

Review

Smoking and Smokeless Tobacco-Associated Human Buccal Cell Mutations and Their Association with Oral Cancer—A Review

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Abstract

Reported herein are the results of a structured literature review that was undertaken to (a) determine if human buccal (mouth) cell changes are associated with smoking and smokeless (“chewing”) tobacco, (b) tabulate different buccal cell alterations that have been reported, (c) delineate buccal cell assays that have been used successfully, (d) determine whether buccal cell changes correlate with oral cancer as defined in clinicopathologic investigations, and (e) assess the feasibility of developing a high-throughput buccal cell assay for screening smokers for the early detection of oral cancer. The results of the studies reported herein have established that diverse buccal cell changes are associated with smoking and smokeless tobacco. This review documents also that buccal cells have been collected in a noninvasive manner, and repetitively for serial studies, from different sites of the mouth (e.g., cheek, gum, and

tongue) and from normal tissue, preneoplastic lesions (leukoplakia), and malignant tumors. Tobacco-associated genetic mutations and nongenetic changes have been reported; a partial listing includes (a) micronuclei, (b) bacterial adherence, (c) genetic mutations, (d) DNA polymorphisms, (d) carcinogen-DNA adducts, and (e) chromosomal abnormalities. Clinical studies have correlated buccal cell changes with malignant tumors, and some oral oncologists have reported that the buccal cell changes are practical biomarkers. Summarily, the literature has established that buccal cells are useful not only for characterizing the molecular mechanisms underlying tobacco-associated oral cancers but also as exfoliative cells that express diverse changes that offer promise as candidate biomarkers for the early detection of oral cancer. (Cancer Epidemiol Biomarkers Prev 2006;15(6):1061–77)

Introduction: Buccal Cells and Oral Cancer

Oral cancer affects as many as 274,000 people worldwide annually, and the frequency of oral cancer around the world is often indicative of the patterns of use of tobacco products (1-4). It has been established that there is a dose-response relationship between the amount of tobacco product used and the development of oral cancer (1, 2, 5, 6).

All parts of the oral cavity are susceptible to cancer from tobacco smoking or chewing, including the lip, tongue, palate, gum, and cheek (reviewed in refs. 5, 6).

The mouth is the only body site that permits viewing with the naked eye the ravages of smoked and smokeless (“chewing”) tobacco. For a given patient, it is often possible to view in the mouth during a clinical examination normal tissue, premalignant lesions (e.g., leukoplakia), and malignant tumors (5, 6).

There exists today a need to identify biomarkers of oral cancer and their association with tobacco smoking (reviewed in refs. 7-10). When compared with other body sites, the mouth offers a unique opportunity for defining biomarkers because the mouth permits noninvasive, repetitive examina-

tions in longitudinal studies of tobacco-associated acute and chronic diseases.

Thirty percent to 80% of the malignancies of the oral cavity arise from premalignant lesions, such as leukoplakia, erythroplakia, and oral submucous fibrosis (1, 11-14).

Currently, the most common type of cancer detection is a visual inspection of the mouth. This, however, has varied results. The low-end range for sensitivity in oral examination is 58%, and specificity is only slightly better at 76% (1, 2). Moreover, there has been very little evidence that visual screening helps to reduce the incidence or mortality from oral cancer. Thus, such methods have not been recommended as screening tools by many government programs around the world (1, 2).

Different *in vivo* and *ex vivo* strategies are being pursued for improving oral cancer mortality (reviewed in refs. 15, 16). One *in vivo* tactic seeks to differentiate normal, precancerous, and cancerous epithelial cells using novel imaging instruments. During the last year, significant advances have been achieved in viewing oral tissue *in situ* with diverse strategies. Examples of the schemes that have proven useful are those that have featured technologies that use fluorescence spectroscopy (17), photodynamic imaging of tumors that have incorporated Photofrin[®] (18), and, in animal studies, multiphoton microscopy of healthy tissue versus malignant lesions as defined in studies of carcinogen-induced tumors (19).

Another approach, introduced in the 1980s (20), that has shown potential clinical utility is the application of different tissue dyes. For example, toluidine blue has been used as a mouth stain for identifying high-risk primary oral premalignant lesions (20-22). Recently, this dye has been used

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successfully in clinical studies that have correlated histologic progression with high-risk clinicopathologic molecular patterns (i.e., loss of chromosomal heterozygosity; ref. 22).

In *ex vivo* strategies, biopsies of normal and malignant tissues, or scrapings containing exfoliated buccal cells, have been explored for many years (6, 15).

The *in vivo* and *ex vivo* strategies are intended to identify the precise location of an oral cancer and thereby enhance the success of different therapies (20-22). These schemes are not without limitations and challenges. For example, screening for premalignant oral tissues by visual examinations with the naked eye, or facilitated with a scope, may be complicated by the presence of nonneoplastic tissue with inflammation or fibrosis (22). Likewise, *ex vivo* assays may identify cellular or genetic changes; however, these alterations may not correlate with oral disease and therefore cannot be used for tumor detection (9).

Study Objective and Rationale

This literature review was undertaken to (a) determine if human buccal (mouth) cell changes are associated with smoking and smokeless (chewing) tobacco, (b) tabulate different buccal cell alterations that have been reported, (c) delineate buccal cell assays that have been used successfully, (d) determine whether buccal cell changes correlate with oral cancer as defined in clinicopathologic investigations, and (e) assess the feasibility of developing a high-throughput buccal cell assay for screening smokers for the early detection of oral cancer.

As will be detailed later, some tobacco-associated buccal cell changes have been reported to be biomarkers of disease progression; other changes are candidate biomarkers that are currently being evaluated. These buccal cell changes include genetic alterations (i.e., mutations). Other changes are nongenetic changes, and these include both clastogenic (i.e., chromosome breaks; e.g., micronuclei) and nonclastogenic (e.g., DNA adducts) changes. In this review, the term mutation is used in a context to denote genetic and nongenetic changes that have been identified as being associated with the use of smoked and smokeless tobacco.

The rationale for undertaking this literature review is derived from (a) a clinical need to define biomarker assays useful for the early detection of premalignant conditions of tobacco-associated mutagenesis of mouth cells, (b) an epidemiologic perspective to use buccal cell biomarkers to identify long-term tobacco users whose habit places them at risk for oral cancer, (c) a prevention prospective to foster smoking cessation, and (d) a molecular biology perspective to link tobacco causation with the molecular events underlying malignant transformation (5-7, 9-11).

It is envisioned that assays for buccal cell changes will be used as an adjunct to clinical examinations of the oral cavity of subjects who may present with premalignant lesions or whose medical and/or smoking history identifies them as being of high risk for oral cancer. Suspect individuals would be monitored clinically; conceivably, those with a positive biomarker profile would be invited to enroll in a chemopreventive intervention modality (5, 6).

The rationale is supported also by expectation that risk markers of oral cancer of subjects with clinically normal mucosa will prove beneficial in smoking cessation counseling.

The impetus for undertaking this review is supported also by the knowledge that numerous human buccal cells can be collected readily, frequently, and in a noninvasive manner. Thus, buccal cells from the same location can be collected for correlating emerging gene alterations with histologic changes. It is also reasonably anticipated that diverse buccal cell changes can be mapped in longitudinal studies to define the molecular mechanisms underlying malignant transfor-

mation. In contrast to other body sites, the mouth is readily accessible; cells can be collected from (a) the same patient; (b) in a noninvasive manner; (c) easily and repetitively; (d) from normal tissue, preneoplastic lesions, and malignant tumors; and (e) for longitudinal studies of drug discovery and assessment.

An example of the research opportunities afforded by buccal cells is that, for a given patient, regulatory mechanisms, signaling pathways, and genetic modulation can be assessed before, during, and following antitumor therapy. Thus, buccal cells not only offer the clinician opportunities for early diagnosis and an aid for smoking cessation counseling but also provide a unique model for mutation research that permits correlating genetic alterations with histopathologic changes and for drug discovery investigations.

Methods: Literature Review and Databases

The scheme used in the literature review was to identify all reports of investigations that had been undertaken to assess buccal cell changes associated with the use of tobacco. A structured search was done of diverse subject-unique databases.

The literature searches were done using short-string Boolean-based methodologies. Different Boolean search phrases were applied. These are denoted within < > brackets. The search phrase "operators" are presented in capital letters (e.g., <buccal cells AND smoking>). Various search terms were incorporated in the searches [e.g., "buccal cells," "smoking," "tobacco," "snuff," "tumor," "lesions," "neoplastic," "leukoplakia," and word variants (e.g., "smok\$" where the dollar sign is used to identify all variants of the word)].

