ARTICLES

Histopathologic and Molecular Alterations in Bronchial Epithelium in Habitual Smokers of Marijuana, Cocaine, and/or Tobacco

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Background: **Tobacco smoking has been observed to cause molecular alterations in bronchial epithelium that antedate the development of lung carcinoma. The rising prevalence of marijuana and cocaine use among young adults in the United States prompted us to investigate whether similar molecular and histopathologic alterations occur in habitual smokers of marijuana and/or cocaine who may or may not also smoke tobacco.** ***Methods:*** **Bronchoscopy was performed in 104 healthy volunteer subjects, including 28 nonsmokers and 76 smokers of one or more of the following substances: marijuana, tobacco, and/or cocaine. Bronchial mucosa biopsy specimens and brushings were analyzed for histopathologic changes, for immunohistopathologic expression of intermediate or surrogate end-point markers that are linked to an increased risk of cancer (Ki-67 [a marker of cell proliferation], epidermal growth factor receptor, p53, Her-2/neu [also known as erbB-2 and ERBB2], globular actin, and abnormal DNA ploidy). Reported *P* values are two-sided.** ***Results:*** **Smokers of any one substance or of two or more substances exhibited more alterations than nonsmokers in five to nine of the 10 histopathologic parameters investigated (all $P<.05$), and they exhibited more molecular abnormalities than nonsmokers. Differences between smokers and nonsmokers were statistically significant (all $P\leq.01$) for Ki-67, epidermal growth factor receptor, globular actin, and DNA ploidy. There was general agreement between the presence of molecular abnormalities and histopathologic alterations; however, when disagreement occurred, the molecular abnormalities (e.g., Ki-67 and epidermal growth factor receptor) were more frequently altered (all $P\leq.01$).** ***Conclusions:*** **These findings suggest that smoking marijuana and/or cocaine, like tobacco smoking, exerts field cancerization effects on bronchial epithelium, which may place smokers of these substances at increased risk for the subsequent development of lung cancer. [J Natl Cancer Inst 1998;90:1198–1205]**

Tobacco smoking is associated with an increased risk for lung cancer *(1)*. In smokers, both histopathologic and molecular alterations have been observed in bronchial epithelium adjacent to the lung cancer *(2,3)*.

Recognition that clinical cancer is the end point of a series of genetic and epigenetic alterations in epithelium at risk is central to the concept of ''field cancerization'' *(4)*. This hypothesis states that, when a given area is exposed to the effects of exogenous carcinogens, it is at increased risk for developing cancer even though cancers that eventually arise are themselves monoclonal in origin (i.e., derived from single transformed cells) *(5)*. This field disease concept can be applied to a number of different human cancers, especially those of the bladder, head and neck, and lung. The genotypic and phenotypic properties of a given tumor represent the end product of a complex process driven by genetic instability and epigenetic factors operating within the cancerization field. This phenomenon provides a model for determining the molecular markers linked to the emergence of the malignant phenotype. Molecular and histopathologic alterations identified in bronchial epithelium of cigarette (tobacco) smokers have shed light on some of the steps of lung cancer progression *(2,3,6–9)*.

Because of the rising prevalence of marijuana and ''crack'' cocaine use among young individuals in the United States *(10)*, we undertook cohort studies to evaluate the impact of habitual smoking of marijuana, cocaine, and/or tobacco on bronchial histopathology. Some of the histopathologic findings from these studies have been reported previously *(11,12)*. Furthermore, in a subset of marijuana and cocaine smokers, we investigated whether molecular alterations similar to those noted in tobacco smokers also occurred in habitual smokers of marijuana and/or cocaine and whether the presence of these molecular changes correlated with histopathologic alterations in these subjects. We also measured associations between new molecular markers and use of marijuana and/or cocaine in these groups.

Molecular markers that have shown promise as intermediate or surrogate end-point markers in lung and other cancers were chosen to supplement traditional bronchial histopathologic stud-

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See ''Notes'' following ''References.''

ies in these smokers. These markers included Ki-67, epidermal growth factor receptor (EGFR), p53, Her-2/neu (also known as erbB-2 and ERBB2), DNA ploidy, and globular actin (G-actin) *(13–17).* Each of these molecular markers has been shown to be altered in field cancerization studies of several different cancers.

Ki-67, a proliferation marker reflecting the percentage of cells in a given tissue actively engaged in cell division, is thought to be a marker of overall cancer risk *(13).*

EGFR, an integral membrane protein that binds epidermal growth factor and other ligands, is a surrogate end-point marker that is not altered by mutation, rearrangement, or amplification but is altered epigenetically. Increased EGFR expression has been observed previously in bronchial epithelium of tobacco smokers *(14).* Because basal cell (reserve cell) hyperplasia was a common histopathologic alteration observed in our cohorts of smokers of any one substance or combination of substances, increased expression of EGFR was considered not only a surrogate end-point marker but also a potential cause of these histopathologic alterations.

Her-2/neu, a member of the class I growth factor receptor tyrosine kinase family, is often amplified and overexpressed in both precancerous and invasive carcinomatous epithelium, especially in the breast but also elsewhere *(15);* therefore, we decided to study its alterations in bronchial epithelium.

p53 is one of the most common suppressor genes altered in human cancers. p53 alterations have been observed in dysplastic and metaplastic epithelium of tobacco smokers *(6),* and so we studied this marker in our cohort of marijuana and cocaine smokers.

