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Research Article

Up-regulation of Inflammatory Signalings by Areca Nut Extract and Role of *Cyclooxygenase-2* −1195G>A Polymorphism Reveal Risk of Oral Cancer

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Abstract

Because the mRNA expression of *cyclooxygenase-2* (*COX-2*) is up-regulated by arecoline in human gingival fibroblasts, as shown in our previous study, we further investigated the mRNA expression level of *COX-2* and its upstream effectors in three oral epithelial carcinoma cell lines (KB, SAS, and Ca9-22) by using areca nut extract (ANE) and saliva-reacted ANE (sANE). A case-control study of 377 oral squamous cell carcinoma (OSCC) patients and 442 controls was conducted to evaluate the gene-environment interaction between *COX-2* promoter polymorphisms and substance use of alcohol, betel quid, and cigarettes (ABC) in risk of OSCC. The heterogeneous characteristics of the oral site and the *COX-2* −1195G>A polymorphism in these cell lines showed diverse inflammatory response (KB ⩾ Ca9-22>SAS) after 24-hour ANE/sANE treatments, and the *COX-2* up-regulation might be mostly elicited from alternative nuclear factor-κB activation. In the case-control study, betel chewing [adjusted odds ratios (aOR), 42.2] posed a much higher risk of OSCC than alcohol drinking and cigarette smoking (aORs, 2.4 and 1.8, respectively), whereas the *COX-2* −1195A/A homozygote presented a potential genetic risk (OR, 1.55). The strongest joint effect for OSCC was seen in betel chewers with −1195A/A homozygote (aOR, 79.44). In the non–betel chewing group, the −1195A/G and A/A genotypes together with the combined use of alcohol and cigarettes increased risk to 15.1-fold and 32.1-fold, respectively, compared with the G/G genotype without substance use. Taken together, these findings illustrate a valuable insight into the potential role of the *COX-2* promoter region in contributing to the development of betel-related OSCC, including ANE/sANE–induced transcriptional effects and enhanced joint effects of *COX-2* −1195A allele with substance use of ABC. [Cancer Res 2008;68(20):8489–98]

Introduction

Oral cavity cancer is the eighth most common cancer in men worldwide, with an age-standardized incidence rate (ASIR) of 6.7 per 100,000 men, and particularly occurs in south central Asia (1). In Taiwan, unfortunately, the ASIR of oral cancer was almost 27 per 100,000 men in 2000, which ranks highly in the world (2). Also, the standardized death rate of oral cavity cancer increased between 1986 and 2006. According to the 2006 annual report of the Department of Health, it is the sixth leading cause of all cancer mortality and the fourth most common cancer in men in Taiwan. Oral squamous cell carcinoma (OSCC) accounts for >90% of oral cancer and can develop from oral precancerous lesions (OPL), such as leukoplakia and erythroplakia. In clinical studies, OSCC is diagnosed as an invasive epithelial neoplasm with various degrees of squamous differentiation characterized by a histopathologic grade of keratinization. We have shown the independent and synergistic effects of alcohol drinking, betel chewing, and cigarette smoking (ABC) on the risk of OPL in southern Taiwan (3). Most relevantly, it is well documented that the prevalent habit of betel chewing is a serious public health concern and an independent environmental risk in developing oral cancer in Taiwan (4–6).

Cyclooxygenase-2 (COX-2) is a prostaglandin synthase that accounts for inflammation response and xenobiotic metabolism. The elevated *COX-2* expression is well recognized as having a potential role in contributing to the progress of head and neck cancer through several biological pathways (7–9). The 5′-coding sequence of the *COX-2* gene contains an epidermal growth factor (EGF)–like domain. EGF can induce COX-2 expression through the EGF receptor signaling pathway (10, 11). Additionally, the *COX-2* promoter contains several putative transcription factor elements (12); thereby, its expression level can be modulated by numerous growth factors, cytokines, and transcription factor binding sites via several signaling pathways. The presence of two nuclear factor-κB (NF-κB) binding sites in the *COX-2* promoter is also relevant on NF-κB activation in proinflammatory and cancerous processes (13, 14). On the other hand, by negative cross-talks with NF-κB, the peroxisome proliferator-activated receptors (PPAR), nuclear retinoic acid receptors (RAR), and retinoid X receptors (RXR) can reduce irregular COX-2 induction and inhibit carcinogenesis on various epithelial tissues (15–17). Although we have reported that arecoline-induced *COX-2* from the oral microenvironment may contribute to the development of OPL or oral cancer (18), the process of cellular signaling pathways between enhanced *COX-2* expression and betel-related OSCC is not yet fully understood. In this study, we first determined the transcriptional changes of upstream inflammatory effectors in a dose-response relationship study with areca nut extract (ANE), including growth factors,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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cytokines, mediators, and regulatory elements. The cytotoxic effect of saliva-reacted ANE (sANE) was also investigated because saliva catalyzes the ingredients of areca nut (AN) into derived nitrosamines, such as 3-(methylnitrosamino)propionitrile (MNPN) and *N*-nitrosoguvacoline (NGL; refs. 19, 20). By this approach, we could further characterize the upstream regulators significantly involved in AN-induced *COX-2* expression and possibly in risk of betel-related OSCC.

Betel chewing with tobacco or smokeless tobacco chewing poses a risk of developing oral cancer, but these are not commonly seen in Taiwan (6, 21, 22). Alternatively, combined substance use of ABC is widely observed in the high-risk Taiwanese population and its synergistic effects have a large role in the etiology of upper aerodigestive cancers (4, 23–25). Moreover, the gene susceptibility from individual genetic variants is well known to contribute to different disease processes and status. This explains why some subjects exposed to a similar time or dose of ABC did not get the corresponding oral lesions and cancer. Therefore, the polymorphic variants in the *COX-2* promoter region are the foremost genetic factors that modify cancer susceptibility by modulating the expression level of *COX-2* in the inflammatory process and tumorigenesis. Consequently, we hypothesized that the different risk levels of developing OSCC might result from varied gene-environment interactions between the *COX-2* promoter polymorphisms and the substance use of ABC. This case-control study investigates the independent and joint effects of *COX-2* promoter variations with or without betel chewing in risk of OSCC.

Materials and Methods

Cell culture and ANEs. Three oral carcinoma cell lines, KB (epidermoid carcinoma), SAS (tongue carcinoma), and Ca9-22 (gingival carcinoma), were used in this study. KB cells were maintained in DMEM-11885 with 1× nonessential amino acid; SAS and Ca9-22 were maintained in DMEM/F12. Each culture medium was supplemented with 10% fetal bovine serum, 100 IU/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Invitrogen). AN was purchased from a local commercial store and homogenized with or without a 20% human saliva–contained solution. The 20% human saliva–contained ANE was then incubated at 37°C for 8 h before harvesting. The aqueous ANE and sANE were immediately passed through 6-μm (Advantec) and 0.22-μm (Millipore) filters and stored in a freeze-drying form at −20°C until use.

