Research Article

Cancer Epidemiology, Biomarkers & Prevention

Acrolein Is Involved in the Synergistic Potential of Cigarette Smoking– and Betel Quid Chewing–Related Human Oral Cancer

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Abstract

Background: Cigarette smoking (CS) and betel quid (BQ) chewing are two known risk factors and have synergistic potential for the development of oral squamous cell carcinoma (OSCC) in Taiwan. The *p53* mutation characteristics in OSCC (G to A or G to T mutations) are similar to that of acrolein-induced DNA damage. Acrolein is a major cigarette-related carcinogen that preferentially causes *p53* mutations and inhibits DNA repair function in lung cancer. We hypothesize that acrolein is associated with OSCC carcinogenesis.

Methods: A total of 97 patients with OSCC and 230 healthy subjects with CS and/or BQ chewing histories were recruited. Slot blot analysis of Acr-dG adducts, an indicator of acrolein-induced DNA damage in buccal DNA, and LC/MS-MS analysis of 3-HPMA levels, urinary Acr metabolites, were performed.

Results: Our results showed that the level of Acr-dG adducts in buccal cells was 1.4-fold higher in patients with OSCC than in healthy subjects with CS and/or BQ chewing histories ($P < 0.001$). In addition, in healthy subjects, CS and BQ chewing were associated with significantly higher levels of 3-HPMA, indicating that CS and BQ chewing promotes acrolein absorption. However, 3-HPMA levels in patients with OSCC were significantly lower than those in healthy subjects, indicating impaired acrolein metabolism.

Conclusions: In this study, we provide a novel mechanism by which increased acrolein uptake and impaired metabolism may contribute to the synergistic potential of CS and BQ-induced OSCC.

Impact: Elevated acrolein-induced DNA damage (Acr-dG adducts) detected in buccal swabs may serve as an early indicator to identify patients at risk of developing OSCC.

Introduction

Oral cancer is a widely prevalent cancer worldwide and a leading cause of mortality in Southern Asia (1–3). Approximately 95% of oral cancer is oral squamous cell carcinoma (OSCC). The prevalence of the disease in different parts of the world reflects different forms and extents of exposure to etiologic agents. While cigarette smoking and alcohol consumption are the major risk factors in Western countries, betel quid (BQ) chewing and cigarette smoking (CS) are the primary causes of OSCC in Taiwan and both south and Southern Asia (4–7). Unfortunately, despite all of the advancements in the understanding of the disease process and recognition of the associated risk factors, the 5-year survival rate remains at 50% (8). The key challenge to reduce the mortality and morbidity of this disease is to develop strategies to identify and detect OSCC when it is present at a very early stage, which will enable effective intervention and therapy. The detection of OSCC is currently based on expert clinical examinations and histologic analysis of suspicious areas, but it may be undetectable in hidden sites. Therefore, sensitive and specific biomarkers for OSCC may be helpful in screening high-risk patients (9).

Previous studies have shown that the incidence of oral cancer in Taiwan was 18-fold higher in patients who smoked than in abstainers and 89-fold higher in patients who smoked and BQ chewing than in abstainers (4). The tumor suppressor gene *p53* is frequently mutated in human cancers (10, 11), and its mutational patterns often bear the fingerprints of the etiologic carcinogens. Most notably, it has been found that >50% of aflatoxin B1-associated liver cancers have mutations in codon 249 of the *p53* gene and that *p53* mutations are concentrated at contiguous pyrimidines in sunlight-associated skin cancers (12, 13). Previous studies have shown that characteristics of *p53* mutations in OSCC are associated with CS and BQ chewing in Taiwan (14). *p53* mutations occur at exons 5–9 in nearly half of patients with OSCC, and G to A or G to T mutations are the predominant mutations that were observed and associated with CS and BQ chewing. Similar *p53* mutations in oral cavity tumors have been found (14–17). These findings suggest an important contributive role of tobacco carcinogens in *p53* mutations in a series of Taiwanese patients with OSCC.

Acrolein (Acr), an α,β-unsaturated aldehyde, is abundant in CS, cooking fumes, and automobile exhaust fumes (18). The main

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route of Acr elimination involves the production of S-(3-hydroxypropyl)-N-acetylcysteine (3-HPMA) by conjunction with glutathione (GSH), the primary metabolite of Acr found in urine (19), which has been used as a biomarker for Acr exposure (20) and GSH has been identified as a protective scavenger of Acr. The free form of Acr induces α- and γ-hydroxy-1,N^2-cyclic propano-2′-deoxyguanosine (α-OH-Acr-dG and γ-OH-Acr-dG) adducts in human cells (21, 22). It has been shown that both types of Acr-dG adducts are mutagenic and that they induce mainly G to T and G to A mutations (21–27). By mapping the Acr-dG adduct distribution at the nucleotide level in Acr-treated normal human bronchial epithelial (NHBE) cells, we found that the Acr-DNA–binding spectrum in the *p53* gene coincides with the *p53* mutational spectrum in CS-related lung cancer (28). A similar *p53* mutational spectrum between CS-related lung cancer and CS-related OSCC, such as mutational hotspots on codons 175, 248, and 273 of the *p53* gene, was observed (Supplementary Fig. S1). These findings raise the question of whether Acr-DNA adducts are responsible for *p53* mutations in CS- and BQ-induced OSCC. Previous studies clearly demonstrated the synergistic effects of CS and BQ in oral cancer (4, 6, 7). However, the underlying mechanism for the synergistic potential of BQ and CS remains elusive. To investigate the role of Acr in OSCC and the synergistic mechanisms of CS and BQ, this study was designed to compare Acr-dG adducts of buccal cells and urinary 3-HPMA levels in CS subjects and patients with OSCC either with or without BQ chewing histories.

