(23) Crouch EC, Stone KR, Bloch M, McDivitt RW. Heterogeneity in the production of collagens and fibronectin by morphologically distinct clones of a human tumor cell line: evidence for intratumoral diversity in matrix protein biosynthesis. Cancer Res 1987;47: 6086–92.
(24) Vince GH, Wagner S, Pietsch T, Klein R, Goldbrunner RH, Roosen K, et al. Heterogeneous regional expression patterns of matrix metalloproteinases in human malignant gliomas. Int J Dev Neurosci 1999;17:437–45.
(25) Anzai H, Kitadai Y, Bucana CD, Sanchez R, Omoto R, Fidler IJ. Intratumoral heterogeneity and inverse correlation between expression of E-cadherin and collagenase type IV in human gastric carcinomas. Differentiation 1996;60: 119–27.
(26) Kuniyasu H, Troncoso D, Johnston D, Bucana CD, Tahara E, Fidler IJ, et al. Relative expression of type IV collagenase, E-cadherin, and vascular endothelial growth factor/vascular permeability factor in prostatectomy specimens distinguishes organ-confined from pathologically advanced prostate cancers. Clin Cancer Res 2000;6:2295–308.
(27) Sekiya S, Oosaki T, Takamizawa H. Tumor angiogenesis activity of human choriocarcinoma cells grown *in vitro*. Gynecol Oncol 1986; 25:271–80.
(28) Gullino PM. Natural history of breast cancer. Progression from hyperplasia to neoplasia as predicted by angiogenesis. Cancer 1977;39 (6 Suppl):2697–703.
(29) Nielsen M, Thomsen JL, Primdahl S, Dyreborg U, Andersen JA. Breast cancer and atypia among young and middle-aged women: a study of 110 medicolegal autopsies. Br J Cancer 1987;56:814–9.
(30) Harach HR, Franssila KO, Wasenius VM. Occult papillary carcinoma of the thyroid. A "normal" finding in Finland. A systematic autopsy study. Cancer 1985;56:531–8.
(31) Montie JE, Wood DP Jr, Pontes JE, Boyett JM, Levin HS. Adenocarcinoma of the prostate in cystoprostatectomy specimens removed for bladder cancer. Cancer 1989;63:381–5.
(32) Hart IR. Perspective: Tumour spread—the problems of latency. J Pathol 1999;187:91–4.
(33) Enzinger FM, Weiss SW. Soft tissue tumors. 3rd ed. St. Louis (MO): Mosby; 1995. p. 331–466.

NOTES

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Long-Term Impact of Smoking on Lung Epithelial Proliferation in Current and Former Smokers

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Background: **Lung cancer risk remains elevated for many years after quitting smoking. To assess using proliferation indices in bronchial tissues as an intermediate endpoint biomarker in lung cancer chemoprevention trials, we determined the relationship between the extent, intensity, and cessation of tobacco smoking and proliferative changes in bronchial epithelial biopsy specimens.** ***Methods:*** **Bronchial biopsy specimens were obtained from up to six epithelial sites in 120 current smokers (median pack-years, 42) and 207 former smokers (median pack-years, 40; median quit-years, 8.1). Sections from the paraffin-embedded specimens were stained with hematoxylin–eosin to determine the metaplasia index and with an antibody to Ki-67 to determine the proliferative (labeling) index for the basal and parabasal (Ki-67 PLI) layers. All statistical tests were two-sided.** ***Results:*** **Biopsy sites with metaplasia had statistically significantly higher Ki-67-labeling indices than those without metaplasia (*P*<.001) in both current and former smokers. Increased proliferation was observed in multiple biopsy sites, with the average Ki-67 PLI of the subject strongly correlating with the metaplasia index (*r* = .72 for current smokers; *P*<.001), even in sites without metaplasia (*r* = .23 for current smokers; *P*<.001). In current smokers, the Ki-67 PLI was associated with the number of packs smoked/day (*P* = .02) but not with smoking years or pack-years. In subjects who had quit smoking, the Ki-67 PLI dropped statistically significantly within 1 year (*P* = .008) but remained detectable for more than 20 years, even in the absence of squamous metaplasia.** ***Conclusion:*** **Smoking appears to elicit a dose-related proliferative response in the bronchial epithelia of active smokers. Although the proliferative response decreased gradually in former smokers, a subset of individuals had detectable proliferation for many years and may benefit from targeted chemoprevention. Bronchial epithelial proliferation, measured by Ki-67, may provide a useful biomarker in the assessment of lung cancer risk and in the response to chemopreventive interventions. [J Natl Cancer Inst 2001;93:1081–8]**