Cell viability. Each cell line was trypsinized and seeded into a 96-well plate at a density of 10^4 cells per well in a total volume of 100 μL. Cells were cultured for 24 h before the addition of the various concentrations (0, 100, 200, 400, and 800 μg/mL) of ANE and sANE. To assess the cell viability through dose-response relationships, the living cell activity via mitochondrial dehydrogenase was quantified using *In vitro* Toxicology Assay kit (MTS-based, Promega). The absorbance of a dual-wavelength reading (490/690 nm) was measured by the Benchmark Plus Microplate Spectrophotometer (Bio-Rad), and the quantification of average cell viability (%) was performed in triplicate at each treatment. The alteration of cellular morphology was visualized on a ×100 magnification level using an inverted microscope (Leica Microsystems).

Real-time quantitative reverse transcription-PCR. After 24-h treatments with ANE/sANE, total RNA was isolated using RNeasy Midi kits with DNase digestion (Qiagen). Total RNA (2 μg) was used to synthesize first-strand cDNA with oligo dT primers using a SuperScript III first-strand synthesis kit (Invitrogen). The cytotoxic effects on *COX-2* mRNA expression of various ANE/sANE treatments (0, 100, 200, 400, and 800 μg/mL) were first determined. Furthermore, 23 selected genes related to proinflammatory signaling were conducted and divided into four signaling groups: (*a*) growth factors (*EGF, TGFB1*, and *GDF15*); (*b*) cytokines/mediators (*TNFA, TNFRSF11A, MAP4K4, IL1B, IL6*, and *IL8*); (*c*) NF-κB signaling (*MAP3K14/NIK, AKT1, RELA, RELB, REL, NFKB1*, and *NFKB2*); and (*d*) PPARs (*PPARA* and *PPARG*), retinoid receptors (*RARA* and *RARB*; *RXRA* and *RXRB*), and cAMP-responsive element binding protein binding protein (*CREBBP*). The primer sets for the Universal ProbeLibrary Set (human) assay were listed in Supplementary Table S1, and real-time quantitative reverse transcription-PCR was performed using the LightCycler 1.2 and 480 System (Roche), as previously described (18). The gene expression level was normalized using *β-actin* (*ACTB*) as an internal reference gene, and the average relative change was calculated from triplicate to quintuplicate determinations by relative quantification, applying the formula $2^{-\Delta\Delta Ct}$. Compared with control, the arbitrary cutoff of >2-fold average change was defined as significantly up-regulated. The strength of fold change was arbitrarily described as minor (2-fold to 5-fold), moderate (5-fold to 10-fold), and strong (>10-fold) up-regulation.

Study population and specimen collection. A total of 377 male patients diagnosed with OSCC were recruited from the Department of Dentistry and the Department of Otorhinolaryngology, Kaohsiung Medical University Hospital. The control group, composed of 442 unrelated males, was frequency-matched with OSCCs by age. This study was approved by the Human Experiment and Ethics Committee of Kaohsiung Medical University (KMU-IRB-950070 and KMU-IRB-950072). The questionnaire data, medical report, whole blood sample, and oral tissue specimens were gathered from volunteers after written informed consents were obtained. The demographic characteristics with regard to personal information, substance use history, and disease status were included in the analysis. An individual without substance use of alcohol, betel quid, and cigarettes was defined as nondrinker, nonchewer, and nonsmoker, respectively. To evaluate the dose-response relationship of ABC in the risk of OSCC, the variable of consumption years was stratified into two subgroups (nonusers and users) and three subgroups (nonusers, ≤20 y, and >20 y) in alcohol drinking and cigarette smoking and four subgroups (nonchewers, ≤10 y, 11–20 y, and >20 y) in betel chewing. The starting age of substance use was divided into three subgroups (nonusers, ≤20 y, and >20 y). For gene expression analysis, pairs of tumor and normal marginal tissues were obtained from nine OSCCs. The tumor grade of oral cancerous tissue was diagnosed by a histopathologist. Frozen tissues were soaked in RNAlater-ICE (Ambion) at −20°C before total RNA isolation. Total RNA was extracted with TRIzol reagent (Invitrogen) and RNeasy mini kit (Qiagen) according to the instruction manual. The method of cDNA synthesis and real-time quantitative PCR was as described previously.

Resequencing of *COX-2* promoter region and genotyping. Briefly, genomic DNA of cell lines and human whole blood were isolated using Puregene DNA purification kit (Gentra). In resequencing, two amplicons expanded from position +56 of exon 1 to −1541 of the 5′ untranslated region in the *COX-2* promoter region (972 bp, forward 5′-catttagcgtccctgcaaat-3′ and reverse 5′-ctgctgaggagttcctggac-3′; 977 bp, forward 5′-tccgtgtctcatgaagaatca-3′ and reverse 5′-tgacgacgcttaataggctgta-3′) were resequenced in 20 pairs of OSCCs and controls. Genetic variation detection and minor allele frequency (MAF) were further analyzed. Genotyping was performed by PCR-based RFLP (PCR-RFLP). PCR products were amplified using 100 ng DNA, 1.5 mmol/L $MgCl_2$, 0.3 units of Super-Therm Gold DNA polymerase (JMR Holdings), and 5 μmol/L each of the forward and reverse primers (−765G>C, forward 5′-ccgcttcctttgtccatcag-3′ and reverse 5′-ggctgtatatctgctctatatgc-3′; −1195G>A, forward 5′-ccctgagcactacccatgat-3′ and reverse 5′-gcccttcataggagatactgg-3′) in a 30-μL reaction volume. The PCR conditions for −765G>C were as follows: an initial denaturation of 94°C for 5 min; 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 45 s; and a final extension of 72°C for 7 min. The PCR condition for −1195G>A was identical, except for the annealing temperature with 45°C in cycling. The genotypes of −765G>C and −1195G>A were distinguished using *Aci*I and *Pvu*II (NEB), respectively, and visualized by electrophoresis on 3% agarose gels stained with ethidium bromide under UV light transilluminator (Alpha Innotech).