Materials and Methods

Subjects and collection of buccal cells and urine

For patients with oral cancer, 97 patients treated at Mackay Memorial Hospital (Taipei, Taiwan) from February 2016 through August 2018 were recruited for participation in the study. All patients were interviewed uniformly by a well-trained interviewer before surgery. For each case, tumor tissue samples containing oral cavity and oropharynx neoplasms were surgically dissected and sent to the Department of Pathology for examination by the Association of Directors of Anatomic and Surgical Pathology (ADASP; ref. 29). Informed written consent was given by each participant or his (or her) relative, and our study protocol was approved by the Institutional Review Board of Mackay Memorial Hospital (Taipei, Taiwan). Experiments were conducted in accordance with the Declaration of Helsinki principles. For healthy participants, 230 healthy subjects, including 111 without a BQ chewing history, 107 with a BQ chewing history, and 12 nonsmokers with a BQ chewing history, were recruited for participation in the study in cooperation with the Department of Public Health, Kaohsiung Medical University (Kaohsiung, Taiwan). All participants were interviewed by a well-trained interviewer. Buccal cells and urine samples were collected during the interviews. The questionnaire used in the interview sought detailed information on current and past cigarette smoking, alcohol consumption, and BQ chewing habits, occupational history, family disease, dietary history, and general demographic data. Informed written consent was given by each participant or his (or her) relative, and our study protocol was approved by the Institutional Review Board of Kaohsiung Medical University (Kaohsiung, Taiwan; IRB # KMUH-IRB-20110270). Experiments were conducted in accordance with the Declaration of Helsinki principles. For the collection of buccal cells, subjects were asked to rinse their mouths with water, followed by rinsing with antiseptic mouthwash and a subsequent water rinse. A trained technician placed one buccal brush against the inside of the cheek and scraped the blush against the center of a subject's cheek, applying firm pressure throughout the process. The brush was moved up and down as well as back and forth to ensure maximum cell collection, and the inside of the mouth was scraped at least 10–15 times. The procedure was repeated to obtain a second buccal swab sample on the other side of the cheek. A total of 2 buccal swabs were collected for each subject. Buccal cell and urine samples were stored at −30°C until use.

Slot blot assay for Acr-dG detection

Analysis of Acr-dG adducts in DNA samples was based on previously described methods (30, 31). Control genomic DNA was modified with Acr (0.5, 1, 2, and 5 mmol/L) at 37°C for 24 hours and purified with repeated phenol/ether extraction as Acr-dG adduct standards. After purification, modified DNA was precipitated with ethanol and dissolved in TE buffer (pH 8.0). Buccal DNA was extracted from buccal cells using Puregene buccal cell core kits (Qiagen) according to the manufacturer's instructions. Modified DNA or buccal DNA (0.25 μg) were loaded onto polyvinylidene difluoride membranes using a Bio-Dot SF microfiltration apparatus (Bio-Rad). WesternDot 625 Western blotting kits (Invitrogen) were used for Western blot analysis according to the manufacturer's instructions. After blocking for 1 hour at room temperature in blocking buffer, the membrane was probed overnight at 4°C with anti-Acr-dG mouse mAbs (32). After washing with washing buffer to remove unbound primary antibodies, quantum dot–conjugated secondary antibodies (1:1,000 dilution) were added for 2 hours at room temperature. Ultimately, Acr-dG adducts were detected using a UVP BioDoc-It imaging system, and band density was quantified with UVP imaging software. After antibody detection, the same membrane was stained with methylene blue (Molecular Research Center) to indicate the amount of DNA.

Detection of 3-hydroxypropyl mercapturic acid and creatinine in urine

Analysis of 3-hydroxypropyl mercapturic acid (3-HPMA) and creatinine in urine was based on methods described previously (33). For analysis of 3-HPMA in urine, solid-phase extraction with Isolute ENV+ cartridges (Biotage) was used to prepare each sample before LC/MS-MS analysis. High-performance liquid chromatography (HPLC) was performed using an Agilent 1100 Series HPLC system with a quaternary pump (Agilent G1311A), a vacuum degasser (Agilent G1322A), and an autosampler (Agilent G1313A). The HPLC was directly connected to a triple quadrupole mass spectrometer (Finnigan TSQ Quantum Discovery MAX, Thermo Electron Corporation) equipped with an electrospray ionization (ESI) interface and a 10-port valve. A Hypersil GOLD aQ C18 2.1 mm x 150 mm, 3 μm column (Thermo Fisher Scientific) was used for LC separation. For analysis of creatinine, HPLC was performed using an Agilent Series 1260 HPLC system with a binary pump (Agilent G1312B), a vacuum degasser (Agilent G1322A), and an autosampler (Agilent G1367E). The HPLC was directly connected to a variable wavelength detector (Agilent G1314F). A NUCLEODUR C18 HTec 250 mm × 4.6 mm × 4.6 μm column was used for LC separation. The samples were analyzed at a wavelength of 240 nm via a variable wavelength detector.