Lung cancer remains the leading cause of cancer death for men and women in the United States, with 169 500 new lung cancer patients and 157 400 lung cancer deaths in the year 2001 *(1)*. Despite improvements in staging and multimodality therapeutic approaches, the overall 5-year survival rate remains at 14% *(1)*. Even if initial treatment is successful, these patients still develop second primary lung cancers at a rate of 1%–5% per year for non-small-cell lung cancer *(2,3)* and 2%–14% per year for small-cell lung cancer *(2)*. Thus, it is important to focus on early detection or chemoprevention.

Several problems confront lung chemoprevention studies. First, it is difficult to identify individuals at highest lung cancer risk. Epidemiologic studies *(4–7)* suggest that lung cancer risk is a function of the cumulative tobacco smoking exposure and the intrinsic susceptibility of the exposed individual. Although individuals with a 20-pack-year smoking history have an approximately 10-fold increased lung cancer risk, only 10% may develop lung cancer in their lifetime. Moreover, lung cancer risk remains considerably elevated for many years after quitting smoking, such that nearly half of the

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newly diagnosed lung cancer cases in the United States occur in former smokers *(8,9)*. Thus, lung cancer prevention studies that use lung cancer incidence as a primary endpoint require tens of thousands of subjects and tens of years of duration for adequate statistical interpretation. Unfortunately, all lung cancer prevention studies reported so far have had negative results and, in some cases, individuals who continued to smoke during the intervention had an adverse outcome *(3,10–12)*. Second, it is difficult to identify potentially efficacious chemopreventive agents *(13,14)*. Unfortunately, little is known about lung tumorigenesis in humans or which molecular pathways to best target for chemoprevention. Therefore, biomarkers are needed to identify individuals at the highest risk for lung cancer and to provide intermediate surrogate markers of response. The development and incorporation of such biomarkers into chemoprevention trials would reduce the numbers of subjects required for adequate statistical evaluation, decrease the time needed to assess biologic efficacy, and help identify new molecular targets for intervention *(15)*.

Lung tumorigenesis involves a field cancerization process whereby the lung is exposed to a carcinogenic insult (e.g., tobacco smoke), setting off a chronic pattern of tissue damage and wound healing *(16)*. Chronic carcinogenic insult initiates a multistep process marked by cumulative histologic and genetic changes and proliferative clonal outgrowths throughout the lung *(17,18)*. The development of metaplasia is one such step that has been used as a biomarker. In a study of 20-pack-year smokers *(19)*, where bronchial biopsy specimens were obtained from six independent sites, squamous metaplasia was evident in at least one of the six sites in nearly 80% of the smokers. However, if the subjects stopped smoking, the metaplasia index (MI) fell from 37% to 17% within 6 months, and squamous metaplasia disappeared altogether in 50% of the subjects. Thus, the use of bronchial metaplasia as a biomarker of risk and response in chemoprevention trials involving former smokers is problematic.

Because abnormal epithelial proliferation is one hallmark of tumorigenesis, it was of interest to explore the feasibility of using a proliferation biomarker to assess tobacco smoke-associated bronchial changes. Although proliferating cell nuclear antigen (PCNA) was shown to correlate well with histologic changes in the lungs of chronic smokers *(20)*, there is some concern regarding its use as a proliferation marker because PCNA is also a component of a DNA repair pathway, which may be active in chronically damaged tissues, such as the smoking-exposed lung *(21)*.