All searches were free of bias and were unrestricted (i.e., excluded limits); thus, the explorations included all (a) fields (e.g., author and title), (b) languages, (c) dates, (d) subjects (e.g., humans and animals), and (e) database subject subsets (e.g., cancer and tobacco).

The first literature search, done using PubMed at <http://www.nlm.nih.gov>, sought all articles published from 1966 to March 2005. This exploration identified 3,049 publications addressing <buccal cells>. The results of other searches were as follows: <buccal cells AND cancer> yielded 494 articles, <buccal cells AND tumors> yielded 475 articles, <buccal cells AND lesions> yielded 175 articles, <buccal cells AND smoking> yielded 104 articles, and <buccal cells AND tobacco> yielded 68 articles.

A search of the database for U.S. Patents for "buccal cells" identified 95 patents. Represented awards included those for "Human buccal cell collection and transport" (U.S. Patent 6,312,395), "Detection of DNA damage" (U.S. Patent 6,548,252), "Methods of newborn identification and tracking" (U.S. Patent 6,187,540), and "High-throughput screening method for genetic alterations" (U.S. Patent 5,849,483). Thus, many patents have been awarded that address buccal cells; however, no patent was identified that was subject specific to this literature review of <buccal cells AND tobacco>.

Two databases have been established for recovering the numerous documents, including original research reports, released by different tobacco companies in response to U.S. and foreign court orders. From the *Tobacco Documents Online* database, 69 articles were retrieved. From the *American Legacy Foundation*, 101 reports were obtained.

Also searched was the database of the international *Human MicroNucleus Project* and publications from participants of this *Human MicroNucleus Project* who have published their observations of cytokinesis-block micronucleus assays of human lymphocytes (reviewed in refs. 23, 24). This assay is dependent on the cells to divide and thereby differs from the buccal cell micronuclei assay. Notwithstanding, many of the guidelines

and recommendations of this *Human MicroNucleus Project* are applicable to the buccal cell assays that are addressed in this review.

Numerous citations ($n = 671$ documents) of buccal cells were identified in the Google database. A search of the Ask Jeeves database yielded 251 articles. For both of these databases, there was frequent duplication of articles that had been found previously in PubMed.

A query for <buccal cells AND smoking> in CRISP, a database of NIH-awarded grants, identified awards that had been issued recently to 22 different scientists. The NIH grant awards not only document the widespread and current interests in studies of buccal cells but also showcase the spectrum of ongoing novel investigations.

The literature searches were extended by using the "Related Articles" link to articles recovered with PubMed. Thereafter, a search was done of the literature references cited in these articles to identify additional writings that reported the results of research defining buccal cell changes associated with tobacco use.

At this juncture, all articles were assembled and arranged in reverse chronological order. The articles were read, and off-topic articles were excluded. By way of example, rejected articles included those that reported research of cells other than buccal cells (e.g., oral leukocytes). Likewise, reports of investigations of buccal cell changes that were not associated with tobacco were excluded (e.g., radiation).

Results

Buccal Cells Changes Associated with Tobacco. A total of 39 topic-specific articles were identified that addressed buccal cell changes associated with the smoking of tobacco. These articles were grouped as smoking specific and are listed in reverse chronological order (refs. 25-63; see Table 1).

To facilitate comparison, the smoking-specific articles in Table 1 are arranged according to the assay used. This classification was selected with the authors' intent of grouping same-type investigations for a meta-analysis.

A further search of the literature was done to identify articles that reported the results of research that had been undertaken to assess changes of buccal cells associated with smokeless tobacco (e.g., chewing tobacco and oral snuff). This grouping was not exclusive to tobacco and was extended to tobacco admixtures and nontobacco concoctions. The reason for this extension was justified on the basis that many chewing concoctions have been identified, or are suspect, as etiologic agents of oral cancer.

Twenty-three articles addressing buccal cell changes associated with the use of smokeless tobacco and other substances were assembled (refs. 64-86; see Table 2).

A literature search was done to identify clinical studies that have been done to correlate buccal cell mutations with premalignant lesions and tumors. Nine publications addressing clinicopathologic studies and buccal cell mutations were found (refs. 52, 87-94; see Table 3).

Collectively, the results of the literature searches identified 61 topic-specific articles (Tables 1-3), and these publications served as the basis for this review.

Buccal Cell Collection and Cytology. Tobacco-associated oral changes have been summarized by Taybos (5). The mucous-secreting epithelial lining (i.e., mucosa) is separated into two types: masticatory (keratinized) and lining (non-keratinized). The masticatory mucosa is thick, with a denser, less vascular connective tissue component. Keratin is a protective barrier against stimuli, such as traumatic forces of the everyday activities of eating foods, drinking liquids, speaking, and swallowing or noxious stimuli from ill-fitting dentures or tobacco use (5).

Stimulation of the masticatory tissue may result in increased keratin formation and the appearance of a white lesion (i.e., leukoplakia). Masticatory mucosa is found on the hard palate, dorsum of the tongue, and keratinized gingival. The lining mucosa will form very little keratin and has a less fibrous, more vascular connective tissue. Lining mucosa is found on the floor of the mouth, ventrolateral surface of the tongue, soft palate complex, labial vestibule, and buccal mucosa (5).

The most common type of oral cancer is squamous cell carcinoma, which develops from the stratified squamous epithelium that lines the mouth and pharynx (5, 6). This form of cancer accounts for ~90% of the oral malignancies (6). Tobacco use affects mainly the surface epithelium, resulting in changes in the appearance of tissues. The changes may range from an increase in pigmentation to a significant thickening of the epithelium (hyperkeratosis), resulting in leukoplakia (reviewed in ref. 5; also see ref. 6).

Buccal cells are shed spontaneously (e.g., exfoliative cells) and daily from healthy buccal mucosa. In this respect, buccal cells are similar to cells shed in the vagina and harvested by scraping (i.e., Papanicolaou smear). The exfoliative buccal cells are end-stage cells of differentiation and seldom display mitotic figures. Buccal cells can be collected from different sites of the mouth. These include the cheek, tongue, including the dorsal, lateral, and ventral surfaces, soft palate, hard palate, and oral pharynx—all of which are sites of oral cancer (5, 6, 11).

Diverse methods have been used successfully for harvesting buccal cells. These include scraping with a wood spatula (31, 34, 48, 51, 52, 60, 62, 91, 92), wood tongue depressor (12, 32, 43, 45, 56), cotton swab (42), short-bristle cytobrush (28, 29, 33, 40, 44, 57, 95, 96), and toothbrush (95, 96).

Buccal cells may also be obtained by gargling using the "swish and spit" method (60, 97). With this method, buccal cells have been harvested successfully using water (45, 95, 96) and mouth washes (e.g., Scope[®]) from different commercial vendors (25, 26, 58, 59). Studies have validated different technical variables and logistical issues for large-scale epidemiologic studies in which participants collected cells from mouth washes and mailed the sample to a laboratory for analysis (reviewed in refs. 95-97).

Samples collected by scraping consist almost exclusively of buccal cells. In some cases, erythrocytes and epithelial cells may be observed. However, the presence of these cells is indicative of a brushing or scraping procedure that was too aggressive. Samples collected using the "swish and spit" method (60, 97) contain two cell types: buccal cells and polymorphonuclear leukocytes. For most cases, approximately equal numbers of buccal cells and polymorphonuclear leukocytes were present. The viability of the polymorphonuclear leukocytes was good (>90%). Similar to the observation of buccal cells collected by scraping, buccal cells in mouth washes also display poor viability. Isolation methods have been reported to characterize cell yield, viabilities, type of cells obtained, and baseline levels of genetic damage (60).

Notable is that for all publications none of the investigators reported difficulty in obtaining a sufficient number of buccal cells for any of the assays. Likewise, in no instance was there a comment that assay results had to be excluded due to the poor quality of the buccal cells due to lysed or damaged cells, clumping, or adherent bacteria.

Morphologically, buccal cells are identified by their relatively large size, flat pancake-like shape, nongranular cytoplasm, centerline located nucleus, and large cytoplasm-to-nucleus ratio (Fig. 1).

