measurements, 11 genes consistently showed at least twofold change as compared to microarray data (Table 1). Focusing on these selective genes significantly affected by arecoline in HGF-1 cell
line, we further examined the dose-response effects of arecoline on the same set of genes in primarily cultured HGF, also by the gold-standard qRT-PCR. As shown in Figure 4, all tested genes were affected in a dose-dependent manner, although PTGS2, DNAJA1, GDF15, S100A12, and CYP26B1 showed saturated response at the concentration of 150 μg/ml. Additionally, GSS was repressed significantly in dose of 150 and 200 μg/ml in primary cultured HGF, and the repression achieved 2.94-fold when cells were treated with the highest dose of arecoline. Interestingly, at the concentration of 100 μg/ml, the level of induction of DDIT4 and repression of CYP26B1 in primarily cultured HGF (about 24- and 15-fold, respectively) were far greater than that observed on HGF-1 cells in stage I experiments. Despite the level of changes between both cell lines of different ethnic groups is variant, generally, the patterns of gene expression change in primarily cultured HGF cells were quite consistent with those in HGF-1 cell line.

## DISCUSSION

The physiological function of fibroblasts includes the maintenance of the extracellular matrix and wound healing in connective tissues and regulates differentiation of adjacent epithelial (Fries et al., 1994; Tomasek et al., 2002). The gingival fibroblasts, the cells used in this study contain