DNA ploidy, a marker of genetic instability reflecting genetic alterations in both cancer and precancer cells, had been predictive of subsequent cancer risk in several studies *(16).* Because of the latter observations and the ease of screening our bronchial brushings for ploidy status, this marker was included in our study.

The cytoskeletal protein actin is involved in regulation of cell morphology, cell–cell interactions, motility, secretion, intracellular transport, endocytosis, exocytosis, and cell division. A complex polymerization–depolymerization reaction governs the relative levels of G-actin monomer and filamentous actin (F-actin) fiber. A growing body of evidence suggests that changes in actin are an important step in malignant transformation. Alterations of G-actin in bladder cancer fields have been shown in normal-appearing transitional epithelium adjacent to the cancer *(16).* These prior studies led to our selection of G-actin as one of the surrogate end-point markers for evaluation.

The results of these molecular studies and their relationship to the histopathologic alterations form the basis of this article.

Subjects and Methods

Healthy male and female subjects, 21–50 years of age, were recruited for bronchoscopic study from two cohorts of participants in two ongoing longitudinal studies *(18,19).* The first study, initiated in 1983, concerned the pulmonary effects of heavy habitual smoking of marijuana with or without tobacco (or the ''marijuana cohort''). The second study, initiated in 1988, examined the pulmonary consequences of smoking cocaine with or without smoking marijuana and/or tobacco (or the ''cocaine cohort''). Participants in both of these studies also included comparison groups of age-matched smokers of tobacco alone, as well as nonsmokers of similar age, residing in metropolitan Los Angeles. Accruals of subjects from these two cohorts for the present study occurred during the period from October 1993 through March 1996.

A total of 241 subjects from the marijuana and cocaine cohorts underwent bronchoscopy and bronchial mucosal biopsy for histopathologic evaluation; the results of these evaluations have been published *(10).* Of these 241 subjects, a subset of 104 (43%) was also evaluated specifically to determine whether particular molecular markers associated with cancer risk were present. Forceps biopsy and /or brush biopsy specimens of bronchial mucosa were used for these determinations and are the experimental samples of this study.

To be included in this bronchoscopic study, marijuana smokers were required to have a current smoking history of an average of 10 or more marijuana cigarettes per week for 5 years or longer. Cocaine smokers were included only if they had a history of current or recent (within 6 months) smoking of alkaloidal cocaine on a regular basis for 9 months or longer and in an average amount of 1.0 g or more per week during the past year. Exclusionary criteria included intravenous drug abuse six times or more per lifetime, smoking of other illicit substances 20 times or more per lifetime, a recent (within 3 weeks) upper or lower respiratory tract infection or a history of chronic lung disease (e.g., asthma or interstitial lung disease), previous or active tuberculosis, pneumonia within the past year, or substantial occupational exposure to dust or fumes.

All subjects answered a detailed respiratory and drug use questionnaire, modified from the respiratory questionnaire used by the American Thoracic Society/National Heart, Lung, and Blood Institute *(20)* and the National Institute on Drug Abuse National Survey on Drug Abuse *(21).* All subjects also underwent routine pulmonary function testing as described previously *(18,19).* Before bronchoscopy, all subjects signed an informed consent form approved by the School of Medicine Human Subject Protection Committee of the University of California, Los Angeles, indicating their willingness to undergo fiberoptic bronchoscopy and bronchial mucosal brush and forceps biopsies. Screening procedures included a medical history, physical examination, 12-lead electrocardiogram, coagulation studies, and human immunodeficiency virus serology. Human immunodeficiency virus-seropositive individuals were excluded from the study.

Subjects were advised not to smoke or eat for at least 8 hours before the bronchoscopy. Topical anesthesia of the upper airway was achieved with 2%–4% lidocaine and 20% benzocaine. Subjects were sedated with midazolam (0.5–1.0 mg, administered intravenously) and meperidine (25–50 mg, administered intravenously) in accordance with the University of California, Los Angeles, Medical Center guidelines for conscious sedation. A flexible fiberoptic bronchoscope was then orally introduced with video monitoring. The larynx, trachea, and both main-stem bronchi and their branches down to the third order were endoscopically examined. Pinch forceps biopsies were performed of the mucosa of the primary carina and up to four secondary or tertiary carinae of the right or left lung, including the medial–basal segmental take-off of the right lower lobe bronchus, the take-off of the right upper lobe bronchus, the anterior–basal segmental take-off of the left lower lobe bronchus, and the bifurcation between the lingula and the left upper lobe bronchus. Bronchial brushings were also obtained from both the right and left main-stem bronchi.

Bronchial biopsy specimens and brushings were fixed separately in buffered 10% formaldehyde or 95% ethanol, sectioned, and processed for histopathologic and cytologic examination. Sections from biopsies obtained from the last 104 subjects who underwent bronchoscopy were examined by light microscopy by a pathologist (S. H. Barsky), who was not informed about the clinical and smoking histories of the subjects. Specimens were systematically evaluated according to criteria modified from those used by Auerbach et al. *(2).* Specifically, tissues were examined to assess proliferative, metaplastic, and dysplastic changes in the epithelium, as well as inflammatory and connective tissue alterations in the epithelium, basement membrane, and subepithelium.