Buccal cells can readily be identified from polymorphonuclear leukocytes and other cells of the mouth (6). For some samples scraped from the oral mucosa, stratified (keratinized) epithelial cells are observed (5). These cells most always

Table 1. Summary of studies to define human buccal cell changes associated with the smoking of tobacco

No.	First author (year) (ref.)	Primary aim (other aims) [confounders]	Smoking history			Buccal cell		Remarks
			Current	Never	Former	Assay*	Change [†]	
1	Belowska (2004) (27)	Smoking and buccal cells (larynx tumors) [air pollution]	49	0	0	Micronucleus	Yes	Patients with malignant tumors of the larynx showed a higher frequency of micronuclei in buccal cells of smokers and individuals exposed to airborne mutagenic substances.
2	Wu (2004) (28)	Smoking, buccal cells, and chewing of betel nut [age, alcohol, tea, diet, occupation, and vitamin supplements]	56	85	0	Micronucleus	Yes	Heavy smoking was positively associated with micronuclei frequency; areca quid chewing was negatively associated. A significant positive trend was shown for the relationship between micronuclei frequency and either daily cigarette consumption or cumulative smoking pack-years. All confounders studied were negative.
3	Montero (2003) (33)	Smoking and buccal cells (urban environment and pollution) [body mass and diet]	10	27	0	Micronucleus	Exc- α , [‡] Exc- β	Studied 11- to 16-year-old girls in urban environment for metabolic polymorphism. Study limitations: few smokers (n = 10), low smoking (thrice weekly; two cigarettes per occurrence). No increase in buccal cell micronuclei.
4	Konopacka (2003) (34)	Smoking and buccal cells [age and gender]	50	70	0	Micronucleus	Yes	Average buccal cell micronuclei frequency ($1.50 \pm 0.47\%$) of smokers was significantly higher than nonsmokers ($0.55 \pm 0.32\%$). Higher frequency of micronuclei was observed for cells collected from female smokers ($1.54 \pm 0.42\%$) than male smokers ($1.31 \pm 0.56\%$). Age and gender did not influence micronuclei frequency of nonsmokers.
5	Stich (1983) (56)	Smoking and buccal cells (oral cancer) [alcohol]	200	200	0	Micronucleus	No	An elevated frequency of micronuclei of buccal cells from the cheek and tongue was observed only for subjects who smoked and drank alcohol. Neither smoking alone or drinking alone was associated with an elevated frequency of micronuclei.
6	Suhas (2004) (31)	Smoking and buccal cells (oral pathology) [bedi smoking]	25	25	0	Micronucleus	Yes	People of India who smoked bedi displayed higher micronuclei frequency of cells harvested from the buccal mucosa and palate ($P \leq 0.01$) but not the tongue. The correlation between micronuclei and age was weak.
7	Piyathilake (1995) (53)	Smoking and buccal cells (vitamin deficiency) [age, gender, race, and diet]	39	60	0	Micronucleus	Yes	Buccal cell micronuclei were more frequent among cigarette smokers (mean = 20 pack-years) than nonsmokers. Micronuclei-positive cells occurred twice as often among Whites compared with Blacks. Age and gender were also important variables affecting micronuclei frequency. None of several dietary variables were associated with the presence of micronuclei in buccal cells.
8	Benner (1994) (52)	Smoking and buccal cells (clinical trials and oral leukoplakia) [alcohol]	12	0	19	Micronucleus	Yes	Higher buccal cell micronuclei frequency was recorded for buccal cells from lesions than normal-appearing mucosa. Observed a significant reduction ($P < 0.01$) in micronuclei frequency in buccal cells from both oral lesions and normal mucosa of subjects in an intervention trial evaluating α -tocopherol for chemoprevention of leukoplakia.

(Continued on the following page)

Table 1. Summary of studies to define human buccal cell changes associated with the smoking of tobacco (Cont'd)

No.	First author (year) (ref.)	Primary aim (other aims) [confounders]	Smoking history			Buccal cell		Remarks
			Current	Never	Former	Assay*	Change [†]	
9	Munoz (1987) (61)	Smoking and buccal cells (clinical trials and esophageal cancer) [alcohol]	200	0	0	Micronucleus	Exc-β	Double-blind intervention trial evaluating the effect of riboflavin, retinol, and zinc prevention trial for reducing precancerous lesions of the esophagus. No difference was observed for the frequency of micronuclei of buccal cells harvested before or after treatment. A significant reduction, however, was observed for esophageal cells in the treated vs placebo group. Study limitations: only two to three heavy smokers per smoking group.
10	Li (1999) (43)	Smoking and buccal cells (precancerous oral lesions and therapy)	46	0	0	Micronucleus	Yes	Double-blind interventional trial conducted in patients with oral mucosa leukoplakia using a mixed-tea product. Increased micronuclei frequency was reported for leukoplakia group and was attributed to smoking. Significant reduction in micronuclei frequency and lesion size observed for the treated patients.
11	Sarto (1987) (62)	Smoking and buccal cells [alcohol and age]	25	25	0	Micronucleus	Yes	Frequency of buccal cell micronuclei that resulted from chromosomal breakage was double in smokers (0.95) compared with nonsmokers (0.41); this was statistically highly significant. No difference was observed for buccal cell spindle disturbances between smokers and non-smokers.
12	Wrubel (1961) (63)	Smoking and buccal cells (oral pathology)	11	0	0	Cytology	Exc-α, Exc-γ	Evaluation of the keratinization patterns in oral mucosa of smokers did not find a decrease when subjects stopped smoking for period of a few weeks. Study limitations: only 11 smokers.
13	Gordon (2002) (37)	Smoking and buccal cells (infant death and sudden infant death syndrome) [passive smoke]	ND	ND	ND	BAC	Yes	Buccal cells from smokers bound significantly more bacteria than those from nonsmokers. Binding of <i>Staphylococcus aureus</i> correlated with tobacco smoke tar levels. Smoking is a major risk factor for sudden infant death syndrome.
14	El Ahmer (1999) (42)	Smoking, buccal cells, and bacterial pathogens (respiratory tract infections)	16	16	0	BAC	Yes	Analysis by flow cytometry showed an increased binding of all tested Gram-positive bacteria (3 species, 8 strains) and Gram-negative bacteria (4 species, 11 strains) to buccal cells from smokers compared with nonsmokers. Low amounts of cigarette smoke extract enhanced bacteria binding to the epithelial cells.
15	Piatti (1997) (50)	Smoking and buccal cells (oral infection)	18	18	0	BAC	Yes	Studies of bacteria that colonize the mucosal surface, in a highly selective manner, showed an increase binding of pneumococcal adherence to buccal cells from smokers (46.92 ± 5.12 bacteria per cell) vs nonsmokers (29.85 ± 4.26 bacteria per cell).
16	Niedermaier (1983) (55)	Smoking and cells of the mouth, nose, and trachea (binding of bacteria)	6	10	0	BAC	Exc-α	Studies using electron microscopy showed that cells from ciliated surfaces bound more bacteria than cells from squamous surfaces. Adherence of bacteria collected from different sites (i.e., trachea, nasal passage, and buccal mucosa) did not differ for smokers vs nonsmokers.
17	Broberg (2005) (25)	Smoking and buccal cells (bladder cancer)	34		122	PCR	Yes	Telomere length was significantly shorter in buccal cells from patients with cancer than in control subjects. When stratifying for smoking, a >6-fold risk was observed among current smokers.

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Table 1. Summary of studies to define human buccal cell changes associated with the smoking of tobacco (Cont'd)

No.	First author (year) (ref.)	Primary aim (other aims) [confounders]	Smoking history			Buccal cell		Remarks
			Current	Never	Former	Assay*	Change [†]	
18	Koppikar (2005) (26)	Smoking, buccal cells, and tumor cells (oral cancer and viruses) [chewing and alcohol]	25	28	0	PCR	Exc- γ	Patients with oral cancer were studied for the presence of human papillomavirus in genomic DNA extracted from their tumor tissues and the corresponding adjacent normal mucosa. Human papillomavirus was detected in 32 of 102 patients.
19	Moore (2004) (32)	Smoking and buccal cells (polymorphism and bladder cancer)	139	76	0	PCR	Yes	Buccal cells were used to define the genetic polymorphism in detoxification enzymes for 102 bladder cancer cases and 109 healthy controls. Increased risk was associated with smoking was found for almost all genotypes studied.
20	Elahi (2003) (35)	Smoking and buccal cells (polymorphisms and orolaryngeal cancer)	156	74	No	PCR	Yes	DNA isolated from buccal cells was used to study the association of polymorphisms of glucuronidating activity against metabolites of the tobacco smoke carcinogen benzo[<i>a</i>]pyrene. Only six case participants were never smokers; thus, it was not possible to determine gene-smoking interactions.
21	Spivack (2004) (30)	Smoking and buccal cells (gene-environment) [gender and diet]	19		23	PCR	Yes	In research to define gene-tobacco smoke interactions, buccal cells were proven useful for evaluating mRNA expression of nine genes encoding carcinogen- and oxidant-metabolism enzymes. Multivariate analyses showed that tobacco smoke exposure was associated with the level of expression of CYP1B1 and GSTP1.
22	Elahi (2002) (36)	Smoking and buccal cells (DNA repair and laryngeal cancer) [alcohol]	ND	ND	ND	PCR	Yes	PCR analysis of buccal cell DNA was studied for 169 orolaryngeal cancer cases and 338 matched controls. No significant risk was observed for subjects with OGG1 genotype in never smokers; increased risk was observed for the cancer subjects (odds ratio, 4.8).
23	Sweeney (2000) (41)	Smoking and buccal cells (polymorphisms and oral cancer)	517	267	No	PCR	Exc- α	Most analyses were done using pathology tissue. Study limitations: of 490 patients studied, buccal cell tests were done for only 9 subjects.
24	Morabia (2000) (40)	Smoking and buccal cells (postmenopausal women and breast cancer)	71	57	207	PCR	Yes	Gene-smoking interaction was conducted using buccal cell DNA from 170 breast cancer cases and 170 age-matched population controls. Active smoking in postmenopausal women who were slow acetylators correlated with an increase risk of breast cancer (odds ratio, 2.5).
25	Liu (1997) (47)	Smoking and buccal cells [chewing of betel leaf]	ND	ND	ND	PCR	Yes	Study showed significant methylguanine-DNA methyltransferase activity of buccal cells and illustrated the potential inhibition of methylguanine-DNA methyltransferase by the habitual tobacco (bedi) smoking and betel quid chewing.