<table>
<thead>
<tr>
<th>Functional group</th>
<th>UniGene</th>
<th>Symbol</th>
<th>Gene description</th>
<th>Average dye-swap fold change</th>
<th>qRT-PCR fold change</th>
</tr>
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<tr>
<td>Phase I biotransformation</td>
<td>AW078972</td>
<td>AKR1A1</td>
<td>Aldo-keto reductase family 1, member A1</td>
<td>3.07 ± 0.81</td>
<td>1.19 ± 0.16</td>
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<td></td>
<td>AO26040</td>
<td>CYP26B1</td>
<td>Cytochrome P450, family 26, subfamily B, polypeptide 1</td>
<td>2.74 ± 0.64</td>
<td>3.44 ± 0.14</td>
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<td></td>
<td>AA700005</td>
<td>S100A12</td>
<td>S100 calcium–binding protein A12</td>
<td>2.74 ± 0.61</td>
<td>2.37 ± 0.15</td>
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<td></td>
<td>R46816</td>
<td>ALDH9A1</td>
<td>Aldehyde dehydrogenase 9 family, member A1</td>
<td>2.48 ± 0.95</td>
<td>1.43 ± 0.09</td>
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<td></td>
<td>AA011096</td>
<td>MAOA</td>
<td>Monoamine oxidase A</td>
<td>2.35 ± 0.81</td>
<td>1.02 ± 0.09</td>
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<td></td>
<td>R98442</td>
<td>UGCG1</td>
<td>UDP-glucose ceramide glucosyltransferase–like 1</td>
<td>2.25 ± 0.77</td>
<td>1.30 ± 0.07</td>
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<td>Phase II Biotransformation</td>
<td>AA463458</td>
<td>GSS</td>
<td>Glutathione synthetase</td>
<td>2.40 ± 0.79</td>
<td>1.48 ± 0.06</td>
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<td></td>
<td>AA461443</td>
<td>LCMT1</td>
<td>Leucine carboxyl methyltransferase 1</td>
<td>2.22 ± 0.60</td>
<td>1.32 ± 0.02</td>
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<td></td>
<td>N58170</td>
<td>NAT8</td>
<td>N-acetyltransferase 8</td>
<td>2.07 ± 0.27</td>
<td>1.19 ± 0.45</td>
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<tr>
<td>DNA damage/repair</td>
<td>AA447746</td>
<td>DDIT4</td>
<td>DNA damage–inducible transcript 4</td>
<td>4.53 ± 1.19</td>
<td>9.69 ± 0.16</td>
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<tr>
<td></td>
<td>R92967</td>
<td>RAD50</td>
<td>Rad50 homolog (Saccharomyces cerevisiae)</td>
<td>7.40 ± 2.88</td>
<td>1.48 ± 0.14</td>
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<td></td>
<td>A680547</td>
<td>BRCA1</td>
<td>Breast cancer 1, early onset</td>
<td>16.80 ± 3.88</td>
<td>26.03 ± 0.01</td>
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<td></td>
<td>AA029300</td>
<td>RAD1</td>
<td>RAD1 homolog (Schizosaccharomyces pombe)</td>
<td>4.12 ± 1.44</td>
<td>1.16 ± 0.10</td>
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<td></td>
<td>AW070763</td>
<td>SMC1A</td>
<td>Structural maintenance of chromosomes 1A</td>
<td>3.78 ± 1.28</td>
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<td></td>
<td>AA281797</td>
<td>GTF2H2</td>
<td>General transcription factor IIIH, polypeptide 2, 44 kDa</td>
<td>3.40 ± 1.48</td>
<td>1.09 ± 0.16</td>
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<tr>
<td></td>
<td>AA478273</td>
<td>APEX1</td>
<td>APEX nuclease (multifunctional DNA repair enzyme) 1</td>
<td>2.90 ± 0.45</td>
<td>1.59 ± 0.05</td>
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<td></td>
<td>R54492</td>
<td>ERCC2</td>
<td>Excision repair cross-complementing rodent repair deficiency, complementation group 2</td>
<td>2.72 ± 0.59</td>
<td>1.59 ± 0.15</td>
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<td></td>
<td>AA219061</td>
<td>MSH2</td>
<td>MutS homolog 2, colon cancer, nonpolyposis type 1</td>
<td>2.71 ± 0.60</td>
<td>1.58 ± 0.12</td>
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<td>R10662</td>
<td>MLH1</td>
<td>MutL homolog 1, colon cancer, nonpolyposis type 2</td>
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<td>1.55 ± 0.07</td>
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<td>AA932402</td>
<td>GTF2H5</td>
<td>General transcription factor IIIH, polypeptide 5</td>
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<td>1.17 ± 0.07</td>
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<td></td>
<td>AA666673</td>
<td>DCLRE1B</td>
<td>DNA cross-link repair 1B (PSO2 homolog, S. cerevisiae)</td>
<td>2.35 ± 0.95</td>
<td>1.32 ± 0.38</td>
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<tr>
<td>Response to stimuli and immune response</td>
<td>AA430675</td>
<td>FANCG</td>
<td>Fanconi anemia, complementation group G</td>
<td>2.31 ± 0.50</td>
<td>2.64 ± 0.07</td>
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<td></td>
<td>AA704459</td>
<td>CHAF1A</td>
<td>Chromatin assembly factor 1, subunit A</td>
<td>2.26 ± 0.52</td>
<td>3.25 ± 0.16</td>
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<tr>
<td></td>
<td>AA454146</td>
<td>CCNH</td>
<td>Cyclin H</td>
<td>2.23 ± 0.39</td>
<td>1.49 ± 0.08</td>
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<tr>
<td></td>
<td>AA426096</td>
<td>CHAF1B</td>
<td>Chromatin assembly factor 1, subunit B</td>
<td>2.19 ± 0.62</td>
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<td></td>
<td>A631910</td>
<td>EEF1E</td>
<td>Eukaryotic translation elongation factor 1 epsilon 1</td>
<td>2.15 ± 0.51</td>
<td>1.15 ± 0.07</td>
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<td></td>
<td>R27615</td>
<td>PRKDC</td>
<td>Protein kinase, DNA-activated, catalytic polypeptide</td>
<td>2.13 ± 0.48</td>
<td>1.11 ± 0.17</td>
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<tr>
<td>TGF-beta pathway</td>
<td>R80217</td>
<td>PTGS2</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
<td>6.38 ± 3.02</td>
<td>4.33 ± 0.18</td>
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<td></td>
<td>AA55563</td>
<td>HSPA14</td>
<td>Heat shock 70 kDa protein 14</td>
<td>3.32 ± 0.64</td>
<td>4.65 ± 0.18</td>
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<td></td>
<td>AA490946</td>
<td>DNAJAI</td>
<td>DNAJ (Hsp40) homolog, subfamily A, member 1</td>
<td>3.25 ± 1.27</td>
<td>2.35 ± 0.13</td>
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<tr>
<td></td>
<td>N26311</td>
<td>GDF15</td>
<td>Growth differentiation factor 15</td>
<td>15.41 ± 4.76</td>
<td>15.45 ± 0.50</td>
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</table>

**Note.** †: induced; ‡: repressed. Average fold changes were estimated from 10 chips in the dye-swap microarray experiments. Average fold changes were estimated from triplicates by qRT-PCR. Compared to control, gene expression change is more than twofold in qRT-PCR validation.
myofibroblasts, play important role in wound closure. Damage of these cell types by intergradient of areca nut might lead to oral pathogenesis. For instance, OSF is a connective tissue injury known to be strongly associated with the habit of betel chewing as evidenced by (1) there was a dose-dependent relationship between increase of incidence of OSF and areca nut consumption (Hazare et al., 1998; Lee et al., 2003; Yang et al., 2001); (2) the increased periodontitis in betel chewers might implicate that some components of areca nut can be absorbed in periodontium (Amarasena et al., 2003) and result in harm of gingival connective tissue, and (3) the initial risk factor of OSF in betel chewers has been seriously considered to be correlated with arecoline exposure (Chang et al., 2002; Shieh et al., 2003; Tsai et al., 2003; Tsai et al., 2005). In an attempt to elucidate the possible molecular damage underlying arecoline-induced lesions on normal HGF, we have profiled the changes in global gene transcription in the established cell line using cDNA microarray technology and validate the results in primary cultured cells using qRT-PCR. A schematic representation of possibly etiological pathways is delineated in Figure 5.