Paraffin-embedded sections (5 μm thick) from bronchial biopsy specimens were processed for immunocytochemical analyses to assess expression of Ki-67, EGFR, p53, and Her-2/neu. Cytology preparations of bronchial brushings were processed for quantitative fluorescence image analysis to determine DNA ploidy and to measure G-actin *(16).* Reagents were purchased from the following sources: HemoDe (product 15-182-507A; Fisher Scientific Co., Pittsburgh, PA), Triton X-100 (product T-6878; Sigma Chemical Co., St. Louis), Crystal Mount (product BM-M02; Fisher Scientific Co.), thimerosol (product T-5125; Sigma Chemical Co.), citric acid (product C-0759; Sigma Chemical Co.), albumin (product A6003; Sigma Chemical Co.), and 30% hydrogen peroxide (product H-1009; Sigma Chemical Co.). Antibodies and other immunohistochemical reagents were purchased from the following sources: Ki-67 (Dako Corp., Carpinteria, CA), p53 Ab2 (Oncogene Science, Inc., Cambridge, MA), c-neu Ab3 (OP15; Oncogene Science, Inc.), EGFR (Ciba-Corning Diagnostic Corp., East Walpole, MA), biotinylated goat anti-mouse immunoglobulin (product 62-6540;

Zymed Laboratories Inc., South San Francisco, CA), and horseradish peroxidase-conjugated streptavidin (SA-5004; Vector Laboratories, Inc., Burlingame, CA). For detection of Ki-67, Her-2/neu, and p53, slides were processed with microwave antigen retrieval *(22–24)* by boiling the sections in 10 m*M* citric acid for 30 minutes. For detection of EGFR, the antigen was retrieved by incubating in Nargase (product P-4789; Sigma Chemical Co.) *(23)* for 5 minutes at room temperature. Sections were then incubated with primary antibody (diluted 1 : 100 for Ki-67, 1 : 300 for Her-2/neu, 1 : 100 for p53, and 1 : 10 for EGFR) overnight in a hydration chamber at room temperature. Sections were incubated with biotinylated goat anti-mouse immunoglobulin G diluted 1 : 200 for 30 minutes at 37 °C followed by incubation with horseradish peroxidase-conjugated streptavidin diluted 1 : 200 for 15 minutes. Antigen-binding sites were then visualized by incubation with a peroxidase substrate, which was hydrolyzed to an insoluble brown precipitate. All bronchial epithelial cells present in the biopsy sample were evaluated; they included several hundred cells over three to five high-magnification fields. Ki-67 measurements were recorded as percentage of bronchial cells that showed nuclear staining (number of positive cells/number of total bronchial cells × 100%). Samples with 5% or more positive cells were interpreted as being abnormal (a positive result). EGFR immunostaining showed weak basal staining along the basement membrane side of the reserve cells (negative staining) or intense circumferential staining throughout most bronchial layers (abnormal staining) (a positive result). Any p53 nuclear staining was interpreted as abnormal (a positive result). Her-2/neu membrane staining was uniformly absent.

Quantitative fluorescence image analysis was used to measure DNA ploidy and G-actin. The slides were double-labeled for DNA with 15 μ*M* Hoechst 33258 and for G-actin with Texas red-conjugated deoxyribonuclease I (Molecular Probes, Inc., Eugene, OR) *(16)*. Corresponding controls included omitting primary antibodies and using cell lines known to be high and low expressors for each marker protein. A total of 50–100 cells per slide were measured by use of image analysis and quantitative fluorescence. Only bronchial cells were analyzed; inflammatory cells, including macrophages, polymorphonuclear leukocytes, and lymphocytes, were specifically excluded. G-actin was measured on a continuous scale of gray levels ranging from 0 (black) to 256. The average gray level of the pixels composing an image is proportional to the average concentration of the target molecule (e.g., G-actin within a cell) and is expressed in arbitrary units. The average gray level was corrected for background fluorescence by subtracting the mean average gray level of approximately 100 cells on negative control slides. Average gray levels greater than or equal to 60 arbitrary units were designated as abnormal. Abnormal DNA ploidy was recorded if any cells were detected with a DNA content of more than 5N (where 2N is the amount of DNA equivalent to the diploid chromosome complement).

The statistical analysis was as follows: Univariate statistics were calculated for baseline characteristics (age, current and lifetime smoking amounts, spirometry, and the presence of chronic respiratory symptoms) for each smoking group (based on tobacco, marijuana, and/or cocaine smoking status). Mean spirometric values were compared between smoking groups by use of Student's *t* test. The prevalence of abnormalities for each of 10 histopathologic features evaluated was also calculated and compared between smoking groups by use of the χ^2 or Fisher's exact test. The percentage of subjects in each smoking group exhibiting an abnormal molecular marker was calculated, and the number of abnormal markers present in each subject (zero to five markers) was tabulated. The prevalence of markers was compared between each pair of smoking groups by use of χ^2 tests. The relationship between smoking and the presence of abnormal molecular markers was further examined by logistic regression, with each marker as a dependent variable in separate analyses and three dummy independent variables representing each subject's smoking status with respect to tobacco, marijuana, and cocaine and with interaction terms for each pair of smoking variables. For bronchial mucosal biopsies, the κ statistic was used to assess intrasubject agreement between abnormalities in molecular markers and histopathologic alterations. Among discordant pairs (i.e., the presence of a normal molecular marker and an abnormal histopathologic feature or vice versa), McNemar's test was used to determine whether abnormalities were significantly more frequent in either parameter. All statistical tests were two-tailed and were considered significant for $P<.05$. SAS software *(25)* and BMDP software *(26)* were used for statistical analysis.