A number of additional proliferation markers are available that identify different aspects of proliferating cells *(22)*. For example, Ki-67 is a proliferation marker that is expressed in all phases of cycling cells but not in resting cells *(23)*. In lung tumors, the fraction of Ki-67-positive cells was generally increased and of some prognostic value, especially when used in combination with other tumor parameters *(24,25)*. Increased numbers of Ki-67-positive cells have also been detected in the aerodigestive tract epithelium of smokers compared with nonsmokers *(26,27)*. However, little is known about the relationship between Ki-67 expression, smoking history, and the long-term consequences of quitting smoking on the proliferation of bronchial epithelium.

In this report, we determined the feasibility of Ki-67 staining as a biomarker for use in chemoprevention trials. In particular, we determined the relationship between the Ki-67-labeling index in the bronchial epithelium of chronic smokers, squamous metaplasia, and the subject's smoking characteristics and determined the impact of quitting smoking on the proliferative activity in the bronchial epithelium.

Subjects and Methods

Subject Selection and Clinical Trial Design

Baseline tissue samples from 120 current and 207 former smokers participating in two randomized chemoprevention trials were analyzed in this report. Current smokers were defined as active smokers or smokers who had quit smoking within 1 year of registration onto the respective chemoprevention trials; former smokers were those who quit smoking for at least 1 year at the time of registration. Smoking history was measured by packs/day, smoke-years, and pack-years, which is the product of packs/day and smoke-years.

The first trial was designed to study the chemopreventive effect of *N*-(4-hydroxyphenyl)retinamide in smokers with a minimum of 20 pack-years of smoking history and detectable bronchial squamous metaplasia and/or dysplasia at the time of the screening bronchoscopy. Study subjects may have had a prior tobacco-related cancer but must have been tumor free for at least 6 months before enrollment *(28)*. One hundred thirty-nine smokers were registered in the first trial from April 1994 through December 1998 and, among them, 137 (120 current smokers and 17 former smokers; 80 randomly assigned and 57 not randomly assigned) had tissue samples available for the biomarker analysis. The presence of squamous metaplasia was required for the subjects to be randomly assigned to receive the chemopreventive agent or placebo but was not necessary for the subjects to be included in this report.

The second ongoing, randomized trial is evaluating the effect of 13-*cis*-retinoic acid plus α-tocopherol versus 9-*cis*-retinoic acid versus placebo in former smokers. To be eligible for receiving randomly assigned treatment in the trial, former smokers needed to have a minimum of 20 pack-years of smoking history but did not need to have squamous metaplasia or dysplasia. One hundred ninety former smokers were registered in the trial from November 1995 through June 2000 (target sample size for registration = 225). All registered subjects in both trials, whether randomly assigned or not, who had available baseline tissue samples were included in this report.

Both trials were approved by the Institutional Review Board, and all study subjects provided written informed consent.

Bronchial Biopsy and Tissue Preparation

A baseline bronchoscopy was performed for all participants at the time of registration for their respective chemoprevention trial. Biopsy specimens were taken at six predetermined sites in the bronchial tree, including the main carina, the bifurcation of the right upper lobe and the mainstem bronchus, the bifurcation of the right middle lobe and right lower lobe, the bifurcation of the left upper lobe and lingula, the medial bronchus of the right lower lobe, and the anterior bronchus of the left lower lobe. The biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. The first 10 4-μm tissue sections from each biopsy site were stained with hematoxylin–eosin and evaluated for the presence of squamous metaplasia and dysplasia. The MI was calculated as the percentage of biopsy sections exhibiting squamous metaplasia out of the total number of sections examined *(19)*.