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Table 1. Summary of studies to define human buccal cell changes associated with the smoking of tobacco (Cont'd)

No.	First author (year) (ref.)	Primary aim (other aims) [confounders]	Smoking history			Buccal cell		Remarks
			Current	Never	Former	Assay*	Change [†]	
26	Liede (1998) (45)	Smoking and buccal cells (leukoplakia) [vitamins]	343	0	ND	PCR	No	Study undertaken to define β -carotene concentrations in buccal cells in smokers who had received long-term β -carotene supplementation in a controlled trial. Observed reduced β -carotene levels in smokers. There was no correlation for smoking, β -carotene levels, and oral dysplasia.
27	Phillips (2002) (39)	Smoking and buccal cells (DNA adducts)	ND	ND	ND	DNA adduct	Yes	Review article on smoking-related DNA adducts and their association with cancer. Cited several other studies that showed higher levels of DNA adducts of buccal cells of smokers than of nonsmokers.
28	Zhang (2002) (38)	Smoking and buccal cells (malondialdehyde-DNA adducts)	25	25	0	DNA adduct	Yes	Malondialdehyde-modified DNA adduct, used as a marker of human cancer risk, found increased levels of damage in smokers versus nonsmokers.
29	Besarati (2000) (57)	Smoking and buccal cells [polycyclic aromatic hydrocarbon (PAH)-DNA adducts]	26	22	0	DNA adduct	Yes	Increased in PAH-DNA adduct formation found in smokers than in nonsmokers. Adduct number was associated with tar content and number of cigarettes consumed per day.
30	Romano (1999) (58)	Smoking and buccal cells (PAH-DNA adducts)	33	64	No	DNA adduct	Yes	Immunohistochemistry study of buccal cells showed a positive correlation between the numbers of PAH-DNA adducts and the number of cigarettes consumed per day.
31	Hsu (1997) (46)	Smoking and buccal cells (PAH-DNA and 4-aminobiphenyl adducts)	20	20	0	DNA adduct	Yes	Smokers had 2- to 3-fold higher levels of PAH-DNA and 4-aminobiphenyl adducts compared with nonsmokers.
32	Stich (1988) (54)	Smoking and buccal cells (intervention trials)	ND	ND	ND	DNA adduct	Yes	Review of author's previous reports addressing DNA adducts, micronuclei, and leukoplakias as intermediate end points in intervention trials. Micronuclei meet many of the prerequisites of a good intermediate end point.
33	Romano (1997) (59)	Smoking and buccal cells (DNA adducts)	12	12	0	DNA adduct	Yes	Immunohistochemistry showed an increased binding of stain to buccal cells of smokers. Stain intensity was related to number of DNA adducts.
34	Stone (1995) (48)	Smoking and buccal cells (DNA adducts in mucosa vs biopsy)	8	10	2	DNA adduct	Yes	Authors found that adduct levels were increased in smokers versus nonsmokers, with results being comparable between buccal mucosa and biopsy samples.
35	Zhang (1995) (49)	Smoking and buccal cells (PAH-DNA adducts)	16	16	0	DNA adduct	Yes	Observed an increase in PAH-DNA adducts in smokers compared with nonsmokers. Variation among smokers was in the 3-fold range, illustrating individual differences in adduct formation.
36	Oßwald (2003) (60)	Smoking and buccal cells (chromosomal damage)	14	9	0	Comet	No	Evaluated the yield and viability of buccal epithelial cells and leukocytes. Comet assay failed to detect a change in buccal cells of smokers.
37	Rojas (1996) (51)	Smoking and buccal cells (head/neck cancers)	10	9	0	Comet	Yes	Used single buccal cell gel electrophoresis test (Comet assay) for detection of DNA strand breaks. The extent of DNA damage was found to be significantly greater in smokers than nonsmokers.

(Continued on the following page)

Table 1. Summary of studies to define human buccal cell changes associated with the smoking of tobacco (Cont'd)

No.	First author (year) (ref.)	Primary aim (other aims) [confounders]	Smoking history			Buccal cell		Remarks
			Current	Never	Former	Assay*	Change [†]	
38	Barrera (1998) (44)	Smoking and buccal cells (head/neck cancers)	30	30	0	FISH	Yes	FISH assay results documented higher chromosomal instability in smokers than in nonsmokers.
39	Orellana-Bustos (2004) (29)	Smoking and buccal cells (pathology and oral disease)	30	30	No	AgNOR	Yes	When compared with nonsmokers, smokers with clinically normal buccal mucosa showed significant cytologic changes, including increased keratinization and higher nuclear (AgNOR) activity that is associated with acrocentric chromosomes.

*Assays for measuring buccal cell changes: BAC, bacteria adherent to buccal cells; DNA adduct, binding of defined carcinogen to DNA; Comet assay, migration of buccal cell homogenate in an electric field; FISH, fluorescence *in situ* hybridization; AgNOR.

[†]Statistically significant association identified between buccal cell changes and tobacco smoking: positive (yes) or negative (no) association.

[‡]Exc, report was excluded from analysis due to small number of subjects (Exc- α), low tobacco exposure (Exc- β), or no assessment presented by investigators (Exc- γ).

present as small tissue fragments consisting of a dozen or fewer cells.⁴

Buccal Cells and Micronuclei—Biological and Genetic Significance. Chromosomal instability is a common feature of human tumors. A review of the causes of chromosomal instability in oral cancer cells and the clinical relevance of factors associated with chromosomal instability as they relate to tumor prognosis and therapy has been published (ref. 98; see also ref. 99). There are several sources of chromosomal instability; the primary causes seem to be defects in chromosome segregation, telomere stability, cell cycle checkpoint regulation, and repair of DNA damage (98).

Squamous cell carcinoma of the head and neck and its subset, oral squamous cell carcinomas, arise through an accumulation of genetic alterations. Oral squamous cell carcinomas are characterized by complex karyotypes that involve many chromosomal deletions, translocations, and structural abnormalities. Cells of this type of tumor often have errors in chromosome segregation that lead to the formation of a lagging chromosome or chromosome parts that become lost during the anaphase stage of cell separation and are excluded from the reforming nuclei (98-100). The laggards are observed in the cytoplasm as micronuclei. Diverse technologies have been used successfully to investigate the mechanisms underlying specific structural defects in the spindle and chromosome segregation machinery (100). Particularly notable is that cigarette smoke has been shown to induce anaphase bridges and genomic imbalances in cultured normal human fibroblasts, which in a short time lead to genomic imbalances (101).

The biological significance of the micronuclei in buccal cells of the oral mucosa is that the micronuclei are a manifestation of a readily identifiable clastogenic event that, as reviewed herein, has been associated with smoke and smokeless tobacco. The clinical significance of micronuclei in buccal cells, as will be described in detail below, is that the appearance of the micronucleated buccal cells is a useful biomarker that correlates with oral cancer.

Buccal Cell Assay. The assay used most frequently for tobacco-associated buccal cell changes was the micronucleus assay. The buccal cell micronucleus assay was introduced >45 years ago (102). Descriptions of the prescribed procedure for assaying buccal cells have been published (reviewed in refs. 13, 60, 62, 103-106; see also ref. 107).

Operationally, the instructions for the buccal cell micronucleus assay is as follows: (a) collect buccal cells by gently scraping the cheek with a wood spatula; (b) transfer the cells from the spatula directly onto a microscope slide; (c) fix the cells, stain with Feulgen, and apply fast green as a counterstain; and (d) enumerate 3,000 cells microscopically for micronuclei using an established morphologic criteria and with attention to defined safeguards (62, 106).