Statistical analysis. The *P* values for linear trend on cell viability (%) and mRNA expression level were analyzed using the *t* test (slope estimate) in simple linear regression models. The difference was considered statistically significant at the $P < 0.05$ or $P < 0.01$ level. The average fold-changes among all treatments ($n = 8$; doses of 100, 200, 400, and

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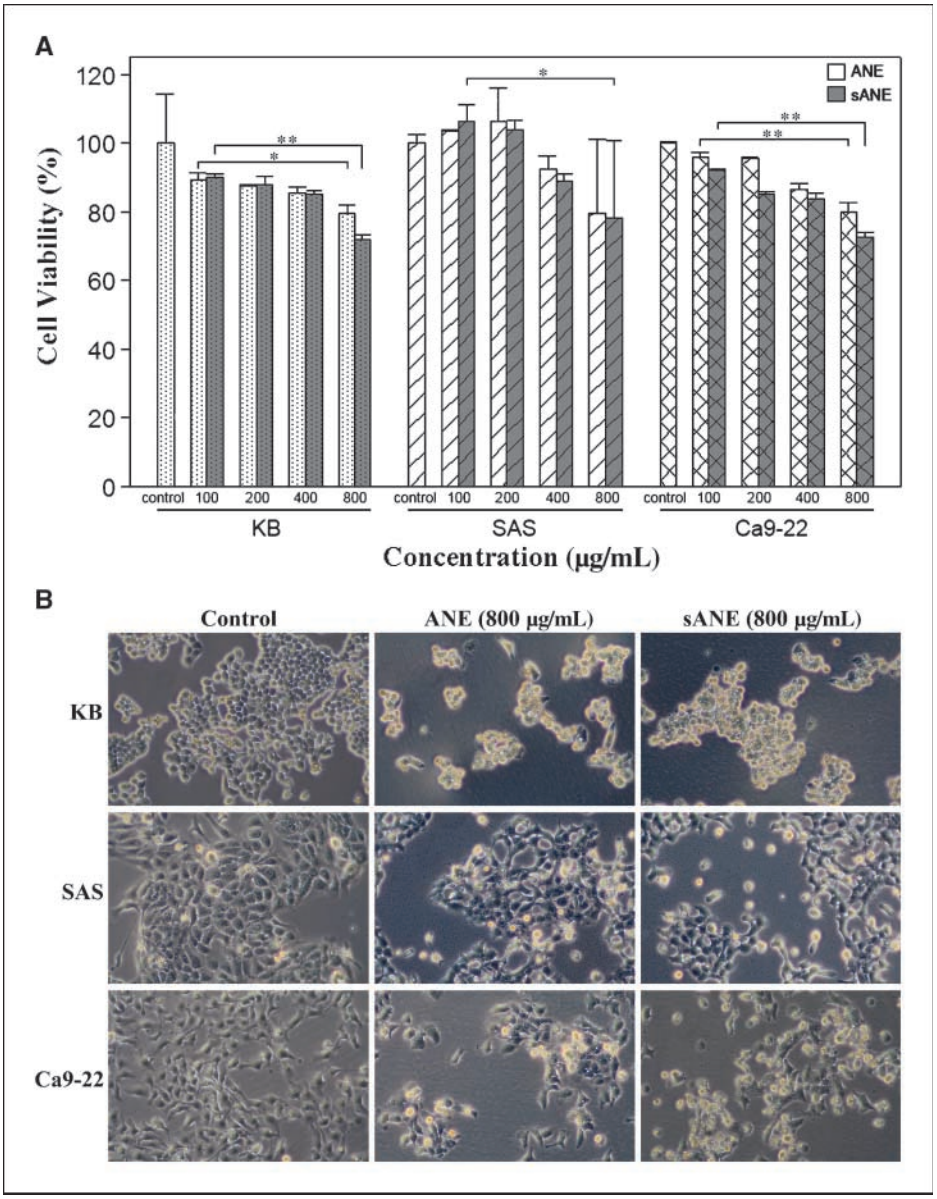

Figure 1. Cytotoxic effects of ANE and sANE on three oral epithelial carcinoma cell lines. *A,* cell lines (10^4 cells) were exposed to 100 to 800 μg/mL of ANE and sANE, respectively, for 24 h. MTS-based cellular viability was detected in triplicate. *Columns,* mean; *bars,* SD. The dose-dependent trend test was performed by a simple regression model. *, $P_{trend} < 0.05$; **, $P_{trend} < 0.01$. *B,* cellular morphologic changes of three cell lines at dose of 800 μg/mL of ANE and sANE for 24-h treatments were observed at ×100 magnification. Nontreated control showed normally polygonal form, whereas numbers of enlarged round form and atrophied form were observed at ANE-treated and sANE-treated cells, especially on KB cells.

800 μg/mL of both extracts) were used to assess the level of gene coexpressions between upstream effectors and *COX-2* by the Pearson's and Spearman's correlation coefficients. In the case-control study, the trend for dose effect on substance use of ABC was calculated by using the Cochran-Armitage trend test. In the genotype analysis, the goodness-of-fit χ^2 test was used to evaluate any deviation from the Hardy-Weinberg equilibrium in OSCCs and controls, respectively. Adjusted odds ratios (aOR), 95% confidence intervals (95% CI), exact *P* values of independent and joint effects, and *P* values for interaction based on a multiplicative interaction model were estimated by a multiple logistic regression model controlling potential confounders. The aORs were adjusted for continuous age and substance use of ABC (yes and no). All analyses were performed using the SAS Statistical Package (version 9.1.3, SAS Institute, Inc.).

Results

Cell viability and morphologic change. After a series of ANE/sANE treatments for 24 hours, sANE decreased cell viability in a dose-dependent manner ($P_{trend} < 0.05$ or $P_{trend} < 0.01$) in all cell lines, whereas ANE significantly decreased the viability of KB and Ca9-22 cells (Fig. 1*A*). The cytotoxic effect of both extracts causes the normal attached cells to change to enlarged round and atrophied forms, especially at the concentration of 800 μg/mL (Fig. 1*B*). In morphologic changes on the concentration of 800 μg/mL of both sANE and ANE, the KB cells were observed to have a higher cellular damage than Ca9-22 and SAS. Although Ca9-22 and SAS were seen to have a higher toxic tolerance for ANE/sANE treatments, the inhibition of cell growth and spreading by both extracts seems to influence the cellular viability.