Detection of NNAL in urine

Analysis of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in urine was based on modified methods previously described by Shah and colleagues (34). Urine samples were frozen and then stored at −30°C until analysis. Initially, urine samples were thawed, equilibrated to room temperature, and vortexed. The urine samples were centrifuged at 3,000 × *g* for 10 minutes at 4°C, and 3 mL of the supernatant was collected. Solid-phase extraction with mixed cation exchange (MCX) cartridges (Waters) was used to prepare each sample before LC/MS-MS analysis. Each cartridge was conditioned with 2 mL of methanol, followed by 2 mL of water. The total NNAL volume, a volume of 1-mL aliquots of urine or water blanks, was added to 15-mL centrifuge tubes. Five milliliters of phosphate buffer solution (pH = 7.4) and 1,000 units of β-glucuronidase (G-0876; Sigma Chemical Co.) were added to each sample. The tubes were incubated in a shaking water bath at 37°C overnight. Spiked [13C6] NNAL (0.39 nmol) was added to each sample. The free NNAL volume, a volume of 2-mL aliquots of urine or water blanks, was added to centrifuge tubes and spiked [13C6] NNAL (0.13 nmol) was added to each sample. This mixture was loaded onto the prepared MCX cartridges. Each cartridge was washed with 1 mL of 1 mol/L HCl, followed by 1 mL of methanol and 1 mL of methanol with 25% ammonium hydroxide in water (5/5/90, v/v/v). Each cartridge was eluted with 3 mL of 25% ammonium hydroxide in methanol (5/95, v/v). The eluates were combined and dried with a rotary evaporator. Each sample was then reconstituted in 50 μL of the mobile phase. LC/MS-MS analysis was conducted with an Agilent 1200 series vacuum degasser, binary pump, well plate autosampler, and thermostatted column compartment and paired with an Agilent 6430 Triple Quad LC-MS operated in electrospray ionization (ESI) mode (Agilent Technologies, Inc.). All data were acquired and analyzed using Mass Hunter software, version B01.03. A Hypersil GOLD aQ C18 2.1 mm × 150 mm × 3 μm column (Thermo Fisher Scientific) was used for LC separation. The buffers used were (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. The linear LC gradient was as follows: time = 0 minutes, 0% B; time = 4 minutes, 95% B; time = 4.5 minutes, 0% B; time = 8 minutes, 0% B. Multiple reaction monitoring was used for MS analysis. The NNAL data were acquired in positive electrospray ionization (ESI) mode by monitoring the following transitions: 210/93.1 with a collision energy of 17 V. The [13C6] NNAL data were acquired in ESI mode by monitoring the following transitions: 216/98.1 with a collision energy of 21 V. The jet stream ESI interface had a gas temperature of 350°C, gas flow rate of 10 L/minute, nebulizer pressure of 30 psi, and capillary voltage of 4,000 V.

Statistical analyses

Descriptive statistics were presented as the mean ± SD or as the number (percentage). Shapiro–Wilk test was used to test for normality. Student *t* tests were used to determine statistical significance. Pearson correlation analysis was used to analyze the correlation between Acr-related metabolites and clinical parameters. A minimum of three independent replicate experiments of slot blot analysis or isotope dilution HPLC mass spectrometry were performed to justify the use of the statistical tests. All calculated *P* values were two-tailed. Statistical significance was defined as a $P < 0.05$. All analyses were performed with the IBM SPSS Statistics software package, version 23.0.

Cell culture and Acr treatment

Human oral squamous carcinoma cells (SAS cells), were provided by Dr. Kuo-Wei Chang at the Institute of Oral Biology, School of Dentistry, National Yang-Ming University (Taipei, Taiwan), and were authenticated by short tandem repeat analysis (35). SAS cells were grown in DMEM supplemented with 10% FBS. Acr stock solution (Sigma-Aldrich) was freshly prepared before use. Cells at 70% confluency were washed with PBS buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 8 mmol/L Na_2HPO_4, and 1.46 mmol/L KH_2PO_4, pH 7.0) and treated with Acr (50 μmol/L) in complete culture medium for 3 hours, as indicated, at 37°C in the dark.

Measurement of total glutathione (GSH) and GSH/GSSG

For total GSH levels and GSH/GSSG, a glutathione fluorometric assay kit (BioVision) was used according to the manufacturer's instructions. Briefly, Acr-treated cells (2×10^6) were lysed in 100 μL of assay buffer, and 60 μL of each homogenate was mixed with perchloric acid and then centrifuged at 13,000 × *g* for 2 minutes to pellet the insoluble materials. The supernatant was collected, neutralized with 20 μL of ice-cold 6 N KOH, and analyzed for GSH assay. Finally, the total amount of GSH was measured using a fluorescence plate reader at excitation/emission = 340/420 nm.