Immunohistochemical Analysis of Ki-67

One 4-μm tissue section from each biopsy specimen was immunocytochemically stained for Ki-67 expression and evaluated for proliferative activity. A positive control section of HeLa cells was placed on each sample slide to serve as an internal control for the immunostaining procedure. The tissue sections were deparaffinized in xylene, rehydrated through a series of alcohols, and immersed in 3% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity. Antigen retrieval was accomplished by placing slides in citrate buffer (pH = 6.0) and heating in a 800-W microwave at 100% power for two 4-minute periods. The slides were then blocked in 2% horse serum in phosphate-buffered saline (PBS) at 37 °C for 30 minutes, treated with a 1 : 10 dilution of MIB 1 mouse anti-Ki-67 antibody (Zymed Laboratories Inc., South San Francisco, CA), and incubated overnight at 4 °C. After the slides were brought to room temperature for 15 minutes and sequentially washed in PBSD

(PBS + 0.1% Tween 20) and PBS, biotinylated anti-mouse immunoglobulin G secondary antibody (Vector Laboratories, Inc., Burlingame, CA) was applied in blocking solution (2% horse serum) and incubated at 37 °C for 30 minutes. The slides were washed sequentially with PBSD and then PBS, processed with the ABC kit (Vector Laboratories, Inc.) according to the manufacturer's recommendation, and reacted with 0.5 mg/mL of diaminobenzidine and 0.6% hydrogen peroxide. The color reaction was stopped by washing the slides in water. The slides were lightly counterstained in hematoxylin, washed in water, allowed to dry, and mounted in Eukit.

The fraction of Ki-67-positive cells was determined separately in the basal, parabasal, and, whenever present, superficial epithelial layers of the bronchial biopsy specimens and were expressed as the percentage of cells with positive nuclear staining (labeling index). Slides with inadequate HeLa staining or bronchial sections lacking epithelial cells (i.e., tangential cuts) were excluded from the analysis. Among 327 subjects, 1558 biopsy specimens could be evaluated for a basal-layer Ki-67-labeling index (Ki-67 BLI), and 1553 biopsy specimens could be evaluated for a parabasal-layer Ki-67-labeling index (Ki-67 PLI). Only 168 biopsy specimens had superficial layers that could be evaluated. Because about 2% of the cells in the superficial layer stained positive, we excluded the superficial layer Ki-67 results from the main analysis. Analyses of the Ki-67-labeling indices are presented both on a per-site basis and on a per-subject basis (the average of all biopsy specimens that could be evaluated within a participant).

Statistical Analysis

Summary statistics, including frequency tabulation, mean, standard deviation, median, and range, were given to characterize the distribution of covariates, as well as the cell proliferation indices (Ki-67 BLI and Ki-67 PLI) and MI. When the subject was used as the analysis unit, the mean Ki-67 value over six biopsy sites was computed for both the basal and the parabasal layers. Box plots were used to display the distribution of the three indices (Ki-67 BLI, Ki-67 PLI, and MI). Scatter plots with added nonparametric regression lines by use of lowess smoothing was applied to show the correlation between continuous variables. The Wilcoxon rank sum test was used to test for equal median between two continuous variables without the Gaussian distribution assumption. Spearman's rank correlation was applied to estimate the association between continuous variables. The between-subject and within-subject variabilities were estimated by the variance component model. The linear mixed-effect model was applied to model the effect of covariates on the three indices that used the biopsy site as the analysis unit but assumed that the site was nested within the subjects. Statistical analysis was performed by use of standard statistical software, including SAS Release 8.1 and S-Plus 2000 *(29,30)*. Only data from baseline specimens were included in this study. No attempt was made to analyze the treatment effect and biomarker modulation in this report. All of the *P* values reported were based on two-sided tests.