FIG. 4. Dose effects of arecoline on selected genes in primarily cultured HGF by qRT-PCR. ACTB was used as internal control gene. The relative expressions were detected in cells treated with four different concentrations of arecoline treatment (50, 100, 150, and 200 μg/ml for 24 h) on (A) five induced genes and (B) seven repressed genes, comparing with control. The dash line indicated a twofold change.

FIG. 5. Model of possible pathways involved in arecoline-induced damage on HGF. Arecoline significantly induces five genes while represses seven genes involved in toxicologically biological pathways, which might contribute to oral pathogenesis on normal HGF. The risk on oral carcinogenesis might be increased upon long-term exposure in betel quid chewers. BR (bradykinin receptor), B (bradykinin).
The metabolism and excretion of arecoline in cellular process requires nonspecific biotransformation enzymes (Nery, 1971). It is unfortunately that there is no particular enzyme to be implicated on xenobiotic metabolism of arecoline in human oral cells, although the carboxylesterase has been reported to be responsible for arecoline metabolism in mouse liver tissue (Patterson and Kosh, 1993). In detoxifying areca nut and related ingredients, two monoxygenase systems (cytochrome P450 and flavin containing monoxygenases) are considered to contribute to phase I metabolism. In our present study, two repressed genes (CYP26B1 and S100A12) were supposed to be involved in phase I metabolic process according to their biological function. The monoxygenase activity of CYP26B1 may involve in detoxification process although it showed to be repressed susceptibly by arecoline treatment in the present study. Moreover, this gene has been reported to take part in retinoic acid metabolism and is believed to play an important role in the protection of specific tissues from exposure to retinoids (White et al., 2000). The S100A12 gene encoding calcitermin, which contains similar zinc-binding domains in C-terminal sequence with S100 family, is proposed to be involved in cellular detoxification in nasal fluid (Cole et al., 2001). Nevertheless, no evidence of both genes directly link to metabolism of arecoline or detoxification of betel chewing. The utility of both genes on betel-related research cannot be ascertained without further study.

In phase II biotransformation, GSH is an ubiquitous thiol for its properties in xenobiotic detoxification. Moreover, the GSH depletion is associated to arecoline-induced chromosomal aberrations (Deb and Chatterjee, 1998). Previous studies have demonstrated that intracellular depletion of GSH is not coupled with oxidation of GSH to oxidized glutathione or oxidative stress during exposure of uninjured buccal fibroblasts to arecoline, and cytotoxicity could be prevented by addition of extracellular GSH or its precursor, L-cysteine (Jeng et al., 1994). This is similar to the finding in human buccal epithelial cells by AN extract treatment (Sundqvist et al., 1989). Therefore, it is considerable that the causation of GSH depletion in oral cells may also arise from direct conjugation (phase II biotransformation) between GSH and arecoline as well as that the A³- ethylenic bounds of arecoline react with thiol groups in the rat model (Boyland and Nery, 1969). The CySH is a precursor of GSH; however, its protective efficiency is obviously lower than extracellular GSH (Jeng et al., 1994). Therefore, we suggest that the level of two important enzymes (γ-glutamycysteine synthetase and glutathione synthetase [GSS]) in γ-glutamyl cycle may affect GSH synthesis, especially the repression of GSS by arecoline in the present study. Our finding provides a new possibility suggesting GSH depletion not only results from conjugation with arecoline but also the repression of GSS in GSH synthesis during arecoline exposure.

Inhibition of the elimination of arecoline may arise from the dysfunction of xenobiotic biotransformation activities, which might further cause substantial intracellular damage of genomic structure as previously described. The continuance of DNA adducts in cells is predominantly the result of the failure of DNA repair as well as the possible formation of arecoline-DNA adducts (Nery, 1971). The reported effects of DNA strand breaks in cells of mouse tissue were not observed in normal human mucosa fibroblasts in vitro, even up to the concentration of 600 µg/ml (Jeng et al., 1994). In our current study, however, DDIT4 showed strong upregulation in HGF after arecoline exposure, even at doses as low as 50 µg/ml. The DDIT4 signaling may be responsible for calling for DNA repair systems. It was reported as a prime transcriptional target for p53 induction following DNA damage, and a strong induction of its expression can induce cellular apoptosis (Ellisen et al., 2002). Following the suspected DNA damage, we noted that four genes related to maintenance of genomic stability and DNA repair were significantly repressed by arecoline. FANCG, also known as XRCC9 that encodes a candidate tumor suppressor capable of correcting chromosome aberrations (Liu et al., 1997), was transcriptionally downregulated here. CHAFIA and CHAFIB, that encode for chromatin assembly factor I (CAF1), have been documented to couple chromatin assembly to DNA repair and replication (Gaillard et al., 1996), and the repression of both CAF1 polypeptide–encoding genes might reflect instability of chromatin reorganization and inhibition of DNA replication process. Intriguingly, BRCA1, a gene implicated in DNA damage response and DNA repair, was repressed by arecoline in a dose-dependent manner. Since there is no report demonstrating the relation between aberration of BRCA1 expression and oral cancers, it would be worthwhile to investigate whether it indeed play a role in formation of OPL and cancer as well as those DNA repair genes in formation of chromosomal aberration and DNA structural damage.