The micronucleus assay, which can be done using cells of human beings and animals, has been proven useful using diverse cell types (e.g., blood lymphocytes and urinary bladder epithelial cells) for measuring the clastogenic effect (i.e., induced chromosome breaks) (reviewed in refs. 13, 23, 24).

Toxins that have been associated with micronucleus are diverse and include airborne pollutants as well as compounds present in various occupational settings. Thus, the micronuclei assay is used widely to test chemicals for the ability to induce numerical chromosomal damage. Tobacco smoke has been associated with the formation of micronuclei in diverse types of human and animal cells. Particularly noteworthy is that micronuclei meet many of the prerequisites of a good intermediate end point in clinical intervention trials (54). The predictive value of micronuclei has been shown using cultured cells exposed to carcinogens and chemopreventive agents and in experiments using human oral mucosa (55).

Buccal Cell Changes and Tobacco Smoking: Results of Literature Review. A total of 39 articles were identified that addressed work that has been done to determine if buccal cell changes were associated with the smoking of tobacco (Table 1). For all articles, the smoking of tobacco addressed cigarette smoking; there were no articles that addressed the smoking of tobacco in cigars or pipes. The smoking of marijuana and hashish was not tobacco specific to the goal of this literature search; accordingly, this topic will not be discussed.

An analysis of these documents revealed that different tests were used. These included studies in which buccal cell changes associated with tobacco smoking were defined using (a) micronuclei assay (Table 1, listings 1-11); (b) changes in cell morphology (e.g., cytology; listing 12); (c) adherence of bacteria (listings 13-16); (d) genomic DNA by amplification using PCR (listings 17-26), DNA adduct (listings 27-35), chromosomal abnormalities as measured using the Comet assay (listings 36 and 37), and fluorescence *in situ* hybridization assay (listing 38); and (e) ribosomal genes of the nucleolar organizer regions as defined with the argyrophilic nucleolar organizer region assay (listing 39).

Of the 39 articles, 33 were topic specific and were suitable for scoring. Six publications were excluded from evaluation. The criterion for excluding an article included (a) small size of

⁴Personal observation, Pauly and co-workers.

Table 2. Summary of studies to define human buccal cell changes associated with smokeless tobacco and similar-use substances

No.	First author (year) (ref.)	Tobacco used or chewing behavior	Buccal cell change* [†]	Remarks
1	Goral (1999) (64)	Moist snuff Chewing tobacco Dip tobacco	Yes Yes Yes	Significant buccal cell alterations were recorded in a study of buccal cells of smokeless tobacco users.
2	Ramaesh (1999) (65)	Smoked tobacco Betel chewers with tobacco Smoke and chew Nontobacco users	Yes Yes Yes No	The effect of tobacco use on buccal mucosa was assessed by cytomorphometry of buccal cells. A significant increase was discovered for cells in all habit groups. There was a significant reduction in the cell diameter of the chewing group and the combined habit group compared with the control nonuser group.
3	Ozkul (1997) (66)	Chewed maras powder Smokers, no maras Nonsmokers, no maras	Yes Yes No	There was no difference in the micronuclei of buccal cells of subjects who smoked or chewed smokeless tobacco (maras powder). Buccal cells of both groups showed a higher frequency of micronuclei than nonusers (nonsmokers, nonchewers).
4	Roberts (1997) (67)	Snuff	Yes	Comparative cytology of the oral cavity was conducted to define the effects of snuff users. The regular use of snuff caused an increase in the incidence of buccal cell micronuclei, loss of cell cohesion, and hyperkeratosis.
5	Desai (1996) (68)	Betel nut Pan masala Mava	Yes Yes Yes	The exfoliated oral mucosal cells had significantly higher numbers of micronuclei compared with those of healthy normal subjects having no chewing or smoking habit.
6	Ghose (1995) (69)	Tobacco smokers with Pika habit Tobacco chewers with Dungia habit	Yes Yes	In India, tobacco smokers with Pika habit showed higher frequency of micronuclei of buccal cells than tobacco chewers with Dungia habit.
7	Kayal (1993) (70)	Areca nut Mava Tamol Tobacco with lime Dry snuff Masheri Tobacco	Yes Yes Yes Yes Yes Yes Yes	All groups studied (see listing) had a significantly higher frequency of micronuclei of buccal cells than nonusers from different parts of India. Patients with oral leukoplakia had a high incidence of micronucleated cells in spite of the fact they had stopped chewing months before collection of the oral mucosa cells.
8	Stich (1992) (71)	Reverse cigar smokers	Yes	An elevated frequency of micronuclei of buccal cells was documented for reverse cigar smokers and Khaini-tobacco chewers; no increase was observed for gudakhu users.
9	Das (1992) (72)	Khaini-tobacco chewers Gudakhu users Gudakhu	Yes No Yes	The incidence of micronuclei was increased in users, and the increased incidence was correlated with the period of use. There were no significant differences between men and women.
10	Dave (1992) (73)	Areca nut	Yes	A clinical study in India showed statistically significant increases in the frequencies of micronuclei among of chewers compared with controls.
11	Adhvaryu (1991) (74)	Mava	Yes	When compared with healthy controls, a clinical study showed a significant increase in micronuclei of buccal cells for all three groups studied (chewers free of oral disease, chewers with oral submucous fibrosis, and chewers with oral cancer).
12	Dave (1991) (75)	Pan masala Pan masala with tobacco	Yes Yes	Buccal cells collected from the site where the pan masala was placed showed a statistically significant ($P < 0.001$) increase in micronuclei of buccal cells compared with nonconsuming controls.
13	Nair (1991) (76)	Betel quid with tobacco Tobacco with lime	Yes Yes	Significantly elevated micronuclei frequencies of buccal cells were observed in the exposed groups compared with the control group. No correlation was seen among age, duration, and frequency of habits.
14	Tolbert (1991) (77)	Snuff	Yes	A study was conducted of subjects who use snuff at levels known to be associated with a significant increase in cancer risk. The prevalence of micronuclei was elevated in the snuff users compared with the nonusers (prevalence ratio = 2.4) and, to a lesser extent, at the contact site compared with a distal buccal site in the snuff users (prevalence ratios = 2-13).
15	Livingston (1990) (78)	Smokeless tobacco	Yes	Cytologic and cytogenetic studies were done to assess the prevalence of somatic cell genetic damage in 48 young adults (college students) equally divided into users and nonusers of smokeless tobacco. The frequency of micronuclei of buccal cells was significantly higher in users than nonusers ($P = <0.01$).
16	Stich (1988) (79)	Betel quid with tobacco	Yes	Remission of oral leukoplakias and micronuclei in tobacco/betel quid chewers treated with β -carotene and with β -carotene plus vitamin A. Remission and inhibition of new oral leukoplakias and reduction of micronuclei of mucosal cells occurred in the groups receiving β -carotene and β -carotene plus vitamin A during the continuous presence of carcinogens derived from tobacco and areca nut.
17	Stich (1985a) (80)	Snuff	Yes	A pilot β -carotene intervention trial with inuits using smokeless tobacco. β -Carotene seemed to be an efficient inhibitor of micronuclei formation in buccal cells of snuff users who do not suffer from any vitamin A deficiency and who had normal levels of retinol.