Results

Most subjects, including nonsmokers and smokers of marijuana, cocaine, and/or tobacco, were male (Table 1). Smokers in all categories were slightly older than the nonsmokers, but smokers of cocaine, marijuana, and/or tobacco were of comparable age. Smokers of tobacco in combination with marijuana and/or cocaine smoked less tobacco than tobacco-only smokers. Similarly, smokers of marijuana who also smoked tobacco and/or cocaine smoked less marijuana than marijuana-only smokers. On the other hand, smokers of cocaine alone and smokers of cocaine along with tobacco with or without marijuana admitted to similar amounts of cocaine use.

Compared with nonsmokers, smokers of all substances, either

Table 1. Characteristics of members of the various smoking groups*

			Tobacco		Marijuana		Cocaine	
Group	No. (% male)	Age, y (SD)	cigs/d	pk-y	mcigs/wk	mcig-y	g/wk	Months
NS	28	30.9	N/A	0.1	N/A	0.0	N/A	N/A
	(64)	(7.9)		(0.5)		(0.2)		
MS	12	38.2	N/A	1.2	20.7	48.5	N/A	N/A
	(83)	(8.7)		(4.0)	(21.7)	(55.0)		
CS	13	36.5	N/A	1.1	N/A	31.1	1.4	110.8
	(77)	(11.3)		(3.7)		(53.7)	(0.9)	(47.9)
TS	14	40.1	25.2	24.0	N/A	7.6	N/A	N/A
	(93)	(8.2)	(13.8)	(19.5)		(25.3)		
CMS	7	37.4	N/A	0.0	13.8	64.5	0.6	80.4
	(100)	(7.6)		(0.0)	(22.7)	(107.2)	(0.7)	(43.5)
CTS	9	41.8	13.9	14.8	N/A	28.3	1.4	111.1
	(67)	(5.6)	(7.7)	(10.5)		(43.9)	(1.2)	(64.9)
MTS	9	35.1	16.0	15.0	11.2	32.1	N/A	N/A
	(100)	(8.6)	(11.4)	(12.9)	(10.7)	(28.3)		
CMTS	12	41.0	21.0	21.3	2.0	20.7	1.2	99.8
	(67)	(7.0)	(21.1)	(23.9)	(2.2)	(18.0)	(1.2)	(91.9)

*Characteristics are number in group (No.) (% male), mean age (±1 standard deviation [SD]), and smoking characteristics (mean ± 1 SD) of subjects by smoking group. Definition of abbreviations: NS = nonsmokers; MS = marijuana-only smokers; CS = cocaine-only smokers; TS = tobacco-only smokers; CMS = smokers of cocaine and marijuana without tobacco; CTS = smokers of cocaine and tobacco without current marijuana; MTS = smokers of marijuana and tobacco without cocaine; CMTS = current smokers of cocaine, marijuana, and tobacco; cigs/d = number of cigarettes currently smoked per day; pk-y = the product of the number of packages of cigarettes smoked per day and the number of years of smoking; mcigs/wk = number of marijuana cigarettes currently smoked per week; mcig-y = the product of the number of marijuana cigarettes smoked per day and the number of years of smoking; g/wk = number of grams of cocaine smoked per week; months = the number of months cocaine was smoked regularly; N/A = not available.

alone or in combination with one or two additional substances, reported a higher frequency of symptoms of chronic bronchitis (cough and sputum for ⩾3 months of the year for ⩾2 years) and/or breathlessness or a higher incidence of acute bronchitic episodes. These findings were similar to those previously reported in different, although overlapping, cohorts of smokers and nonsmokers *(12)*.

Compared with nonsmokers, smokers of all substances exhibited essentially normal pulmonary function tests. Mean values for spirometric indices were within normal limits for subjects in all smoking groups. No differences were noted between groups of smokers and nonsmokers in either percent predicted values or in the frequency of abnormality (data not shown) for any of the lung function measures, except for lower mean values of the forced expired volume in 1 second/forced vital capacity ratio in smokers of marijuana and tobacco ($P = .005$) and smokers of cocaine, marijuana, and tobacco ($P = .045$) and of the percent of forced expired volume in 1 second predicted in smokers of cocaine, marijuana, and tobacco ($P = .018$). These results were also similar to those previously reported *(12)*.