COX-2/PTG2 is well-known in cancer initiation and progression through several pathways including apoptosis, angiogenesis, inflammation, and immune surveillance. The COX-2 mRNA and protein expression both showed dose- and time-dependent increase in human gingival keratinocytes following AN extract treatment (Jeng et al., 2000). The arecoline-induced COX-2 expression also suggests to contribute to pathogenesis of OSF in betel chewers (Tsai et al., 2003). Consistently, the elevated expression level of COX-2 was also observed in arecoline-treated HGF-1 cells and primarily cultured HGF cells in our study, suggesting these cells of different origins derived from oral cavity might have similar inflammatory responses upon exposure to arecoline, which in turn promote oral lesions and tumorgenesis. Also, it is important to take notice of bradykinin receptors spreading on plasma membrane of HGF. When HGF pretreated with inflammatory cytokine (IL-1β) couple with endothelium-dependent bradykinin, the level of COX-2 mRNA expression in HGF can quickly induce via Ca²⁺ mobilization (Nakao et al., 2001). Although the mRNA level of IL-1β is no significantly changed in HGF-1 by arecoline treatment in the present study (data not shown), we
speculate that the higher COX-2 mRNA expression may occur in betel chewers who use slaked lime in the form of chewing. Cellular stress response is thought to be a part of intracellular repair system. In our data, HSPA14 and DNAJA1 were induced coordinately in gene expression level, and both were upregulated by arecoline in dose-dependent manner. The HSPA14 and DNAJA1 belong to HSP70 (DnaK) family and HSP40 (DNAJ) family, respectively. HSP70 is a well-known stress-induced protein and can be strongly upregulated in toxic exposure. In addition, HSP70 is usually not expressed in the most healthy tissues, while it is highly expressed in tumor tissues. Consistently, elevated protein level of HSP70 is implicated in betel- and tobacco-related oral tumorigenesis (Kaur and Ralhan, 1995). Furthermore, HSP40 protein is known to play a role in regulating the molecular chaperone activity of the HSP70 proteins through stimulating their ATPase activity. The overexpression of HSP70 and HSP40 detected by immunoblotting has been reported in other tumor tissue (Isomoto et al., 2003). Lastly, we described GDF15/MIC-1 to be a noticeably induced gene following arecoline exposure. It is a member of the TGF-β superfamily with a specific function to regulate tissue differentiation and maintenance. The expression of MIC-1 is regulated by p53 due to the presence of p53-binding site in its promoter region (Yang et al., 2003), although p53 expression did not show a significant relationship with malignant transformation in oral precancerous lesions (Murthi et al., 1998). However, the elevated gene expression and serum protein levels of MIC-1 have been reported in various carcinomas (Welsh et al., 2003). One might consider the GDF15 gene to be one of the biomarkers for characterizing gene expression of betel chewers with or without oral cancer in further studies.

In summary, we proposed that the important toxic effects of arecoline could be attributed to its wide impact on various biological processes, such as repression of gene involved in xenobiotic metabolism/detoxification, chromosomal structure stability, and DNA repair system. Our findings provide a new basis for improving understanding of arecoline-induced effects in contribution to transcriptional downregulation on normal HGF. Interestingly, a recent concept related to the tumor microenvironment proposed that tumorigenesis occurs not only in target cells but also in various cell types surrounding the lesion site (Albini and Sporn, 2007; Kalluri and Zeisberg, 2006; Karring et al., 1975). This perspective implicates that both oral epithelial and adjacent fibroblasts might initially undergo alternations that promote carcinogenesis of oral epithelial cells during chronic cellular damage and inflammation. Therefore, it would be also interesting to investigate whether such a theme of exposure response in HGF will assist to activate transformation of buccal epithelial cells in the microenvironment by further study, especially on COX-2 and GDF15 mRNA overexpression. In the present study, these candidate genes would serve as novel genetic markers to delineate molecular pathogenesis of betel-related oral lesions and to explore the association between preclinical biological effect and individual gene susceptibility in further molecular epidemiologic research.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