Results

The long-term impact of smoking on lung epithelial cell proliferation was assessed with 327 participants (120 current and 207 former smokers) (Table 1). Both sex and race were evenly distributed between current and former smokers. Former smokers were slightly older than current smokers (median, 57.4 years versus 52.0 years, respectively). There were 48 subjects (15%) with a prior smoking-related cancer history (34 lung, seven oral, four larynx, and three others) similarly distributed between current and former smokers. Current smokers had slightly higher smoke-years and pack-years than former smokers (median, 30 and 42 versus 28 and 40, respectively) but slightly lower smoking intensity (median packs/day, 1.3 versus 1.5, respectively). Except for age, no statistically significant difference was found in any of the medical, demographic, or smoking variables between the current and former smokers. There were 15 subjects in the current-smokers group who quit smoking within 1 year before registration onto the chemopreventive trials. Former smokers had quit smoking for 1.0–48.1 years (median, 8.1 years).

Fig. 1 shows that Ki-67 labeling was readily apparent in the basal and parabasal layers of the bronchial epithelium in both current and former smokers, even in the absence of squamous metaplasia. Current smokers had higher Ki-67-labeling indices and MIs than did former smokers on a per-subject basis ($P<.001$; Wilcoxon rank sum test; Table 1). The scatter plots in Fig. 2 show Ki-67-labeling indices in the basal and parabasal layers were highly correlated both in current (Spearman rank correlation, $r = .70$) and former ($r = .74$) smokers. However, the mean Ki-67-labeling index in the parabasal layer was approximately twofold higher than that in the basal layer (Table 1). Although the basal and parabasal Ki-67-labeling indices were highly correlated with MI (Fig. 2), the correlation was stronger for the parabasal layer (in current smokers, $r = .59$ versus .72 for basal versus parabasal layers, respectively; in

Table 1. Demographics and Ki-67-labeling and metaplasia indices in current and former smokers*

Demographic	Current smokers (n = 120)	Former smokers (n = 207)	All subjects (n = 327)
Sex			
Female	52 (43%)	87 (42%)	139 (43%)
Male	68 (57%)	120 (58%)	188 (57%)
Ethnicity/race			
White	110 (92%)	188 (91%)	298 (91%)
Other	10 (8%)	19 (9%)	29 (9%)
Age, y	51.9 ± 9.9 52.0 (30–77)	57.6 ± 9.7 57.4 (34–78)	55.6 ± 0.1 55.5 (30–78)
History of smoking-related cancers			
Yes	15 (13%)	33 (16%)	48 (15%)
No	105 (88%)	174 (84%)	279 (85%)
Packs/day	1.5 ± 0.6 1.3 (0.5–3.0)	1.8 ± 0.8 1.5 (0.8–4.0)	1.7 ± 0.7 1.5 (0.5–4.0)
Smoke-years	32.7 ± 10.0 30 (14–55)	28.1 ± 8.8 28 (12–50)	29.8 ± 9.5 30 (12–55)
Pack-years	48.5 ± 25.3 42 (15–150)	50.1 ± 29.0 40 (20–152)	49.5 ± 27.7 40 (15–152)
Quit-years	0.37 ± 0.28 0.26 (0.01–0.83)†	10.51 ± 9.03 8.09 (1.01–48.06)	9.82 ± 9.09 7.48 (0.01–48.06)‡
Basal layer Ki-67-labeling index§	6.3 ± 4.2 6.0 (0.4–22.7)	2.4 ± 2.2 1.9 (0–16.7)	3.8 ± 3.6 2.6 (0–22.7)
Parabasal layer Ki-67-labeling index§	12.7 ± 8.2 10.7 (0.6–38.1)	5.7 ± 5.7 3.6 (0–35.6)	8.3 ± 7.5 5.8 (0–38.1)
Metaplasia index§	27.9 ± 26.7 18.7 (0–100)	5.9 ± 11.2 0 (0–66.7)	14.0 ± 21.2 0 (0–100)

*For the continuous variables, mean ± standard deviation and median (range) are shown in the 1st and 2nd line, respectively.

†Summary statistics were based on 15 subjects who quit smoking within 1 year.

‡Summary statistics were based on all 222 subjects who quit smoking.