Discovery of *COX-2* promoter polymorphisms. The valid resequencing in the *COX-2* proximal promoter region in the three cell lines and 20 pairs of subjects was expanded from position −1 to −1463. The RFLP-based *COX-2* −1195G>A genotype of KB, SAS, and Ca9-22 showed A/A, A/G, and G/G, respectively; other positions of genetic variation using resequencing were identical in the *COX-2* promoter region (Fig. 2*A* and *B*). In the case-control study, the positions of −285A>C, −765G>C, −1195G>A, and −1290A>G were discovered by MAFs with 50% (A/C, 50:50), 5.1%

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(G/C, 74:4), 46.2% (G/A, 36:42), and 3.9% (A/G, 73:3), respectively (Supplementary Fig. S1). The polymorphisms with MAF of >5% were further included in genotyping analysis. However, −285A>C was excluded due to the entire heterozygote in all subjects.

mRNA expression levels with ANE and sANE treaments. We examined three OSCC cell lines, KB, SAS, and Ca9-22, for the mRNA expression level in inflammatory signaling pathways with ANE/sANE treatments (Fig. 2*C*). In the analysis of overall

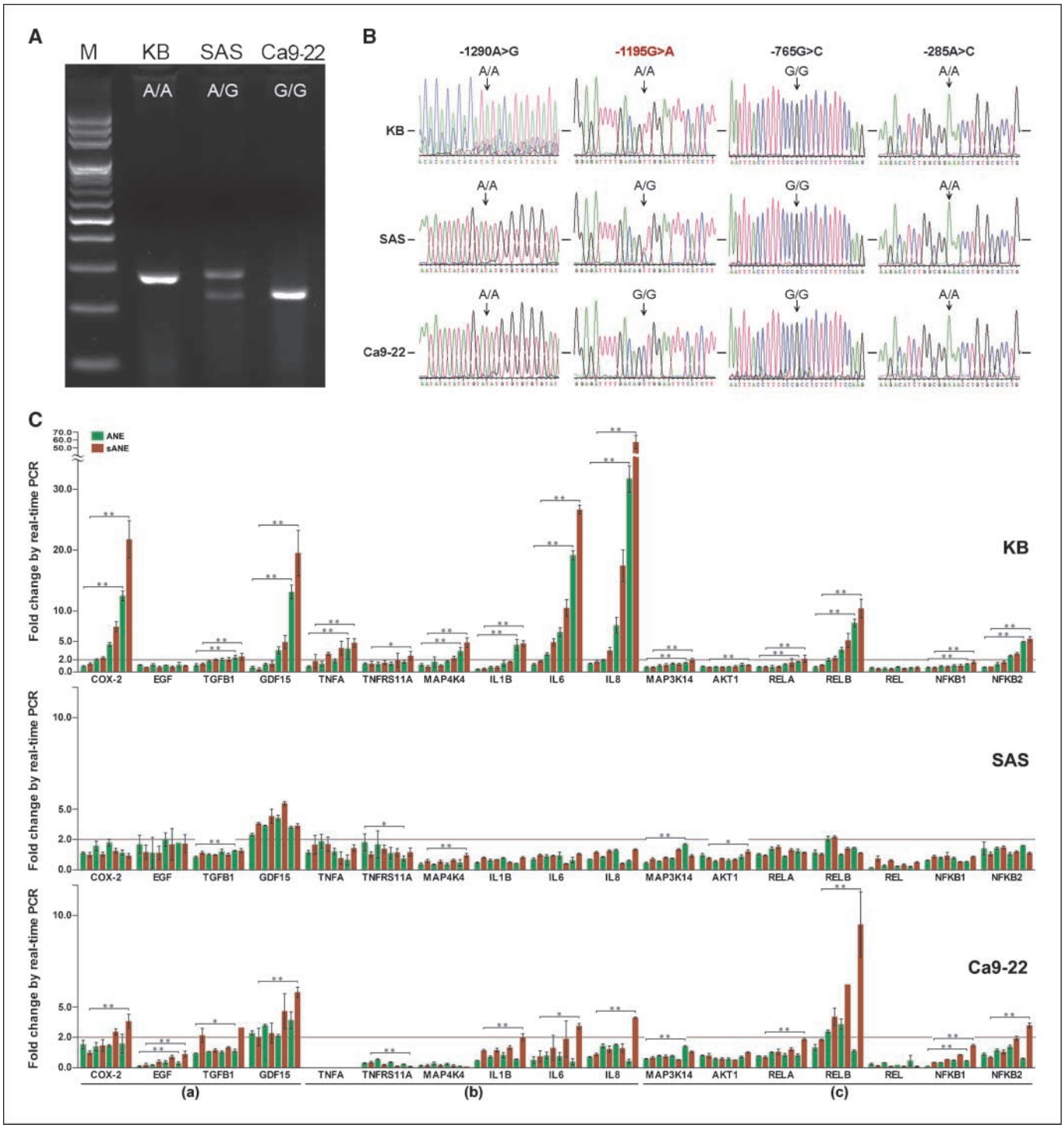

Figure 2. *COX-2* promoter polymorphisms and ANE/sANE–induced mRNA expression in a dose-response analysis using three oral carcinoma cell lines. *A,* RFLP-based *COX-2* −1195G>A genotypes of the three cell lines. *B,* determination of genetic variations from position −1 to −1463 in the *COX-2* promoter region in the three cell lines. Reverse sequencing (3′→5′ direction) was performed at −1290A>G and −1195G>A except for −1290A>G of KB cells; forward sequencing (5′→3′ direction) was performed at −765G>C and −285A>C. *C,* the selected genes involved in inflammatory signaling were subgrouped into *COX-2* and growth factor signaling (*a*), cytokines and its mediators (*b*), and NF-κB signaling (*c*). The treated doses of ANE and sANE were increased from left to right columns (100, 200, 400, and 800 μg/mL). Compared with nontreated cells, the average fold change (*columns,* mean; *bars,* SD) of each gene was in triplicate, except for *COX-2* (quintuplicate) and TNFA (quadruplicate) on KB cells. *Gray line,* 2-fold relative ratio. Linear trend: *, $P < 0.05$; **, $P < 0.01$.