Results

Subjects and collection of buccal cells and urine

Baseline sociodemographic variables are shown in Tables 1 and 2. Of the 327 participants recruited, 97 were patients with OSCC and 230 were healthy subjects. All patients with OSCC have been clinically followed for 2–3 years. The demographic data of

Table 1. Baseline sociodemographic variables of patients with OSCC (*n* = 97)

Characteristics	
Age, y	
Mean ± SD	59.5 ± 11.0
Range	29–89
Sex [*n* (%)]	
Male	82 (84.5)
Female	15 (15.5)
Site of primary tumor [*n* (%)][a]	
Lip	4 (4.35)
Tongue	22 (23.9)
Buccal mucosa	27 (29.3)
Gingiva	23 (25.0)
Hard palate	6 (6.52)
Retromolar trigone	4 (4.35)
Oropharynx	5 (5.43)
Hypopharynx	1 (1.09)
Clinical stage [*n* (%)][a]	
Stage I	18 (19.1)
Stage II	16 (17.0)
Stage III	6 (6.38)
Stage IV	54 (57.4)
Cigarette smoker [*n* (%)][b]	75 (77.3)
Alcohol drinker [*n* (%)][b]	40 (41.2)
Betel quid chewer [*n* (%)][b]	74 (76.3)

[a]Diagnosis case number for site of primary tumor is 92, and the case number of clinical staging is 94.

[b]Study participants were asked if they had ever smoked cigarettes, chewed BQ, and had alcohol on a regular basis (at least once a week). Those who responded "yes" to these questions were classified as tobacco, BQ, and alcohol users.

Table 2. Baseline sociodemographic variables of healthy subjects with CS or BQ chewing histories

	CS		CS + BQ		BQ	
N	**111**	***P*[a]**	**107**	***P*[a]**	**12**	***P*[a]**
Male (%)	88.3	—	88.8	0.47	83.3	0.31
(M/F)	99/12		95/12		10/2	
Age, *y*	41.5	—	42.7	0.23	44.8	0.22
Range	19–70		21–64		28–67	
Smoking (%)	100		100		—	
(±)	111/0		107/0		0/12	
Smoking years	23.1	—	27.1	<0.01	—	
Range	1–53		5–50			
Drinking alcohol (%)	29.7		70.1		58.3	
(±)	33/78		75/32		7/5	
Chewing betel quid (%)	—		100		100	
(±)	—		107/0		12/0	
Chewing betel days	—		34.9		23.4	

[a]The $P < 0.05$ versus smoking (CS) by Student *t* test.

the patients are shown in Table 1. Advanced stage III and IV cancer was diagnosed in 63.8% (60/94) of patients. The most common primary tumor site was the buccal mucosa. A total of 77.3% (75/97) of the patients smoked, 41.2% (40/97) were users of alcohol and 76.3% (74/97) chewed BQ. The healthy subjects, including smokers without a BQ chewing history ($N = 107$), smokers with a BQ chewing history ($N = 111$), and nonsmokers with a BQ chewing history ($N = 12$), were matched for age and gender for the buccal cell and urine sample studies (Table 2). The number of years as a smoker with a BQ chewing history (27.1 years) was significantly greater than that of smokers without a BQ chewing history (23.1 years; $P < 0.01$).

Increased Acr-dG adducts of buccal DNA in patients with OSCC compared with healthy subjects with CS and/or BQ chewing histories

We examined buccal DNA samples collected from healthy subjects and OSCC subjects by slot blot analysis using anti-Acr-dG antibodies to analyze Acr-induced DNA adducts [α- and γ-hydroxy-1,N^2-cyclic propano-2′-deoxyguanosine (α-OH-Acr-dG and γ-OH-Acr-dG) adducts] that have been described previously (refs. 30, 31; Supplementary Fig. S2; Fig. 1A–C). We found that the Acr-dG levels in the buccal DNA of patients with OSCC were 1.4-fold higher than those of healthy subjects with CS or BQ chewing histories ($P < 0.001$; Fig. 1D; Table 3); however, there was no significant difference in healthy subjects with different CS or BQ chewing histories (Supplementary Fig. S3). To analyze the CS exposure spectrum between OSCC and healthy subjects, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a urinary biomarker for assessing tobacco exposure, was used (refs. 36–38; Supplementary Fig. S4). We found that Acr-dG levels in buccal DNA of patients with OSCC with CS and BQ chewing histories were 1.3 higher than those of healthy subjects with CS and BQ chewing histories ($P < 0.05$; Fig. 1E), while there was no significant difference in NNAL/Cre between OSCC and healthy subjects (Supplementary Table S1). These results partially excluded the possibility of different CS exposure spectra between healthy subjects and patients with OSCC. Interestingly, we further found that Acr-dG levels in tumor tissue DNA were 1.8-fold higher than those from buccal DNA in the same group of patients with OSCC ($P < 0.01$; Supplementary Fig. S5A and S5B). Overall, we conclude that the levels of Acr-dG adducts in buccal DNA or tumor DNA of patients with OSCC were higher than those in healthy subjects, suggesting a higher degree of Acr-induced DNA damage in patients with OSCC compared with normal subjects.