§Basal- and parabasal-layer Ki-67-labeling indices were calculated as the percentage of positively stained cells. Metaplasia index was calculated as the percentage of tissue sections having metaplasia. All three indices were statistically significantly different between current and former smokers ($P<.001$; Wilcoxon rank sum test).

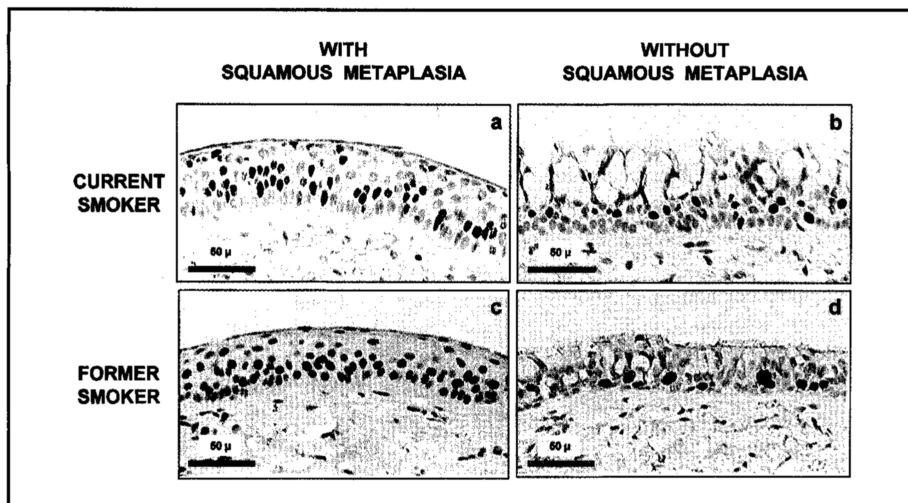

Fig. 1. Immunohistochemical detection of Ki-67 in bronchial biopsy specimens of current and former smokers. Lesions showed (**a**) squamous metaplasia in a current smoker, (**b**) goblet cell hyperplasia in a current smoker, (**c**) squamous metaplasia in a former smoker, and (**d**) goblet cell and basal cell hyperplasia in a former smoker. Note the differential labeling of basal and parabasal epithelial layers and the presence of measurable Ki-67 labeling in lesions without squamous metaplasia.

former smokers, r = .40 versus .47 for basal versus parabasal layers, respectively). Despite this strong correlation, there were many outliers with high Ki-67 PLI, especially in former smokers with low MI, suggesting that Ki-67-labeling indices may offer additional information beyond the MI.

In current smokers, 34.5% (205 of 595) of the biopsy sites had squamous metaplasia, compared with only 7.8% (75 of 958) in former smokers (Fig. 3, A). Biopsy sites with squamous metaplasia consistently had higher Ki-67 PLI than those without metaplasia in both current (median, 20.5 versus 6.3; P<.001; Wilcoxon rank sum test) and former (median, 21.6 versus 4.6; P<.001) smokers (Fig. 3, A). For biopsy sites with squamous metaplasia, there was no difference in Ki-67 PLI between current and former smokers (P = .35). By contrast, for biopsy sites without squamous metaplasia, current smokers had higher Ki-67 levels than former smokers (median, 6.3 versus 2.7; P<.001) (Fig. 3, A). Of interest, sites with increased Ki-67 PLI were observed frequently, even when these sites did not exhibit squamous metaplasia. In fact, in sites without squamous metaplasia, the Ki-67 PLI was still statistically significantly correlated with the MI in both the current and former smokers (r = .23 [P<.001] and r = .15 [P<.001], respectively; *see* supplementary Fig. 1 at the *Journal's* Web site [http://jnci.oup-journals.org]), suggesting that the Ki-67 PLI at any one site reflects the level of tobacco-induced, abnormal changes present throughout the lung. Within each subject, the variability in the Ki-67-labeling index increased with the mean Ki-67 value. Variance component analysis on the log-transformed Ki-67 PLI found that the between-subject and within-subject variances were 0.099 and 0.214, respectively, suggesting that multiple biopsy specimens from each individual are required to best estimate the proliferative activity present throughout the lung.