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Table 1. Selected characteristics and relative risks (aORs) of substance uses of the study group

Characteristics	OSCCs, *n* (%)	Controls, *n* (%)	aOR* (95% CI)
Age (mean ± SD, y)	52.0 ± 10.4	53.2 ± 12.0	
<40	41 (11.4)	50 (11.9)	$P = 0.2990$ †
40–49	116 (32.1)	124 (29.6)	
50–59	121 (33.5)	125 (29.8)	
≥60	83 (23.0)	120 (28.6)	
Ethnicity			
Fukienese	317 (88.3)	367 (83.0)	$P = 0.0513$ †
Hakka	15 (4.2)	17 (3.9)	
Mainlander	21 (5.9)	37 (8.4)	
Aborigines	6 (1.7)	21 (4.8)	
Alcohol drinking (consumption years)			
Nondrinkers	96 (26.6)	316 (75.6)	1.00
Drinkers	265 (73.4)	102 (24.4)	2.42 (1.49–3.93)
≤20	95 (26.3)	46 (11.0)	2.03 (1.06–3.87)
>20	170 (47.1)	56 (13.4)	2.70 (1.55–4.70)
P_{trend} ‡			<0.0001
Start age (y)			
Nondrinkers	96 (26.6)	316 (75.6)	1.00
≤20	122 (33.8)	56 (13.4)	1.55 (0.85–2.82)
>20	143 (39.6)	46 (11.0)	3.62 (2.01–6.51)
Betel chewing (consumption years)			
Nonchewers	41 (11.3)	377 (88.9)	1.00
Chewers	321 (88.7)	47 (11.1)	42.19 (25.48–69.86)
≤10	42 (11.6)	14 (3.3)	17.61 (8.09–38.36)
11–20	109 (30.1)	12 (2.8)	56.67 (26.43–121.49)
>20	170 (47.0)	21 (5.0)	49.09 (26.87–89.69)
P_{trend} ‡			<0.0001
Start age (y)			
Nonchewers	41 (11.3)	377 (88.9)	1.00
≤20	155 (42.8)	28 (6.6)	34.85 (19.25–63.09)
>20	166 (45.9)	19 (4.5)	51.39 (27.53–95.94)
Cigarette smoking (consumption years)			
Nonsmokers	46 (12.7)	229 (54.0)	1.00
Smokers	316 (87.3)	195 (46.0)	1.83 (1.06–3.16)
≤20	68 (18.8)	71 (16.8)	1.16 (0.56–2.41)
>20	248 (68.5)	124 (29.3)	2.14 (1.21–3.77)
P_{trend} ‡			<0.0001
Start age (y)			
Nonsmokers	46 (12.7)	229 (54.0)	1.00
≤20	210 (58.0)	139 (32.8)	1.60 (0.89–2.86)
>20	106 (29.3)	56 (13.2)	2.42 (1.22–4.78)

*Odds ratios were adjusted for age and substance use in the table.
† χ^2 test.
‡Cochran-Armitage test for trend (two-sided).

inflammatory response, KB (*COX-2* −1195 A/A type) showed a greater response, in a dose-dependent manner, of various inflammatory genes than Ca9-22 (G/G) and SAS (A/G) after a 24-hour exposure. Numerous genes in groups a to c displayed a higher mRNA expression level in sANE treatments than at the same dose of ANE treatments on KB and Ca9-22 cells. Most of the selected genes showed significantly positive linear trends ($P < 0.05$ or $P < 0.01$) in treatments using both extracts on KB cells, whereas several genes had significant positive trends in sANE treatments on SAS and Ca9-22.

The mRNA expression level of *EGF* was not significantly up-regulated (<2-fold) by both extracts among those cell lines, even at the concentration of 800 μg/mL. The *COX-2* gene expression level presented minor to strong up-regulation (2.04-fold to 12.49-fold and 2.28-fold to 21.76-fold) at concentrations of 200 to 800 μg/mL of ANE and sANE, respectively, and minor up-regulation (2.36-fold and 3.05-fold) at concentrations of 400 and 800 μg/mL of sANE on Ca9-22. However, no significant up-regulation of the *COX-2* gene expression was observed on SAS. Furthermore, we observed that *GDF15* [a member of transforming growth factor-β (TGF-β) superfamily] was significantly induced by both extracts on those cell lines, especially at 800 μg/mL of ANE and sANE (13.13-fold and 19.51-fold, respectively) on KB cells. In the betel-related and well-discussed cytokine genes (TGFB1, TNFA, IL1B, IL6, and

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IL8), the three former genes presented minor up-regulation at concentrations of 400 to 800 μg/mL on KB cells, whereas the two latter genes showed extremely strong up-regulation. Nevertheless, all of these presented no significance to minor up-regulation on SAS and Ca9-22. In addition to two other genes involved in the TNFα signaling pathway, *MAP4K4* and *TNFRSF11A* only showed a minor up-regulation on KB cells. In upstream regulators of NF-κB signaling pathway, *MAP3K14* and *AKT1* showed no significant up-regulation on all treatments in these OSCC lines. Remarkably, in the NF-κB family members, the ANE/sANE–treated mRNA expression level of *RELB* and *NFKB2* displayed higher up-regulation than *RELA* and *NFKB1* on KB and Ca9-22.

Compared with untreated control, the mRNA expression level of group d showed no significant change on these cell lines in spite of positively increased trends on most genes in sANE treatments (Supplementary Fig. S2). Unexpectedly, the crossing point values of *RARB* and *TNFA* were undetermined in low abundant cDNA of all Ca9-22 samples, whereas *RARB* was undetermined on the 800 μg/mL–treated KB samples. With regard to parallel coexpression with *COX-2* on KB cells, the up-regulated level of 17 upstream effector genes was significantly associated with the increased level of *COX-2* in accordance with the Spearman's rank correlation coefficients and *P* values, but not *EGF, REL, RXRA, RARA, RARB*, and *CREBBP* (Supplementary Table S2).

Characterization of study population. The selected characteristics of age, ethnicity, and substance use were summarized in Table 1. No statistically significant differences were observed between OSCCs and controls in terms of age and ethnicity distribution. The adjusted estimates of substance use of ABC were 2.4-fold, 42.2-fold, and 1.8-fold at risk of OSCC on the study group, respectively. The results, as expected, indicated betel chewing as the strongest risk factor accounting for OSCC in Taiwan. In addition, the dose-response relationship showed a positively increased risk in consumption years of substance use of ABC (all $P_{trend} < 0.0001$). Compared with nonusers, the study subjects with late starting age (>20 years) among all substance use represented a higher risk than those with early starting age (≤20 years). In the study group of non–betel chewers, at controlling age, the independent risk of cigarette smoking (aOR, 1.82; 95% CI, 0.90–3.67) had similar OSCC risk with the betel chewing group; however, the risk was almost twice in the alcohol drinking group (aOR, 4.41; 95% CI, 2.21–8.79). Compared with nonusers, the subjects who had a combined use of both substance increased risk to 8.70-fold (95% CI, 3.30–22.94; table not shown).