(Continued on the following page)

Table 2. Summary of studies to define human buccal cell changes associated with smokeless tobacco and similar-use substances (Cont'd)

No.	First author (year) (ref.)	Tobacco used or chewing behavior	Buccal cell change* [†]	Remarks
18	Stich (1985b) (81)	Areca nut	Yes	Defined the frequency of micronuclei of buccal cells from areca nut and/or tobacco users. When compared with subjects who used areca nut only, all others (see listing to left) showed a significant increase in micronuclei frequency. Authors review critical buccal cell assay variables and statistical methodologies to be used in future large-scale screening programs.
		Areca nut + lime + betel leaf	Yes	
		Areca nut + lime + betel leaf + tobacco	Yes	
		Tobacco + lime + ash + oil	Yes	
19	Stich (1984a) (82)	Tobacco + lime	Yes	The frequency of exfoliated cells with micronuclei in buccal mucosa swabs was used to estimate the protective effect of vitamin A, β -carotene, and canthaxanthin on the buccal mucosa of betel (areca) nut/tobacco chewers. The betel (areca) nut/tobacco chewers served as their own controls. Following a 9-week ingestion of vitamin A and β -carotene the frequency of micronuclei-positive cells decreased significantly.
		Snuff	Yes	
		Betel (areca) nut/ tobacco chewers	Yes	
20	Stich (1984b) (83)	Betel nut and tobacco	Yes	Reduction with vitamin A and β -carotene administration of proportion of micronuclei in Asian betel nut and tobacco chewers.
21	Stich (1983) (84)	Betel nut ("pan")	Yes	The frequency of micronuclei of buccal cells was significantly elevated in all examined betel quid chewers. The betel nut/tobacco mixture of a "pan" seems to be more genotoxic than the chewing of raw betel nut alone. Khaini tobacco only produced less micronuclei in cells than the betel nut with or without tobacco.
		Betel nut and tobacco	Yes	
		Khaini tobacco only	Yes	
22	Stich (1982a) (85)	Betel quid	Yes	Elevated frequency of micronuclei in buccal cells of individuals at high risk for oral cancer-betel quid chewers. The micronuclei frequency was lower among individuals chewing a raw betel nut, betel leaf, and lime mixture compared with those using quids with tobacco, betel nut, lime, or betel leaf.
23	Stich (1982b) (86)	Betel quid	Yes	The frequency of micronuclei of buccal cells in all three groups of chewers compared with a group of nonchewing subjects.
		Khaini (tobacco and lime)	Yes	
		Raw betel nut	Yes	

*For all reports, buccal cell changes were evaluated using the micronuclei assay.

[†]Statistically significant association identified between buccal cell changes and chewing; positive (yes) or negative (no) association.

the study population (<10 smokers), (b) low smoke exposure (<1 pack-year), or (c) the authors did not state whether observed buccal cell changes correlated with tobacco use. Most of the excluded articles were pilot studies or preliminary reports (Table 1).

Of the 33 topic-specific articles, 30 (91%) reported a positive correlation. Three publications reported an absence of correlation.

Several reports are particularly noteworthy. For example, Wu et al. (28) reported in 2004 a positive trend between micronuclei frequency and either smoking intensity (e.g., daily cigarette consumption) or cumulative smoking (e.g., pack-years). All confounders studied were negatively associated with the frequency of micronuclei in buccal cells.

In a study of 120 healthy subjects, Konopacka (34) reported in 2003 that the frequency of micronuclei of oral epithelial cells was three times greater in smokers ($n = 50$) than nonsmokers ($n = 70$); recorded micronuclei frequency values were $1.50 \pm 0.47\%$ and $0.55 \pm 0.32\%$, respectively ($P < 0.05$). Neither age nor gender correlated with the level of micronuclei.

Sarto et al. (62) reported that the frequency of micronuclei resulting from chromosome breaks, as well as micronuclei derived from spindle disturbances, for healthy subjects. The frequency of micronuclei result was approximately twice as high in smokers compared with nonsmokers ($P < 0.01$). No statistically significant correlation was found for any of the variables examined, including alcohol consumption, gender, age, coffee, hot food, spicy food, teeth brushing, oral antiseptics, oral prosthesis, and oral infection.

In addition to the morphology-based micronuclei assay, comparative microscopic studies of buccal cells of smokers and nonsmokers were undertaken to determine whether there was an association of bacteria binding to buccal cells with tobacco use (37, 42, 50, 55). One group reported no association (55).

Three other groups, however, documented an increase in the binding of bacteria. For all studies, the adherence of bacteria to buccal cells (see Fig. 1D) was established with the use of a conventional white-light microscope (37, 42, 50).

It is recognized that bacteria bind and colonize mucosal surfaces (50). The mechanisms underlying the adherence of bacteria to epithelial cells have been reviewed in ref. 108. Bacterial adherence is thought to be the first important step in the pathogenesis of infection (50), and it is known to involve both nonspecific and receptor-mediated binding (108).

It has been shown that tar-resistant bacteria, with carcinogenic potential, are present in the buccal cavity of some individuals and that cigarette smoking simultaneously inhibits the growth of most bacteria and selects carcinogenic bacteria (109). These findings are consistent with the observation that persistent bacterial infections are associated with chronic inflammation (109) and that chronic inflammation is often associated with human carcinogenesis (reviewed in refs. 110-112). Further, recent molecular studies on the relationship between solid malignancies and the surrounding stroma have given new insight to suggest that chronic inflammatory process per se contributes to cancer initiation, progression, invasion, and metastasis (110-112).

In addition to the aforementioned morphology-based buccal cell assays, 19 reports that used biochemically based tests were identified. The results of these reports may be of particular importance because the assays used may be less subjective than the microscopic enumeration of cells for cytoplasmic micronuclei or adherent bacteria. The tests for assessing buccal cell changes associated with smoking included procedures that employed PCR ($n = 10$ articles; refs. 25, 26, 30, 32, 35, 36, 40, 41, 45, 47) and DNA adduct technology ($n = 9$; refs. 38, 39, 46, 48, 49, 54, 57-59; Table 1).

Table 3. Summary of studies to define buccal cell changes associated with clinicopathologic observations of patients with oral cancer

No.	First author (year) (ref.)	Clinical correlate ^{a,t}		Remarks
		Assay	Change	
1	Mager (2005) (87)	Bacteria counts	Yes	Evaluation of salivary bacteria counts of oral bacteria in individuals with cancerous lesions ($n = 229$) versus healthy controls ($n = 45$). Of 40 common oral bacteria, significantly higher counts ($P < 0.0001$) were observed for <i>Capnocytophaga gingivalis</i> , <i>Prevotella melaninogenica</i> , and <i>Streptococcus mitis</i> . When tested as diagnostic markers, the three species were found to predict 80% of cancers (sensitivity). The authors concluded that salivary counts of certain bacteria may be diagnostic indications of oral squamous cell carcinoma.
2	Maraki (2004) (90)	Cytology and DNA	Yes	A study of 98 patients with clinically suspicious oral lesions was undertaken to evaluate the diagnostic accuracy of exfoliative cytology and DNA image cytometry for the very early diagnosis of oral cancer. Brushings and scalpel biopsies were obtained from 98 patients with suspicious oral lesions. The authors concluded that cytology with DNA image cytometry is a highly sensitive, specific, and noninvasive method for the early diagnosis of oral epithelial neoplasia.
3	Cheng (2004) (89)	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling	Yes	Apoptotic cells in whole saliva were detected in four groups of study subjects using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. The apoptotic cells showed morphology similar to normal exfoliated epithelial (buccal) cells of oral mucosa. The fraction of apoptotic cells in treated malignant patients was significantly higher ($P < 0.05$) than that of healthy volunteers, premalignant patients, and untreated malignant patients.
4	Lopez (2004) (88)	<i>p53</i>	Yes	A study of 34 patients with leukoplakia was undertaken to assess the merit of using cytologic specimens for defining <i>p53</i> gene alteration in oral squamous cell carcinoma risk patients. Using an oral rinse and brush swabbing method to collect buccal cells, 11 mutations in the <i>p53</i> gene were detected in the oral cytologic specimens. The authors concluded that the noninvasive method may be useful in the follow-up of at-risk patients and introduces new possibilities to analyze molecular markers before malignant lesions are clinically apparent.
5	Ramirez (2002) (91)	Micronuclei	Yes	The micronucleus test was used as an indicator of genotoxic exposure and clastogenic action of alcohol because it is associated with chromosome aberrations and is related to the development of oral cancer. In this study of buccal cells of 30 alcoholics with carcinomas, the frequency of micronuclei was 7-fold, highly significant ($P = 0.0005$) for regions around the lesions. A 3-fold increase was observed for the opposite nonmalignant region, and no difference was found for upper gingival-labile gutter.
6	Casartelli (2000) (92)	Micronuclei	Yes	Evaluation of micronuclei of buccal cells of different areas of the patients. When compared with healthy tissue, the study documented a significant increase in micronuclei of preneoplastic lesions ($n = 47$) and for carcinomas ($n = 21$). With all samples, micronucleus frequencies were systematically higher when cells were collected by vigorous rather than light scrapings, suggesting a decreasing gradient from basal to superficial layers of mucosa. The micronuclei frequency did not vary with the sex or age of patients, although it did vary with the anatomic site of the lesions. The observed gradual increase in micronuclei counts from normal mucosa to precancerous lesions suggests a link of this biomarker with neoplastic progression.
7	van Oijen (1998) (93)	Cell proliferation	Yes	A study was undertaken to assess the number of proliferating cells in the oral epithelium (buccal mucosa) from smokers and ex-smokers. The proliferation index was assessed by an indirect assay for the proliferation biomarker <i>Ki-67</i> . The epithelium from both smoking cancer patients and health smoking individuals showed an increase in cell proliferation compared with epithelia from nonsmoking subjects ($P < 0.001$). Further, ex-smokers in both groups showed a trend toward increased cell proliferation. Increased cell proliferation after cessation of smoking would indicate permanent epithelial alterations. Ex-smokers also showed a greater proliferation index than nonsmokers, which showed that permanent changes had occurred.