Increased urinary 3-HPMA/Cre in healthy subjects with CS and BQ chewing histories compared with healthy subjects with only CS histories

We analyzed the levels of urinary 3-HPMA, the major metabolite of Acr, using isotope dilution HPLC mass spectrometry that has been previously established (33) in urine samples of patients with OSCC and healthy subjects. The results show that 3-HPMA levels in healthy subjects with CS and BQ chewing histories (8.9 ± 9.3 μmole/g creatinine) were significantly higher compared with those in healthy subjects with only CS histories (5.8 ± 4.7 μmole/g creatinine; $P < 0.01$) or only BQ chewing histories (3.6 ± 6.1 μmole/g creatinine; $P < 0.01$; Fig. 2A; Table 3). Furthermore, we found that in healthy subjects with CS and BQ chewing histories, 3-HPMA levels (9.2 ± 9.7 μmole/g creatinine) were significantly higher than those in healthy subjects with only CS histories (6.0 ± 5.0 μmole/g creatinine; $P < 0.01$) in matched smoking years (smoking years >15 years; Fig. 2B). The correlation between urinary 3-HPMA and smoking years of healthy subjects was studied. We found that an increase in urinary 3-HPMA/Cre was strongly correlated with an increase in the number of smoking years in healthy subjects with smoking histories without BQ chewing histories ($P = 0.0027$) or with a BQ chewing histories ($P = 0.0389$; Fig. 2C).

Decreased urinary 3-HPMA/Cre in patients with OSCC compared with healthy subjects

Interestingly, we found that urinary 3-HPMA levels in patients with OSCC (0.7 ± 0.7 μmole/g creatinine) were significantly lower than those in healthy subjects with CS or BQ chewing histories (7.1 ± 7.4 μmole/g creatinine; $P < 0.001$; Fig. 3A). A similar phenomenon was observed in patients with OSCC (0.6 ± 0.6 μmole/g creatinine) with CS and BQ chewing histories and healthy subjects with CS and BQ chewing histories (9.0 ± 9.5 μmole/g creatinine; $P < 0.001$; Fig. 3B), while there were no significant differences between NNAL/Cre levels in patients with OSCC and healthy subjects (Supplementary Table S1). 3-HPMA is a detoxifying metabolite of Acr when conjugated with GSH, and it has been shown that intracellular GSH levels are inversely correlated with Acr-induced cytotoxicity (39). Furthermore, we found that Acr decreased total intracellular GSH levels or GSH/GSSG levels in SAS cells (Fig. 3C). These results indicate that cellular GSH levels determine the detoxifying activity of Acr, and chronic exposure of Acr through CS may reduce cellular protection for Acr-induced DNA damage.

Discussion

The importance of the *p53* tumor suppressor gene in the process of carcinogenesis is well established, and a high incidence of *p53* mutations has been demonstrated in tobacco-related cancers (10, 40). Previous studies have shown that a high incidence (48.66%, 91/187) of *p53* mutations in OSCC exhibited mutations in the conserved exons of the *p53* gene, which is similar to reports from Western countries (16, 41). The most prevalent types of *p53* mutations found in these studies were G to A transitions and G to T transversions. Acr induces mutagenic Acr-dG adducts and mainly G to T and G to A mutations in human cells (21–27). By mapping the Acr-dG adduct distribution at the nucleotide level in

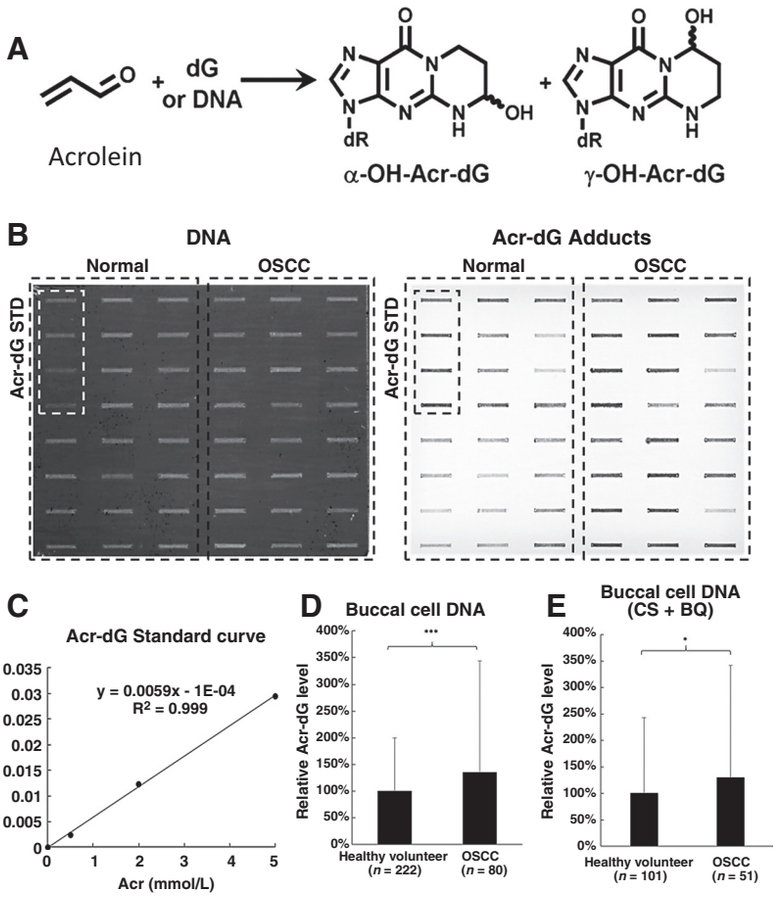

Figure 1.