Compared with nonsmokers, smokers of all substances in each category exhibited a number of histopathologic alterations of the tracheobronchial mucosa, including basal cell hyperplasia, stratification, goblet cell hyperplasia, squamous cell metaplasia, cell disorganization, nuclear variation, mitotic figures, increased nuclear/cytoplasmic ratio, basement membrane thickening, and subepithelial inflammation (Table 2). Smokers of marijuana alone or tobacco alone exhibited more frequent alterations than nonsmokers for all 10 histopathologic parameters measured, and the differences from nonsmokers were statistically significant for the majority of these features. Marijuana-only smokers exhibited at least as frequent, if not more frequent, alterations compared with tobacco-only smokers, but the differences were not statistically significant. Smokers of cocaine alone showed more frequent alterations than nonsmokers in all 10 features ($P = .002$; sign test), and the difference in the prevalence of alterations from nonsmokers was statistically significant (all $P<.05$) for five features (basal cell hyperplasia, cell disorganization, nuclear variation, basement membrane thickening, and subepithelial inflammation) and nearly statistically significant ($.05<P<.1$) for three additional features (stratification, increased nuclear/cytoplasmic ratio, and squamous cell metaplasia). Alterations were noted as frequently or more frequently in smokers of marijuana and tobacco than in any other smoking category for nine of the 10 features ($P = .02$; sign test for smokers of marijuana and tobacco versus all other smoking categories).

Compared with nonsmokers, smokers of all substances exhibited significantly more frequent abnormalities in Ki-67, EGFR, and p53 (Table 3). Her-2/neu was not observed to be abnormal in any of the subjects in this study. Ki-67 (Fig. 1, A) and EGFR (Fig. 1, B) were abnormal in biopsy specimens from a significantly greater proportion of subjects in all smoking categories than in biopsy specimens from nonsmokers. More frequent abnormal expression of Ki-67 occurred in smokers of marijuana, smokers of cocaine and marijuana, smokers of marijuana and tobacco, and smokers of cocaine, marijuana, and tobacco compared with nonsmokers (all $P<.01$), and expression of this marker in the smokers of marijuana and tobacco was significantly more common than in smokers of tobacco alone ($P<.048$). Abnormal expression of EGFR was more commonly noted in all groups of smokers compared with nonsmokers (all $P<.01$). Abnormal p53 (Fig. 1, C) was expressed in one of nine smokers of tobacco and marijuana, one of nine smokers of tobacco and cocaine, and one of 12 smokers of tobacco, marijuana, and cocaine. Thus, abnormal p53 was seen only in smokers of tobacco combined with one or two additional substances.

Compared with nonsmokers, a higher proportion of subjects in all smoking categories demonstrated abnormal DNA ploidy (Fig. 2, A and C) and G-actin (Fig. 2, B and D) in their bronchial cytologic brushings (Table 4). The differences in proportion of smokers and nonsmokers exhibiting these abnormalities were statistically significant for abnormal DNA ploidy among tobacco-only and cocaine-only smokers ($P = .01$ for both) and for abnormal G-actin among smokers of tobacco and marijuana and smokers of tobacco, marijuana, and cocaine ($P = .011$ for all).

Agreement or disagreement between abnormal molecular markers and histopathologic alterations was evaluated by the use of the κ statistic and McNemar's test (Table 5). A significant κ statistic implies greater agreement between the measures than would be expected by chance. A significant McNemar's test

Table 2. Percentage of subjects with histopathologic abnormalities in bronchial mucosal biopsy specimens by smoking group*

	% subjects with abnormalities							
Abnormality	NS (n = 28)	MS (n = 12)	CS (n = 13)	TS (n = 13)	CMS (n = 7)	CTS (n = 9)	MTS (n = 7)	CMTS (n = 12)
Basal cell hyperplasia	10.7	66.7†	46.2§	53.8‡	71.4‡	100.0†	100.0†	83.3†
Stratification	7.1	66.7†	30.8¶	46.2‡	28.6	77.8†	100.0†	66.7†
Goblet cell hyperplasia	7.1	50.0‡	15.4	84.6†	14.3	66.7†	85.7†	83.3†
Cell disorganization	3.6	50.0†	46.2‡	46.2‡	14.3	77.8†	100.0†	58.3†
Nuclear variation	7.1	50.0‡	46.2‡	38.5¶	14.3	77.8†	100.0†	58.3†
Mitotic figures	3.6	16.7§	7.7	15.4	0.0	33.3§	42.9§	33.3§
Increased nuclear/cytoplasmic ratio	3.6	50.0†	23.1¶	23.1¶	0.0	66.7†	71.4†	33.3§
Basement membrane thickening	10.7	50.0†	69.2†	53.8‡	42.9¶	77.8†	85.7†	91.7†
Subepithelial inflammation	42.9	83.3	100.0†	84.6‡	100.0‡	77.8	100.0‡	83.3§
Squamous cell metaplasia	3.6	25.0†	23.1¶	38.5§	28.6¶	66.7†	42.9§	33.3§

*Definition of abbreviations: NS = nonsmokers; MS = marijuana-only smokers; CS = cocaine-only smokers; TS = tobacco-only smokers; CMS = smokers of cocaine and marijuana without tobacco; CTS = smokers of cocaine and tobacco without current marijuana; MTS = smokers of marijuana and tobacco without cocaine; CMTS = current smokers of cocaine, marijuana, and tobacco.

†All $P<.001$; ‡all $P<.01$; §all $P<.05$; ¶all $P<.1$; comparison with percentage of abnormality among never smokers, by χ^2 or Fisher's exact test (both two-sided).