(Continued on the following page)

Table 3. Summary of studies to define buccal cell changes associated with clinicopathologic observations of patients with oral cancer

No.	First author (year) (ref.)	Clinical correlate ^{*†}		Remarks
		Assay	Change	
8	Babu (1996) (94)	Cytology and cell proliferation	Yes	A prospective study examined the <i>in vivo</i> effects of pan masala/gudakhu and betel quid chewing on buccal mucosal cytology in 50 patients with oral submucous fibrosis and 40 controls. The study found a significantly higher ($P < 0.05$) percentage of nucleolated intermediate cells or a proliferative fraction of buccal mucosa cells was significantly higher in all habitual chewers than in controls.
9	Benner (1994) (52)	Micronuclei	Yes	Studied buccal cell micronuclei frequency of specimens from both visible lesions and normal-appearing mucosa ($P < 0.01$; $n = 24$ subjects).

*Buccal cell changes were evaluated using the micronuclei assay and other tests.

†Statistically significant association identified between buccal cell changes and tobacco smoking: positive (yes) or negative (no) association.

Of the 10 articles in which the investigators used PCR, 7 were identified as suitable for evaluation. Of the 7 articles, all reported identifying genetic mutations that were associated with smoking (refs. 25, 30, 32, 35, 36, 40, 47; Table 1).

Of 9 articles that evaluated buccal cells for DNA adducts, all were suitable for evaluation and all reported a positive correlation between buccal cell changes and smoking (refs. 38, 39, 46, 48, 49, 54, 57, 58, 59; Table 1).

A positive association of buccal cell mutations with tobacco smoking was shown with the use of fluorescence *in situ* hybridization (44) and argyrophilic nucleolar organizer region (29) assays. With the Comet assay, conflicting results were obtained; a positive association (51) and a negative association (60) between buccal cell mutations and smoking were reported (Table 1).

Thereafter, the buccal cell articles that had been classified based on the assay used were examined with the intent of doing a structured analysis of the data. An examination of the studies identified significant variations that precluded a meta-analysis. The study variations included research scheme, cohort characteristics, smoking histories, assay modifications, data presentation, and incomplete information of statistical analyses. Only methodologically sound studies should be included in a meta-analysis. Our attempts to craft predictive scales for assessing these variables could not be achieved. Thus, a systematic technique could not be crafted for resolving the apparent contradictions in some research findings.

Despite the inability to address all opposing findings, it is to be noted that the predominance of articles reported a positive correlation of buccal cell changes with smoking. This conclusion is in agreement with reviews published previously that have shown a positive association of human lymphocyte changes with tobacco smoking (refs. 4, 14, 23, 24, 113, 114; reviewed in refs. 4, 14, 24).

Animal Studies of Buccal Cells and Tobacco Smoking.

Animals have been studied as potential surrogates to define human health risks associated with smoking. Accordingly, a search of the literature was made to determine whether studies had been published in which an investigation has been undertaken using different animals to define tobacco-associated mutations. Several relevant articles were identified and assessed. Studies have documented a substantial increase in micronuclei in different animal species and for various tissues after exposure to tobacco smoke (reviewed in refs. 115, 116). Notable is that in studies of lung macrophages and blood lymphocytes a significant increase in micronuclei frequencies was observed for exposed animals compared with nonexposed animals (refs. 115-119; see also ref. 24). These reports are significant in that tobacco-associated mutations were observed in the absence of confounders that

often complicate human studies. No articles were found, however, that addressed changes in buccal cells of animals exposed to tobacco smoke.

Buccal Cell Mutations and Smokeless Tobacco. The adverse effects on the buccal mucosa of smokeless tobacco, betel quid, and diverse admixtures have been reported (64-86). The results of these studies were remarkable—all 23 articles reported a positive correlation of buccal cell changes with chewing (Table 2).

The most significant changes in buccal cells and oral tumor formation were associated with the chewing of betel quid with or without tobacco (65, 68, 76, 79, 82-86). The association of chewing on oral submucous fibrosis and oral mucosal lesions and related clinical findings has been documented (reviewed in refs. 120-122).

Animal Studies of Buccal Cells and Smokeless Tobacco.

Observations similar to those of human beings (see above) have been reported in studies of animals (118, 119). Chen (118) reported that 25% of the buccal epithelial cells of the tobacco-treated rats were tetraploid and 5% octaploid compared with only 11% tetraploid and no octaploid in control rats. Chen concluded that the effects of smokeless tobacco markedly affected cell mitosis and that the response of the rat buccal mucosa was similar to those observed for humans (118). In studies of the hamster cheek pouch, Stich et al. showed that snuff enhanced significantly herpes simplex virus-associated development of microinvasive squamous cell carcinomas in the cheek pouch epithelium (119).

Buccal Cell Changes and Clinicopathologic Observations.

Nine investigative teams have reported the results of buccal cell changes in clinical studies of patients with premalignant lesions or tumors (refs. 52, 87-94; Table 3). In these studies, different buccal cell assays were used (bacterial counts, DNA image cytometry, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, *p53*, micronuclei, and cell growth). Also notable was that for several reports comparative studies were made of buccal cells harvested from different regions of the oral cavity (e.g., normal tissue, precancerous lesions, and malignant tumors). For all nine studies, a positive association was observed between buccal cell changes and oral disease (Table 3).

Concluding Comments and Future Opportunities

In this literature review, we have identified shortcomings that we believe should be acknowledged and that may provide guidance in undertaking future investigations. Of these shortcomings, two issues were identified as being particularly important.

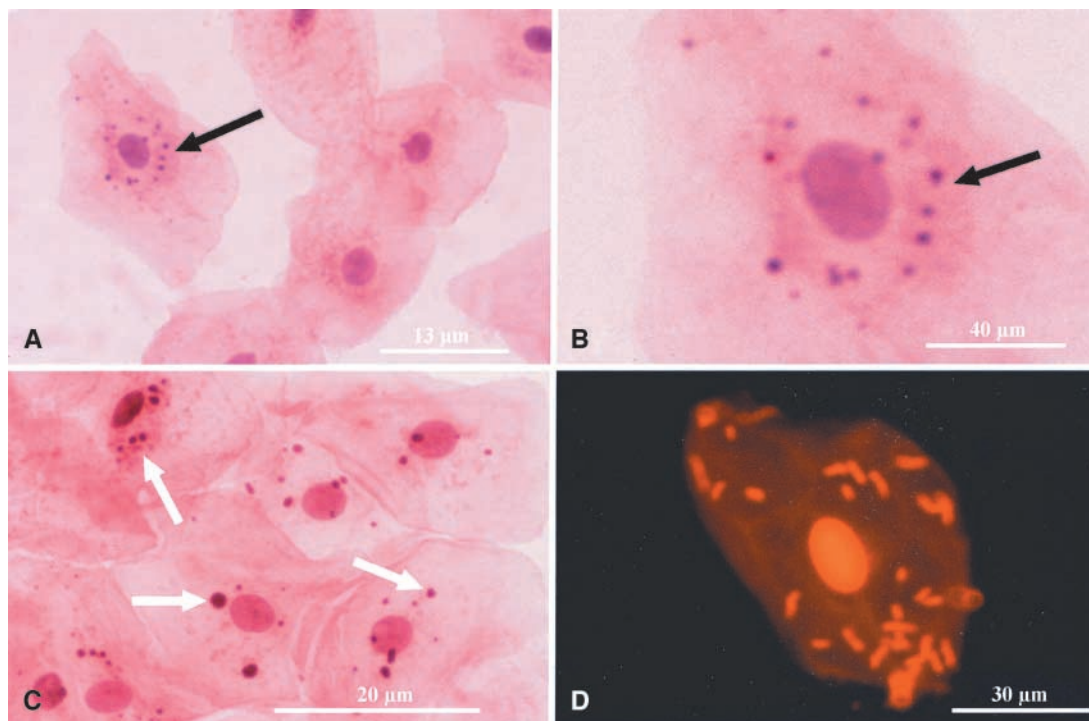


Figure 1. Microscopic views of human buccal cells. The cells were collected by scraping the cheek of the mouth lightly with a toothbrush. Touch imprints were made by stroking, up and down, the bristles of the toothbrush lightly onto a glass microscope slide and in a manner as to make five serial tracks. The deposited buccal cells were air-dried. **A.** Low-power view with a white-light microscope of buccal cells of a 76-year-old male who smoked nonfilter cigarettes for 60 years. One cell has ~20 micronuclei (*arrow*) in the cytoplasm. **B.** Enhanced view of the buccal cell in **A**. In this buccal cell, the micronuclei are uniform in size and morphology and surround the prominent nucleus. **C.** Buccal cells from a 48-year-old female who smoked for 30 years. In contrast to the micronuclei in **A** and **B**, the cells in **C** illustrate micronuclei (*arrows*) that vary in size, morphology, and spatial distribution in the cytoplasm. The cells in **A** to **C** were stained with H&E. **D.** View with a fluorescent microscope of a buccal cell that has ~30 adherent bacteria (propidium iodide stain).