Independent and joint effects of genetic variants and substance use. Compared with the wild type, the A/A homozygote of *COX-2* −1195G>A was significantly associated with OSCC (A/A versus G/G; OR, 1.85; 95% CI, 1.05-3.28; data not shown) in the pilot study, whereas the −765G>C variant showed no statistically significant difference (Table 2). In expanding analysis, 177 of OSCCs were recruited from a previous study (26); however, these were only genotyped for −1195G>A in this study. In the genetically polymorphic distribution of −1195G>A, there was no deviation from the Hardy-Weinberg equilibrium in both OSCCs and controls (Table 2). A significant difference was observed in the allele frequencies of −1195G>A between OSCCs and controls ($P = 0.036$), and allele A appeared to be in risk of OSCCs (OR, 1.23). Compared with subjects holding the G/G homozygote, those with the A/A homozygote had a 1.55-fold increased risk of OSCC but those with the A/G heterozygote displayed no statistically significant risk (OR, 1.13; 95% CI, 0.80–1.60). In OSCCs, we observed no statistical difference in the consumption years and the starting age of substance use of ABC among the three genotypes of −1195G>A by ANOVA (data not shown).

Controlling for the effects of alcohol drinking and cigarette smoking, non–betel chewers with the A allele had a 1.75-fold

Table 2. Distribution of *COX-2* −765*G>C* and −1195*G>A* genotypes between OSCCs and controls

Characteristics	OSCCs, *n* (%)	Controls, *n* (%)	OR (95% CI)
−765*G>C*			
Allele frequency			
G	314 (88.2)	371 (90.5)	1.00
C	42 (11.8)	39 (9.5)	1.27 (0.80–2.02)
HWE (χ^2/*P* value)	3.185/0.07	2.265/0.132	
Genotype frequency			
GG	136 (76.4)	166 (81.0)	1.00
CG+CC*	42 (23.6)	39 (19.0)	1.32 (0.80–2.15)
−1195*G>A*			
Allele frequency			
G	347 (47.2)	463 (52.4)	1.00
A	389 (52.9)	421 (47.6)	1.23 (1.01–1.50)†
HWE (χ^2/*P* value)	0.142/0.707	1.911/0.167	
Genotype frequency			
GG	80 (21.7)	114 (25.8)	1.00
AG	187 (50.8)	235 (53.2)	1.13 (0.80–1.60)
AA	101 (27.5)	93 (21.0)	1.55 (1.04–2.31)†

Abbreviation: HWE, Hardy-Weinberg equilibrium test.
*None of CC homozygote was observed in OSCC or control subjects.
† $P < 0.05$.

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Table 3. Joint aORs of OSCC with regard to *COX-2* −1195G>A variants and substance use of ABC

Genetic variant	Substance use	OSCCs, *n* (%)	Controls, *n* (%)	aOR* (95% CI)	Stratified aOR† (95% CI)
Study group with betel chewing habit					
Allele	B				
G	−	33 (4.6)	401 (47.3)	1.00	
A	−	49 (6.8)	353 (41.6)	1.75 (1.09–2.81)‡	
G	+	309 (42.7)	50 (5.9)	52.52 (31.64–87.18)§	
A	+	333 (46.0)	44 (5.2)	60.18 (36.45–99.36)§	
P for interaction between betel chewing and allele‖				0.457	
Genotype	B				
GG	−	7 (1.9)	100 (23.6)	1.00	1.00
AG	−	19 (5.3)	201 (47.4)	1.42 (0.57–3.55)	1.42 (0.57–3.55)
AA	−	15 (4.1)	76 (17.9)	3.05 (1.16–8.04)‡	3.05 (1.16–8.04)‡
GG	+	71 (19.6)	14 (3.3)	59.87 (21.07–170.08)§	1.00
AG	+	167 (46.1)	22 (5.2)	65.79 (26.30–164.63)§	1.10 (0.49–2.47)
AA	+	83 (22.9)	11 (2.6)	79.44 (28.22–223.65)§	1.33 (0.51–3.43)
P for interaction between betel chewing and genotype‖				0.441	
Study group without betel chewing habit					
Genotype	A/C				
GG	−/−	1 (2.4)	48 (12.9)	1.00	1.00
	+/−, −/+	4 (9.8)	36 (9.7)	3.94 (0.39–39.62)	3.94 (0.39–39.62)
	+/+	2 (4.9)	15 (4.0)	5.73 (0.48–68.28)	5.73 (0.48–68.28)
AG	−/−	4 (9.8)	105 (28.2)	1.76 (0.19–16.20)	1.00
	+/−, −/+	8 (19.5)	72 (19.4)	5.01 (0.61–41.47)	2.85 (0.82–9.86)
	+/+	7 (17.1)	22 (5.9)	15.09 (1.74–130.77)‡	8.59 (2.29–32.21)‡
AA	−/−	2 (4.9)	40 (10.8)	2.14 (0.19–24.67)	1.00
	+/−, −/+	8 (19.5)	27 (7.3)	13.67 (1.61–115.71)‡	6.38 (1.24–32.70)‡
	+/+	5 (12.2)	7 (1.9)	32.13 (3.22–320.80)‡	14.99 (2.37–94.79)‡
P for interaction between both substance use and genotype‖	0.492				

Abbreviations: B, betel chewing status; A/C, alcohol drinking and cigarette smoking status.
*Odds ratios in the study group with betel chewing were adjusted for continuous age, alcohol drinking, and cigarette smoking. Odds ratios in the study group without betel chewing were adjusted for continuous age.
†Odds ratios were adjusted for continuous age in a stratified analysis.
‡$P < 0.05$.
§$P < 0.0001$.
‖*P* for interaction based on a multiplicative interaction model.

increased OSCC risk compared with those with the G allele, and the risk was found to be enhanced to 60.18-fold among betel chewers (Table 3). A similar risk-enhanced pattern with regard to the *COX-2* A/A genotype was identified among betel chewers, with the highest risk (aOR, 79.44) observed in *COX-2* A/A chewers. Although this joint risk was greater than the expected combined risk estimated from an additive interaction model {synergy index = (79.44 − 1)/[(59.87 − 1) + (3.05 − 1)] = 1.29}, as shown in the stratified aOR, the multiplicative interaction between *COX-2* −1195G>A and betel chewing was not significant ($P = 0.441$). Among the non–betel chewers (OSCCs/controls, 41:372), the significant joint risk of contracting OSCC (aOR, 15.09) for alcohol drinking and cigarette smoking was found among subjects who carried the *COX-2* A/G heterozygote, and a higher risk (aOR, 32.13) was recognized among subjects who carried the A/A homozygote. Although the stratified aOR for the combined use of the two substances increased with the increase in number of the A allele, no significant interaction in the multiplicative model was found ($P = 0.492$).