Slot blot analysis of acrolein-induced DNA (Acr-dG) adducts in buccal DNA of healthy subjects and patients with OSCC. **A,** The chemical reaction of Acr with deoxyguanosine (dG) residues of DNA to form α- and γ-hydroxy-1,N^2-cyclic propano-2′-deoxyguanosine (α-OH-Acr-dG and γ-OH-Acr-dG) adducts. **B,** A typical slot blot result is shown. DNA was spotted on the membrane, hybridized with the Acr-dG antibody, then with the quantum dot–conjugated second antibody, and the binding of Acr-dG antibody was analyzed using fluorescence development. First, four bands of the first lane, human genomic DNA isolated from cultured OSCC cells (SAS), were modified with different concentrations of Acr (0–5 mmol/L) at 37°C for 24 hours as Acr-dG standards (Acr-dG STD); the second and third lanes include DNA from healthy subjects; and the fourth, fifth and sixth lanes include DNA from patients with OSCC. Left, equivalent amounts of DNA loaded in the membrane that was stained with methylene blue; right, fluorescence development and the band intensity was quantified with a UVP image analyzer. **C,** Relative Acr-dG levels were calculated by the fluorescence intensity of Acr-dG stained with an anti-Acr-dG antibody normalized to the amount of loaded DNA stained with methylene blue. The standard curve was determined by the fluorescence intensity of the relative Acr-dG DNA adduct level in genomic DNA modified with different concentrations of Acr (0, 0.5, 2, and 5 mmol/L) as Acr-dG adduct standards. Relative Acr-dG adduct levels in buccal cells of healthy subjects ($n = 222$) and OSCC patient buccal cells ($n = 80$; **D**) and relative Acr-dG adduct in healthy subjects with CS and BQ chewing histories ($n = 101$) and OSCC patients with comparable CS and BQ chewing histories ($N = 51$) as detected by the slot blot analysis described above (**E**). Bar graphs of data were collected from three independent slot blot experiments. Data were presented as the mean ± SD. Student *t* tests were used to determine statistical significance, and two-tailed *P* values are shown (*, $P < 0.05$; ***, $P < 0.001$).

Acr-treated normal human bronchial epithelial cells, we found that the Acr–DNA binding spectrum in the *p53* gene coincides with the *p53* mutational spectrum in lung cancer (28). Interestingly, there are some similar *p53* mutation hotspots and mutational spectra between CS-induced lung cancer and OSCC (Supplementary Fig. S1). On the basis of these findings, we hypothesized that Acr is a major etiologic agent in OSCC induced by CS and BQ chewing. In this study, we found that the levels Acr-dG adducts were significantly higher in patients with OSCC compared with those in healthy subjects with comparable CS and BQ chewing histories (Fig. 1D and E). These results suggest that increased Acr-dG levels are due to differences in Acr processing other than different CS exposure spectra between healthy subjects and patients with OSCC. In addition, we found substantially increased Acr-dG adduct levels in tumor tissue DNA compared with those in buccal DNA in the same population of patients with

Table 3. Urinary 3-HPMA levels in healthy subjects with CS or BQ chewing histories and patients with OSCC

	Creatinine Mean ± SD (mg/dL)	3-HPMA Mean ± SD (μmol/L)	3-HPMA/Cre Mean ± SD (μmole/g)
CS ($n = 111$)	71.9 ± 65.7	4.3 ± 6.5	5.8 ± 4.7
CS+BQ ($n = 107$)	167.4 ± 117.9	16.1 ± 23.2	8.9 ± 9.3
BQ ($n = 12$)	196.3 ± 154.8	9.0 ± 15.7	3.6 ± 6.1
Oral cancer (OSCC; $n = 97$)	96.8 ± 67.8	0.6 ± 0.7	0.7 ± 0.7

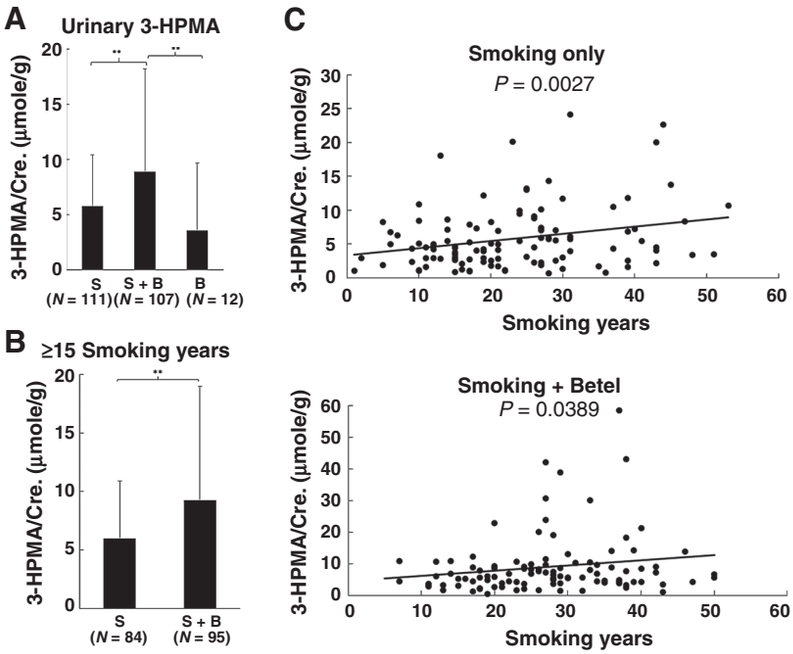

Figure 2.