Different assays have been used successfully for documenting tobacco-associated chromosomal and genetic changes in buccal cells. The assay used most frequently was the buccal cell micronuclei assay (Table 1). The popularity of this assay has prompted the authors to review this assay as applied to the study of buccal cells. Concern is expressed in that many investigators have modified the recommended procedure in a manner that they perceive as beneficial. A partial listing of these changes includes (*a*) prerinsing the mouth with water or other fluids, (*b*) selecting different buccal cell collection sites, (*c*) introducing diverse buccal cell collection tools and cell harvesting procedures, (*d*) using buccal cell washing procedures that often include repetitive centrifugation, (*e*) applying diverse stains and microscopy viewing methods, and (*f*) reducing the number of cells to be examined.

Today, buccal cell micronuclei assays are being used that differ significantly among different laboratories. The observed variance from prescribed methods discloses that a standardized protocol has not been universally accepted. Thus, there exists a need to review the micronuclei assay protocols that are being used. Some of the assay changes introduced by different investigators have been reported to be advantageous. Notwithstanding, alterations in the assay procedures are likely to yield different results. This has precluded our efforts to undertake a meta-analysis of data from different laboratories. Conceivably, the diversity of the assay procedures noted by the reviewers may be responsible for the discordance noted among articles addressing the same question. There exists a need to review the buccal cell micronuclei assay and then reissue a standardized protocol.

Further, the examination and scoring of 5,000 cells per sample is labor intensive; it would not be unexpected to find that the counting procedure is prone to error. The frequency of micronuclei in buccal cells of healthy subjects is low (<0.5%); thus, a few misread false-positive cells would be statistically significant.

Some investigations failed to incorporate quality controls; a partial listing of those that have been recommended include (*a*) using coded slides, (*b*) evaluating the variance of intra-assay and interassay readers, (*c*) doing assay reproducibility from day-to-day, and (*d*) establishing baseline micronuclei frequency values in longitudinal studies of healthy persons (13, 60, 103, 104, 106).

Some technical shortcomings can be rectified. By way of example, one impediment is the laborious and tedious enumeration of 3,000 buccal cells per sample. Others have recognized this problem and have been successful in capturing the advantages afforded by flow cytometry for determining micronuclei present in other types of cells (106, 123, 124). In evidence of this is that human peripheral blood reticulocytes with micronuclei have been evaluated using a conventional cytometer (124, 125). For these cells and this technology, the investigators found a significant increase in micronuclei frequency in smokers compared with nonsmokers. Smokers who smoked >20 cigarettes had a trend of a proportional increase in micronuclei frequency (124).

When compared with microscopic examinations, state-of-the-art multispectral flow cytometry is expedient, permitting the analysis of >10,000 cells per minute for various attributes (e.g., membrane antigens and DNA synthesis cytoplasmic inclusion bodies; reviewed in ref. 126).

In addition, cytometry is cost-effective and objective and offers a database that is readily amenable for statistical analysis. We have shown recently the feasibility of using cytometry for assessing buccal cells and have documented that the cells display a high level of autofluorescence.⁵

Important are technological achievements that have produced cytometers that combine real-time, high-throughput, multispectrum flow cytometry with digital morphology imaging on a cell-by-cell basis (127). The utility of this technology has been shown recently in studies that have profiled and distinguished live, necrotic, and early and late apoptotic cells using a combination of photometric and morphometric features (127).

There remains, therefore, a need to establish a standardized buccal cell assay that is rapid, inexpensive, quantitative, reproducible, technologically simple, and applicable for monitoring longitudinal studies with relatively large cohorts of subjects who have been identified as being at high risk for developing oral cancer.

Secondly, the authors have noticed that many investigators made little or no attempt to quantify tobacco smoke exposure (i.e., dose). Mainstream tobacco smoke and environmental (e.g., secondhand or passive) tobacco smoke are classified by the U.S. National Toxicology Program as a class I "known human carcinogen" (128). Efforts should be made to measure tobacco smoke with the same rigor used for measuring other human carcinogens. The authors make this recommendation for all tobacco smoke-related research regardless of the study objectives or assays used. At a minimum, tobacco smoke exposure should be identified with respect to both long-term exposure (i.e., cigarette pack-years) and current intensity (i.e., cigarettes per day; reviewed in ref. 129).

In none of the reports cited was tobacco smoking behavior assessed biochemically. This is of concern because self-reported cigarette consumption is an insufficient measure of tobacco exposure (129-131). This discrepancy has been identified for teen smokers (129) as well as for men, ex-smokers, and patients without malignant respiratory disease (131).

Cotinine is a metabolite of nicotine that is present in different body fluids (e.g., saliva, plasma, and urine), and cotinine is a useful biomarker of tobacco smoke exposure (reviewed in ref. 132). The cotinine assay has been proven to be a sensitive and reproducible method for assessing smoking of both adults and smoke exposure to nonsmokers and children (129, 130). For many reports, study subjects were designated as current smokers or nonsmokers. Concern is expressed in that designation of "nonsmokers" failed to distinguish never smokers and former smokers. Individuals should be classified as a never smoker, former smoker, or current smoker. Current smokers should be classified as (a) minimal (<15 cigarettes per day), (b) moderate (15-25 cigarettes per day), and (c) heavy (>25 cigarettes per day) smokers.

Not all cigarettes are alike. For most reports reviewed, the cigarettes used were not defined. No articles were identified in this review in which cigarette usage was defined by nationally defined categories (133-136). In future studies, it is recommended that smokers are distinguished by the Federal Trade Commission classification (e.g., regular, >14.5 mg tar; light, 6.5-14.5 mg; ultralight, ≤6.5 mg tar; ref. 136); see also ref. 129.

Other cigarette features that may play a role in assessing buccal cell mutations may include cigarette filtration (e.g., nonfilter versus filter), filter modification (e.g., charcoal filter), flavor (e.g., menthol), tobacco type (e.g., "American" blend), cigarette origin (e.g., Japan), and brand (e.g., Winston).

⁵ Sullivan MA, Paszkiewicz GP, Mahoney MC, Loree TR, Rigual NR and Pauly JL. The Unique autofluorescence of human buccal cells defined by high throughput multispectral flow cytometry. 2006 American Head and Neck Society Annual Meeting and Research Conference, August 17-20, 2006, Chicago, IL.

During the last several years, leading companies of the tobacco industry have introduced to the market cigarettes and cigarette-appearing nicotine delivery articles that are potentially reduced exposure products (reviewed in ref. 137). Potentially reduced exposure products are currently being marketed in the United States and other countries. There is a need to establish an organ- or tissue-specific biomarker of tobacco exposure and mutagenesis (4, 137). Mutation assays evaluated in this review are good candidates to address this subject.

True to all literature reviews is the fact that some articles escape identification. The search effort was without bias, and all identified articles have been included in this report. Individual studies differed greatly with regard to study purpose, scheme, assay procedures, study population, and other critical features. This precluded a meta-analysis of the data.

Despite these and other limitations, the preponderance of the information identifies a positive association of tobacco smoking with buccal cell mutations. This conclusion is consistent with previous reviewers who have shown a correlation between tobacco smoking and mutations of lymphocytes (reviewed in refs. 23, 24) and urinary bladder cells (reviewed in ref. 13). Accordingly, buccal cell mutations are useful biomarkers that are a useful adjunct to current and emerging clinical screening procedures.

Oral cancer is a lifestyle-related cancer with tobacco as a primary factor. Oral cancer, however, is the result of a long-standing process that progresses over several decades. Behavior intervention to quit smoking may be greatly facilitated if the subject is aware of risk markers that have been identified in the clinically normal oral mucosa. Thus, the identification of risk markers of oral cancer may serve as an aid in smoking cessation counseling.

Summarily, this literature review has identified many that document diverse tobacco-associated genetic and nongenetic buccal cell changes, that these changes have been assessed using different assays, that these changes may prove useful for smoking cessation and cancer prevention, that buccal cell micronuclei have been identified as useful biomarkers in clinicopathologic investigations, and that a high-throughput assay can be developed for screening smokers for the early detection of oral cancer.

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