To better understand the factors influencing the correlation between proliferation and MI, we next examined their relationship with medical/demographic variables and smoking history in active smokers. Sex, ethnicity/race, and age did not correlate with proliferation or MI, but a trend toward higher Ki-67 BLI was observed in subjects with a history of smoking-related cancer (P = .10; Wilcoxon rank sum test). In current smokers, proliferation did not correlate with smoke-years or pack-years, and MI weakly correlated with pack-years (r = .19; P = .054). Moreover, in active smokers, smoking intensity (packs/day) positively correlated with Ki-67 BLI, Ki-67 PLI, and MI (P = .01, P = .02, and P =.001, respectively). However, the degree of variability between individuals who smoked comparable numbers of packs/day was high (*see* supplementary Fig. 2 at the *Journal's* website), suggesting that there were large differences in susceptibility to tobacco exposure between individuals. The association between smoking intensity and proliferation or MI was not seen in former smokers.

Of interest, in subjects who quit smoking, Ki-67 BLI, Ki-67 PLI, and MI were negatively correlated with number of years of smoking cessation (r = −.16 [P = .02], r = −.15 [P = .02] and r = −.15 [P = .02], respectively). We then further examined the kinetics between these parameters and years of smoking cessation. After adjustment for the squamous metaplasia status, the proliferation indices were statistically significantly decreased within the first year of smoking cessation (Q [quit]<1; P = .008; linear mixed-effect model for Ki-67 PLI), further decreased in years 1–2 (Q1–2) and in years 2–5 (Q2–5) after quitting smoking, and decreased only slightly thereafter (Fig. 3, B). In addition, the kinetics of the reduction in the MI after quitting smoking were more rapid than that of the proliferation indices, with the median MI decreasing from 26.7 to 0 within the first year and remaining at 0 thereafter. Only 28% of the former smokers had evidence of squamous metaplasia. The strong association between the reduction in Ki-67 PLI and quitting smoking after adjustment for squamous metaplasia supports that notion that Ki-67 assessment provides additional information beyond that of histology. More important, Ki-67 PLI remained measurable for many years after smoking cessation, and high Ki-67 PLI and Ki-67 BLI could still be detected even 20 years after quitting smoking in a subset of former smokers.

Discussion

The goal of this study was to explore the potential for use of proliferative activity in bronchial epithelium as a biomarker for lung cancer risk and chemopreventive responses in chronic smokers. The results provided two important findings. First, Ki-67-labeling indices were increased throughout the bronchial epithelium of active smokers and were correlated with smoking intensity (packs/day) and the presence of squamous metaplasia. Second, Ki-67-labeling indices were decreased in former smokers in a time-dependent manner. However, high Ki-67-labeling indices could still be detected in the bronchial epithelium in some former smokers long after they had quit smoking.