Table 3. Prevalence of abnormal amounts of Ki-67, epidermal growth factor receptor (EGFR), and p53*

Group	N	% of subjects with abnormality		
		Ki-67	EGFR	p53
NS	28	29	7	0
MS	12	92†	58†	0
CS	13	77	92	0
TS	14	57	50‡	0
CMS	7	57†	57†	0
CTS	9	89	67‡	11
MTS	9	100†	89†	11
CMTS	12	75†	67†	8

*Definition of abbreviations: NS = nonsmokers; MS = marijuana-only smokers; CS = cocaine-only smokers; TS = tobacco-only smokers; CMS = smokers of cocaine and marijuana without tobacco; CTS = smokers of cocaine and tobacco without current marijuana; MTS = smokers of marijuana and tobacco without cocaine; CMTS = current smokers of cocaine, marijuana, and tobacco. N = number in group.

†All $P<.001$; ‡all $P<.01$; comparison with percentage of abnormality among never smokers, by χ^2 or Fisher's exact test (both two-sided).

indicates that, when there is disagreement between the two measures, the disagreement tends to be in the direction of more abnormalities in either the molecular marker or the histopathologic feature. For Ki-67, good agreement was noted between this marker and most histopathologic features. When disagreement occurred, the molecular marker was more frequently abnormal than the histopathologic feature. For EGFR, good agreement was noted between this marker and several of the histopathologic features (especially those characterized by proliferative and metaplastic alterations). When disagreement was noted, the molecular marker was more commonly abnormal. Abnormal DNA ploidy showed good agreement with only three of the 10 histopathologic features (including mitotic figures and cell disorganization). When disagreement occurred, histopathologic features (including hyperplasia) were more commonly abnormal.

Discussion

This study extends our previous observations of bronchial histopathologic alterations in habitual smokers of marijuana, cocaine, and/or tobacco *(12)* by identifying significant molecular alterations within the bronchial epithelium of smokers of these same substances compared with findings in nonsmoking control subjects. Our results, therefore, suggest that smoking marijuana and/or cocaine, like smoking tobacco, exerts field cancerization effects on bronchial epithelium. Because the phenomenon of field cancerization and the multipath process of carcinogenesis are not regarded as a series of linear steps but rather are regarded as overlapping networks, multiple surrogate end-point markers instead of a single surrogate end-point marker should be used in any study designed to identify potential genetic or epigenetic alterations leading to cancer. Surrogate end-point markers that have been used in recent studies include histologic and morphometric markers, specific oncogenes/growth regulators, markers of genetic/epigenetic instability, proliferation, and differentiation *(27,28).* Surrogate end-point markers that belong to the latter two classes, in general, have proven to be the ideal biomarkers to study because they can be used to detect ''precancer'' cells before they become histopathologically evident. In our study, several of the surrogate end-point markers chosen (Ki-67 and EGFR) were in fact more commonly abnormal than the histopathologic alterations observed. Other surrogate end-point markers examined (DNA ploidy and G-actin) generally were not as abnormal as the histopathologic alterations studied. The reasons for this differing sensitivity in the surrogate end-point markers are not clear but may reflect the particular actions of the