mRNA gene expression of selected genes on OSCCs. In consideration of the influential factors related to tissue mRNA expression level, the personal information on tumor site, tumor stage (based on tumor-node metastasis grade), and substance use of ABC and *COX-2* −1195G>A was exhibited according to the interview data, medical record, and genotyping (Fig. 3*A*). Seven patients showed a significant up-regulation (>2-fold) in *COX-2* mRNA expression, ranging from 2.51-fold to 7.82-fold, except for O132 (1.52-fold) and O086 (1.24-fold). With regard to NF-κB signaling elements, the significant up-regulation of RELB and NFKB2, which had higher expression levels than RELA and NFKB1, was observed in six OSCCs (C089, C099, CA056, O130, O136, and KO012), but two of them (O130 and KO012) did not have the habit of betel chewing. Remarkably, O130 patient was noted as a heavy drinker based on his personal record, whereas O132 patient presented with necrotizing inflammation of oral lesions based on his histopathologic record and abstained from betel chewing 10 years before surgical operation. Based on tumor sites, the

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betel-related buccal OSCCs showed a higher fold change of *COX-2* expression level (4.36 ± 3.21-fold) than betel-related tongue OSCCs (1.87 ± 0.85-fold; Fig. 3*B*). The *COX-2* expression level of betel-related OSCCs had a positive increase in cancer stage, except for stage I. However, the sample size was insufficient to provide a powerful evidence in clarifying the association between *COX-2* −1195G>A variants and inflammatory level of betel-related OSCC in each cancer stage.

Discussion

Betel chewing with or without tobacco has been a well-declared carcinogenic for human oral cavity (6, 27). Accordingly, in 2004, AN is declared to be carcinogenic to humans (group 1) based on an indirect evaluation from sufficient evidence of carcinogenicity of betel quid. In addition, the AN-derived nitrosamines, including NGL and MNPN, can be detected in the saliva of betel chewers without tobacco (19, 28). In our findings, the higher cytotoxic

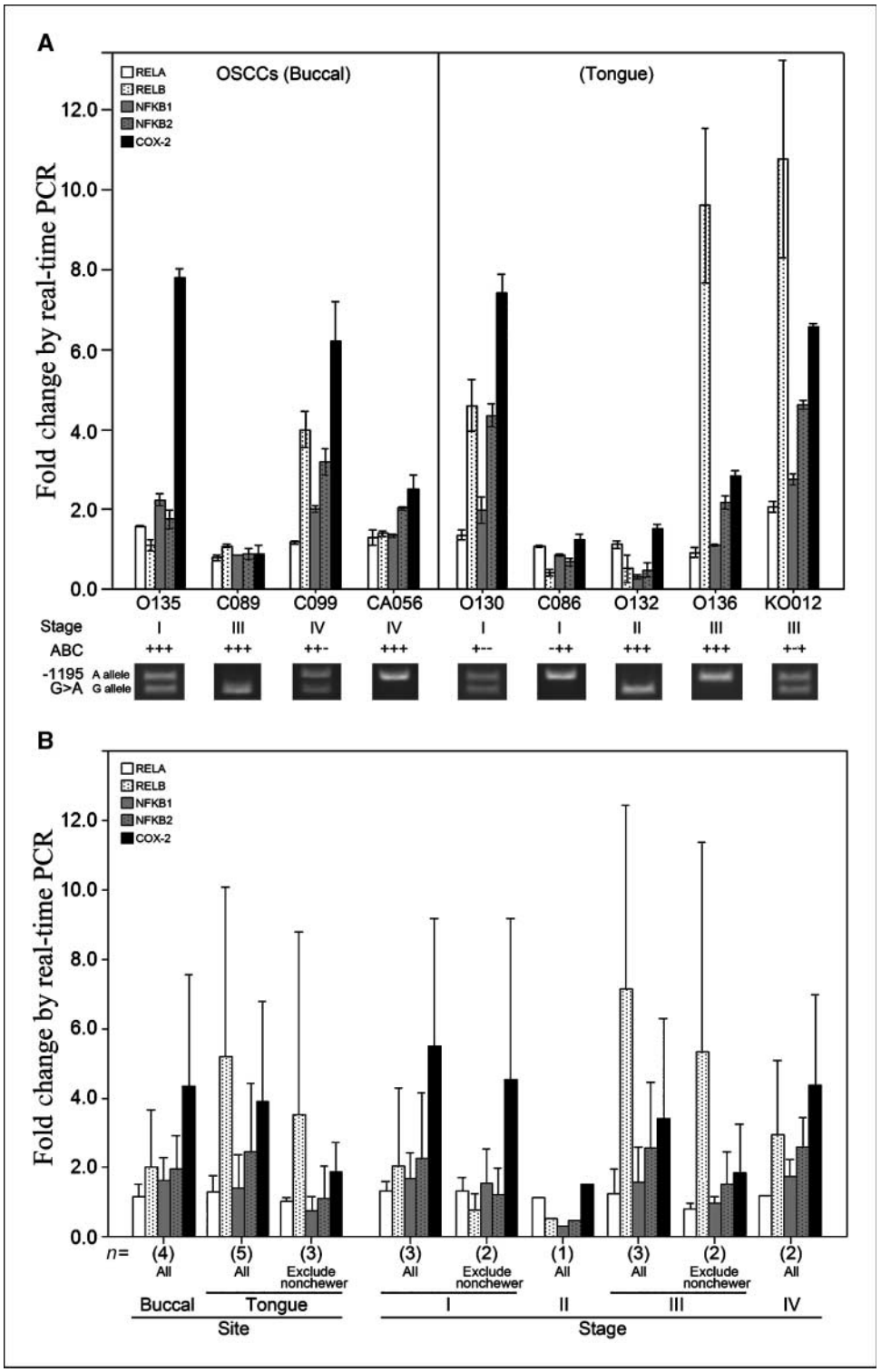

Figure 3. The mRNA expression level of *COX-2* and NF-κB signaling elements in OSCC patients. Paired tissue samples (tumor and normal marginal tissues) without chemotherapy of nine OSCCs were analyzed, including four betel-related buccal OSCCs, three betel-related tongue OSCCs, and two tongue OSCCs without betel chewing. *A,* compared with normal marginal tissue, the relative fold-change was calculated in triplicate (*columns,* mean; *bars,* SD) by using the formula $2^{-\Delta\Delta Ct}$. The mRNA expression level was presented in individuals with important influential factors, including personal substance use of ABC and genotype of *COX-2* −1195G>A. *B,* the average mRNA expression level of all OSCCs was shown at two conditions: tumor site (buccal and tongue) and cancer stage (I–IV). The fully betel-related OSCCs on two conditions were also displayed in extra columns.

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effects at cell morphologic changes and mRNA expression level were observed in sANE treatments. However, whether the ingredients of sANE contain any AN-derived nitrosamine requires further analysis.