Levels of 3-HPMA/Cre in the urine of healthy subjects with CS or BQ chewing histories. 3-HPMA and Cre levels of healthy subjects were measured using isotope dilution HPLC mass spectrometry, as described in the Materials and Methods section. **A,** 3-HPMA and creatinine (Cre) levels of 230 healthy subjects with CS or BQ chewing histories included smokers without BQ chewing histories (S, $n = 111$), smokers with BQ chewing histories (S+B, $n = 107$), and nonsmokers with BQ chewing histories (B, $n = 12$). **B,** 3-HPMA and Cre levels of healthy subjects with matched CS histories (>15 smoking years) included smokers without BQ chewing histories (S, $N = 84$) and smokers with BQ chewing histories (S+B, $N = 95$). Bar graphs of data were presented as the mean ± SD. Student *t* tests were used to determine statistical significance, and two-tailed *P* values are shown. **, $P < 0.01$. S indicates smokers without BQ chewing histories; S+B indicates smokers with BQ chewing histories; and B indicates nonsmokers with BQ chewing histories. **C,** The correlation between urinary 3-HPMA/Cre levels and smoking years of healthy subjects. Urinary 3-HPMA/Cre levels in 111 smokers without BQ chewing histories (top) and 107 smokers with BQ chewing histories (bottom) were evaluated by Pearson correlation analysis.

OSCC (Supplementary Fig. S5A and S5B). Furthermore, we also did sequencing analysis on exon 5–9 of *p53* gene. Results showed that in 12 of 26 samples, mutations were found on *p53* gene and the major types of *p53* mutation were G to T and G to A mutation (66.7%, 8/12 samples; Supplementary Fig. S5C). Taken together, these results indicate that the accumulation of Acr-dG adducts

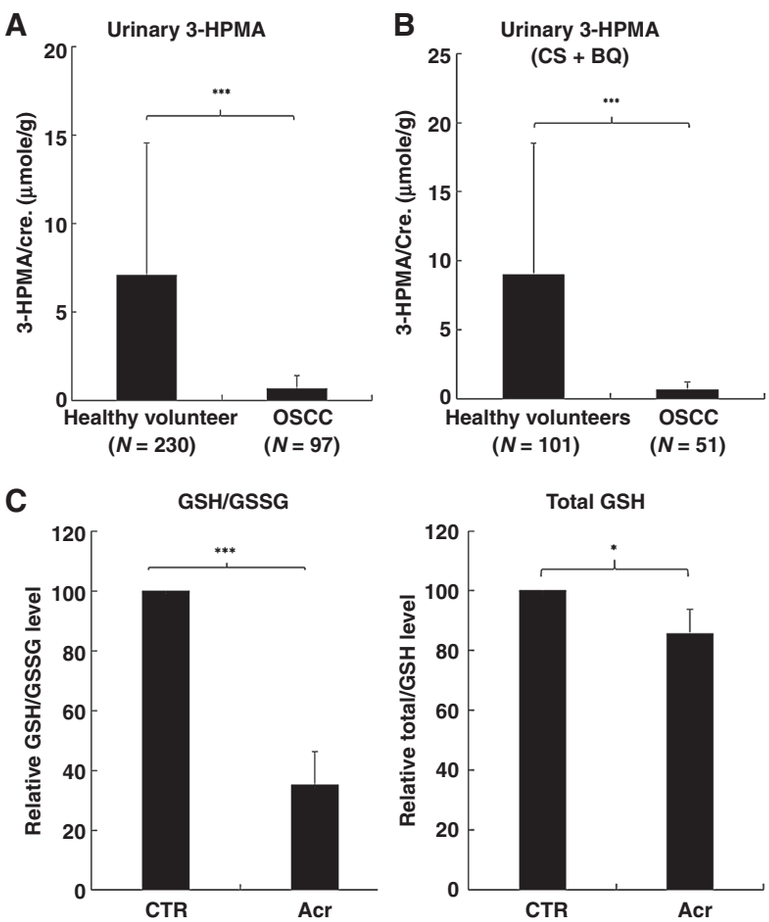

Figure 3.

Levels of 3-HPMA/Cre in the urine of healthy subjects and patients with OSCC. 3-HPMA/creatinine (Cre) levels of 230 healthy subjects with CS or BQ chewing histories and 97 patients with OSCC (**A**) and 3-HPMA/Cre levels in healthy subjects with CS and BQ chewing histories ($n = 101$) and OSCC patients with comparable CS and BQ chewing histories ($n = 51$) were measured (**B**) as described in Fig. 2. Bar graphs of data were presented as the mean ± SD. Student *t* tests were used to determine statistical significance, and two-tailed *P* values are shown. ***, $P < 0.001$. **C,** Total GSH and GSH/GSSG levels in SAS cells treated with Acr (50 μmol/L, 3 hours) were analyzed using a glutathione fluorometric assay kit (Biovision Inc.). Bar graphs of data were collected from three independent experiments. Data were presented as the mean ± SD. Student *t* tests were used to determine statistical significance, and two-tailed *P* values are shown (***, $P < 0.001$; *, $P < 0.05$).

may induce *p53* mutations and contribute to oral carcinogenesis. To the best of our knowledge, this is the first study to hypothesize Acr etiology in OSCC.