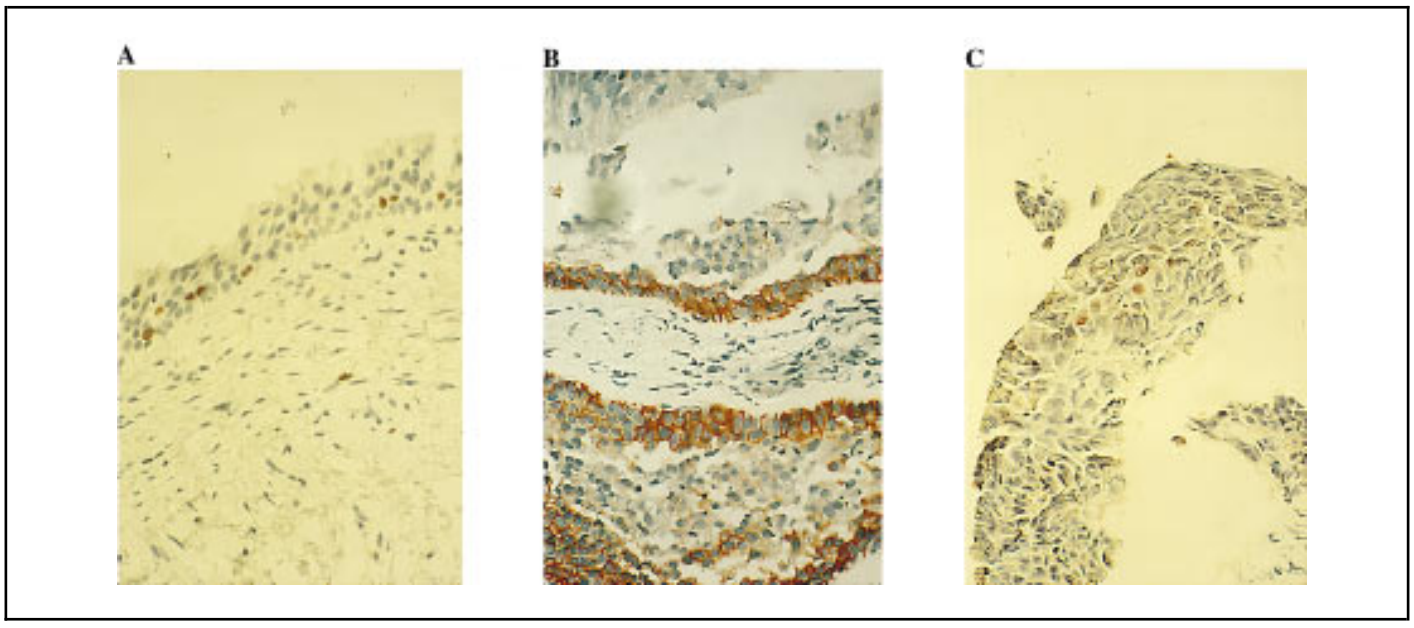

Fig. 1. Immunohistochemistry detection of Ki-67, epidermal growth factor receptor (EGFR), or p53 proteins in bronchial epithelium from representative cases. **A)** Ki-67 immunoreactivity is shown as a brown nuclear staining (original magnification ×250). **B)** EGFR immunoreactivity is shown as circumferential membrane staining in all layers of maturing bronchial epithelium (original magnification ×250). **C)** p53 immunoreactivity is shown focally as nuclear staining (original magnification ×400).

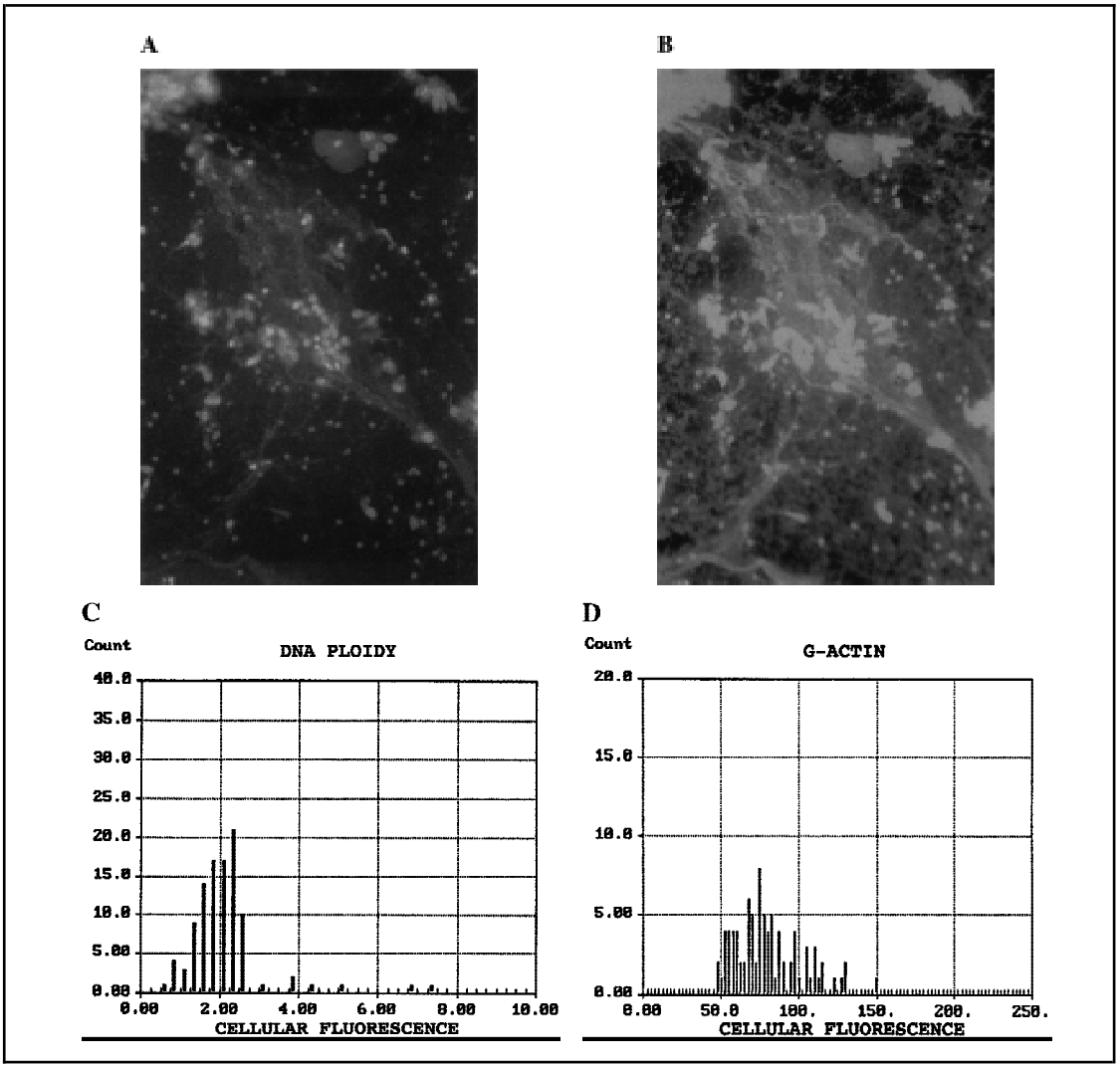

Fig. 2. A) DNA ploidy is measured as Hoechst 33258 nuclear fluorescence displayed by bronchial cells in bronchial brush cytologic preparations. **B)** This preparation was simultaneously double-layered with deoxyribonuclease I conjugated to Texas red to show cytoplasmic globular actin (G-actin). **C)** Histogram of DNA ploidy showing cellular populations containing a DNA content of more than 5N. Data are from one case subject exhibiting abnormal ploidy. **D)** Histogram of G-actin levels from the same case subject also exhibiting abnormal amounts of G-actin (⩾60 arbitrary units).