The biological function of COX-2 is notable not only for recalling the inflammation response but also for involvement in cooxidation during xenobiotic biotransformation (29). The arecoline-induced *COX-2* up-regulation on human gingival fibroblasts has been characterized in our previous study (18), and the higher increased trend of *COX-2* induction on KB cells by ANE/sANE treatments was found in the present study. *COX-2* up-regulation in betel-related OSCC can be enhanced via a variety of upstream effectors, which are released from oral epithelial cells or microenvironment under betel quid/AN exposure by binding to their recognition sequences in the COX-2 coding region or promoter. Consequently, we selected 23 candidate genes to characterize the potential regulators in ANE/sANE–induced COX-2 signaling by using a dose-response analysis in mRNA expression change.

Despite EGF having a critical role in enhanced COX-2 expression, the mRNA expression of *EGF* was not significantly induced by ANE/sANE in OSCC lines. With regard to the proinflammatory regulators, the AN-induced *TNFA, IL6, IL1B*, and *IL8* had a significant parallel effect with *COX-2* up-regulation on KB cells. The findings are also responding to the important roles of TNFA, IL6, and PGE_2 in ANE-induced keratinocyte inflammation (30) and the detectable up-regulation of *IL1B* and *IL8* in OSCCs by a salivary transcriptome analysis (5.48-fold and 24.3-fold, respectively; ref. 31). Within the oral microenvironment, we suggested that ANE/sANE–induced *IL1B* from the oral mucosa may enhance more *COX-2* induction in the gingival region via Ca^{2+} mobilization in a long-term betel quid (contained slaked lime) chewing, as described previously (18).

In TGF-β signaling, the mRNA expression of *GDF15* is strongly induced by arecoline on human gingival fibroblasts in our previous study (18). The similar effect was also found on KB cells by 24-hour ANE/sANE treatments, whereas minor up-regulation was seen on SAS and Ca9-22. Interestingly, it has been shown that the *GDF15* promoter region contains two p53 response elements and plays a role in signaling cell cycle arrest and apoptosis (32). Recently, its transcriptional activation shows involvement in the regulation of transactivating p63-mediated keratinocyte differentiation (33). Although the functional role of the unprocessed form of GDF15 in tumorigenesis remains unknown, the overexpression of *GDF15* might associate with abnormal keratinocyte differentiation toward keratinization, which we can observe in the keratinizing OSCC.

NF-κB is suggested to involve in tumorigenesis (14), and a parallel effect between NF-κB and COX-2 protein expression has been reported in smokeless tobacco-related OPL and oral cancer (9). Dependent on the induction of proinflammatory regulators and the activation of IκB kinase complex, NF-κB signaling pathways are divided into classic [RELA-p50 (NFKB1)] and alternative [RELB-p52 (NFKB2); ref. 34]. We thought that both NF-κB signaling pathways may coexist in the betel-induced NF-κB activation on oral epithelial cells, although the higher mRNA expression of *RELB* and *NFKB2* may imply that the alternative pathway plays an important role in most *COX-2* up-regulation. Furthermore, we attempted to validate the mRNA expression level of *COX-2* and NF-κB signaling elements in OSCC patients. Although it indicated that *COX-2* is up-regulated in malignant tissues, the accumulative dosage and combination of substance use and tumor histologic grade lead to much more complexity on elucidation of *COX-2* −1195 variants into the role of inflammatory mechanisms. This needs to be clarified by a big enough sample size in further studies, and it is important to prove whether *COX-2* −1195A/A has a functional role in contributing to higher inflammatory levels on OSCCs.

In negative cross-talks with NF-κB, PPARγ can inhibit inflammatory transcriptional responses mediated by activator protein 1 (AP-1) and NF-κB transcriptional factors (35). Furthermore, it can also repress the phorbol ester–mediated *COX-2* transcriptional activation by inhibiting the activity of AP-1 to the cAMP-responsive element–binding site of the *COX-2* promoter (15). Therefore, it is also important to explore whether ANE/sANE enhances the *COX-2* expression level by down-regulating cellular PPARs in the meantime. The mRNA expression levels of *PPARA, PPARG*, and *CREBBP* were induced in a dose-dependent manner on KB and SAS cells by sANE, but none of them were significantly up-regulated. Nevertheless, further investigation is needed to determine whether they are significantly up-regulated or down-regulated in betel-related OPLs or OSCCs. Moreover, compared with normal tissues, RARα immunopositivity has shown a significant increase in OSCCs with substance use of ABC, whereas it shows a significant decrease in RARβ immunopositivity (36–38). Although no significant up-regulation of *RARα* and down-regulation of *RARβ* are observed in this study, the resembled effects linking to the findings of both immunoreactivities in OSCCs are particularly noteworthy.

The cytotoxicity of betel chewing is shown on the basis of *in vitro* findings of significant up-regulation in *COX-2* and numerous upstream effectors in oral epithelial cells by ANE/sANE; however, some betel chewers do not develop OSCC even after having a long-term or high-frequency usage of betel nut. Therefore, we hypothesized that individual polymorphic variants in the *COX-2* promoter region may contribute to gene susceptibility in the development of OSCC. In addition, gene-environment interactions between the *COX-2* promoter polymorphisms and the substance use of ABC are also required in risk assessment to OSCC. The findings indicated that the *COX-2* −1195A allele was positively associated with the risk of OSCC. The −1195 A/A homozygote presents a stronger risk of OSCC than A/G and G/G genotypes, especially when joined with the habit of betel chewing. Based on both *in vitro* experiments and population association study, the *COX-2* −1195 A/A variant seems to increase susceptibility to betel-related OSCC. Furthermore, in betel nonchewers, the strongest joint effect is also seen in A/A homozygote carriers who have a combined use of alcohol drinking and cigarette smoking. Intriguingly, the −1195A allele has been shown to increase a potential risk of developing esophageal squamous cell carcinoma in a Chinese Han population (39). Additionally, compared with the −1195G allele, the −1195A allele reveals a higher promoter activity and *COX-2* mRNA expression.

Thus far, several studies illustrate that the risk of OPL or oral cancer is definitely increased in accordance with the length or dose of substance use of ABC (3, 40), as well as the findings in this study. However, the molecular mechanism of OSCC occurrence is more complex in clinical verification with regard to more confounders and unexpected conditions. Although this study cannot faithfully reflect the exact cytotoxicity of oral cavity in betel chewers under long-term, low-dose exposure, the findings provide an important insight into the critical role of *COX-2* promoter at risk of betel-related OSCC. Here, we suggest that this study could offer a basic

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evidence in alternative prevention of the occurrence of betel-related OSCC by using selective COX-2 inhibitors in heavy betel chewers, as previously described (41).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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