Urinary 3-HPMA, the primary metabolite of Acr found in urine (19), has been used as a biomarker for Acr exposure through cigarette smoking or cooking fumes (20). Our results showed that 3-HPMA levels were closely correlated with the number of smoking years of healthy subjects with smoking histories (Fig. 2C). However, the correlation is stronger in smokers without BQ chewing histories than smokers with BQ chewing histories. This result suggested that BQ chewing probably influences the metabolism of Acr inside the human body. In the matched smoking years (>15 smoking years), we found that 3-HPMA levels are much higher in smokers with BQ chewing histories than those in smokers without BQ chewing histories (Fig. 2B). BQ is mixed with the betel nut and its additives. Commonly mixed additives include the areca nut, unripened fruit of *piper betel linn*, betel leaf of *piper betel linn*, *piper betel linn*, slaked lime, and other spices. In 1987, the International Agency for Research on Cancer (IARC) stated that the use of BQ with tobacco is a carcinogen in humans (Group 1; ref. 42). The addition of slaked lime to the betel nut block neutralizes the astringency of a large amount of tannic acid and polyphenolic compounds in the betel nut, but it causes the oral cavity to be alkaline. Studies have shown that the pH of the oral cavity after chewing the betel nut can reach 8.2–11 (43, 44). The alkaline oral environment (pH 8–11) following the chewing of BQ-containing slake lime might facilitate the absorption of Acr (*p*Ka 9.6) during CS (45). This finding provides a possible explanation for the synergistic effects of CS and BQ chewing in oral cancer.

In addition, we found that urinary 3-HPMA levels were significantly lower in patients with OSCC than in healthy subjects with comparable CS or BQ chewing histories (Fig. 3A and B; Table 3; Supplementary Table S1). Previous studies have shown decreased GSH levels in the blood of patients with oral cancer (0.69 ± 0.11 mmol/L) compared with those in healthy subjects (2.54 ± 0.33 mmol/L; ref. 46). The reduced level of GSH in these patients may have been the cause of reduced urine 3-HPMA levels in patients with oral cancer, reflecting their reduced capacity to systemically neutralize Acr toxicity. However, GSH levels in oral tissues directly affect Acr-induced DNA damage in oral tissues. Acr has been shown to reduce intracellular GSH rapidly and further induce oxidative stress (47, 48), which is similar to our results shown in Fig. 3C. In addition, Acr has been shown to reduce DNA repair capacity (21, 22, 39). These results suggest that chronic exposure to Acr may reduce cellular detoxifying activity and repair capacity against Acr. Furthermore, oral tissue GSH levels may change due to pathologic changes. Previous studies have shown significantly increased levels of GSH in tumor tissue compared with those in tumor-free tissue of patients with oral cancer ($P < 0.001$; refs. 46, 49). These results indicate that enhanced GSH levels or antioxidant capacities in tumor tissue can make them less susceptible to oxidative stress, conferring a selective growth advantage on tumor cells. Taken together, these results suggest that increased Acr-dG levels in buccal cells of patients with OSCC, coupled with the reduced repair and detoxifying capacity of Acr-induced damage, may contribute to oral carcinogenesis.

One limitation of our study is that we could not exclude other exposure possibilities to Acr other than CS. Acr is an important pollutant that is widely distributed in the environment because it is a prominent byproduct of the incomplete combustion of organic matter and is also formed from carbohydrates, vegetable oils, animal fats, and amino acids during the heating of foods (18, 50). Therefore, humans are exposed to Acr by the inhalation of incompletely combusted organic matter and overheated cooking oil fumes or by the ingestion of many foods (51). However, smoking of tobacco products is equivalent to or exceeds the total human exposure to Acr from all other sources (52). Previous studies have suggested that a high level of oral exposure to Acr through these sources induces detrimental effects on the oral cavity, including salivary quality and contents, oral resistance to oxidative stress, and stress mechanism activation in a variety of oral cells (50). Therefore, protective pathways against Acr-induced cytotoxicity in the oral cavity need to be further investigated.

OSCC is one of the most common epithelial malignancies with significant morbidity and mortality (1–3). The key challenge to reduce the mortality and morbidity of this disease is to develop strategies to identify and detect OSCC when it is present at a very early stage, which will enable effective intervention and therapy. The detection of OSCC is currently based on expert clinical examinations and histologic analysis of suspicious areas (53, 54), but it may be undetectable in hidden sites. Therefore, sensitive and specific biomarkers for OSCC may be helpful in screening high-risk patients (9). These results provide a novel mechanism by which increased Acr uptake and impaired metabolism may contribute to OSCC carcinogenesis. Elevated Acr-dG levels in buccal swabs may serve as an early indicator to identify patients at risk.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H.-T. Wang, H.-H. Tsou, C.-J. Liu, T.-Y. Liu
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.-H. Tsou, C.-H. Hu, J.-H. Liu, C.-J. Liu, C.-H. Lee, T.-Y. Liu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.-T. Wang, H.-H. Tsou, C.-H. Hu, C.-H. Lee
Writing, review, and/or revision of the manuscript: H.-T. Wang, H.-H. Tsou, C.-J. Liu, C.-H. Lee
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-T. Wang, T.-Y. Liu
Study supervision: H.-T. Wang, T.-Y. Liu

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