Table 4. Prevalence of abnormal DNA ploidy and globular actin in habitual smokers of marijuana, cocaine, and/or tobacco*

	DNA ploidy		Globular actin	
Group	N	%	N	%
NS	21	5	15	7
MS	8	13	8	13
CS	13	23†	12	50
TS	14	43†	14	14
CMS	7	43	7	29
CTS	8	38	8	25
MTS	6	33	6	67‡
CMTS	9	22	9	22‡

*Definition of abbreviations: NS = nonsmokers; MS = marijuana-only smokers; CS = cocaine-only smokers; TS = tobacco-only smokers; CMS = smokers of cocaine and marijuana without tobacco; CTS = smokers of cocaine and tobacco without current marijuana; MTS = smokers of marijuana and tobacco without cocaine; CMTS = current smokers of cocaine, marijuana, and tobacco; N = number in each group; % = percentage of abnormality.

†$P<.01$; ‡$P<.05$; comparison with percentage of abnormality among never smokers, by χ^2 or Fisher's exact test (both two-sided).

specific carcinogens and tumor promoters present in marijuana, cocaine, and/or tobacco.

In the field cancerization concept, key molecular and biochemical events are thought to occur before altered cellular morphology is apparent. Identification of abnormal molecular markers in individuals at a high risk for cancer suggests possible candidate markers that can better define high-risk subsets of individuals and can be used as surrogate end points in a chemoprevention study. In our study, we evaluated the occurrence of abnormal molecular markers in cohorts of smokers of marijuana and/or cocaine who also did or did not smoke tobacco for a different purpose, namely, to determine whether these substances also exert field cancerization effects on bronchial epithelium that may place smokers of these substances at increased risk for the subsequent development of lung cancer. Although the case subjects whom we studied were relatively young (mean age, 39 years old) and otherwise healthy (mostly asymptomatic with normal lung function), our results suggest that habitual smoking of marijuana, cocaine, and/or tobacco, either singly or in combination, has induced in the bronchial epithelial cells of these smokers molecular derangements that are associated with precancerous progression *(2,3,6,7)*. The effects of smoking marijuana and/or cocaine in combination with tobacco also show a trend toward additivity, suggesting that smoking more than one substance may be especially injurious to bronchial epithelium. In this regard, it is noteworthy that abnormal p53 expression was found only in combination smokers of tobacco and marijuana and/or cocaine.

Table 5. Comparison of molecular markers in bronchial epithelium with histopathology for smokers of marijuana, cocaine, and/or tobacco*

	All *P* values							
	Ki-67		EGFR		DNA ploidy		Globular actin	
Abnormality	κ statistic	McNemar's test	κ statistic	McNemar's test	κ statistic	McNemar's test	κ statistic	McNemar's test
Basal cell hyperplasia	<.01		<.01			<.001 (H)		<.001 (H)
Goblet cell hyperplasia	<.05	<.001 (M)	<.01			<.01 (H)		<.01 (H)
Cell disorganization	<.001	<.001 (M)	<.01		<.05	<.01 (H)		<.05 (H)
Nuclear variation	<.001	<.001 (M)	<.05		<.05	<.05 (H)		<.05 (H)
Mitotic figures		<.001 (M)		<.001 (M)				
Increased nuclear/cytoplasmic ratio	<.001	<.001 (M)		<.001 (M)	<.05			
Squamous cell metaplasia	<.001	<.001 (M)	<.05	<.001 (M)				

*Data were compared by using the κ test of agreement between measures and McNemar's test of direction of disagreement in discordant pairs. The *P* values are two-sided and refer to the statistical significance of the κ statistic or the McNemar's test for each pair of histopathologic and molecular variables for all smoking groups. For example, in the comparison involving goblet cell hyperplasia and Ki-67, the κ is significant (*P*<.001), meaning that there is greater agreement between the presence of goblet cell hyperplasia and the presence of an abnormal amount of Ki-67 in the bronchial biopsy specimens from all study subjects than would be expected by chance. In the same comparison, McNemar's test is also significant (*P*<.001), indicating that, for the discordant pairs (irrespective of the number of concordant pairs), Ki-67 (the molecular marker, or M) is more often abnormal than is goblet cell hyperplasia (the histopathologic alteration, or H) present. Definition of abbreviations: M = molecular marker more commonly abnormal; H = histopathology more commonly abnormal; EGFR = epidermal growth factor receptor.

Several lines of evidence implicate smoking of marijuana as a carcinogenic risk factor *(29)*. For example, the tar phase of marijuana smoke contains many of the same carcinogenic compounds present in tobacco smoke, including nitrosamines, reactive aldehydes, and up to 50% more polycyclic aromatic hydrocarbons, such as benz[*a*]anthracene and benz[*a*]pyrene *(30,31)*. The latter compound was found to cause mutations in the p53 gene *(32)*. On the other hand, little is known about the carcinogenic potential of crack cocaine, aside from studies suggesting an immunosuppressive effect of cocaine *(33,34)* and crack-related impairment in the tumoricidal activity of alveolar macrophages *(35)*. Further studies are required to investigate the role of cocaine on tumorigenesis.

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Notes

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