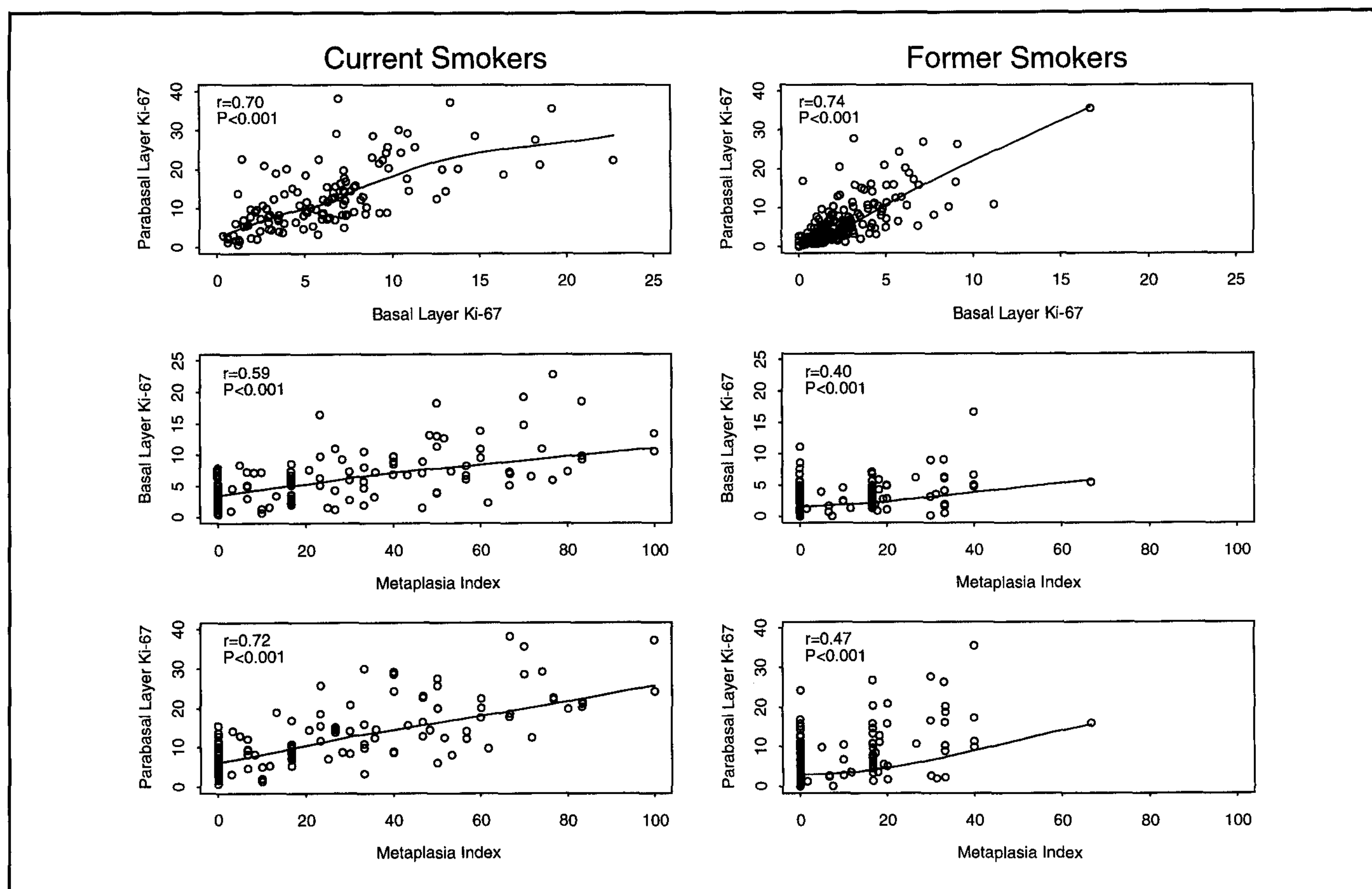

Fig. 2. Pairwise scatter plots for basal-layer Ki-67-labeling index, parabasal-layer Ki-67-labeling index, and metaplasia index in current and former smokers. The subject was used as the analysis unit. The nonparametric regression lines with lowess smoothing are shown in the plot. The Spearman's rank correlation coefficient (r) is indicated on the Fig. For all plots, the P value for testing zero correlation between any two markers was <.001.

Our results have several implications for the role of smoking in the pathogenesis of lung cancer *(31,32)*. The finding that a dose–response relationship exists between Ki-67-labeling indices and the intensity of smoking exposure measured by the number of packs smoked per day in active smokers suggests that smoking exposure elicits proliferative activity in the lung, perhaps associated with chronic wound healing that occurs after smoking-induced tissue damage. Moreover, the findings that 1) the levels of proliferation decreased in a time-dependent fashion after quitting smoking and 2) the relationship between the level of prior smoking intensity and proliferative activity was not seen after quitting smoking further supports a direct relationship between smoking exposure and proliferative activity.

Although the proliferative activity differed greatly among individuals who had comparable smoking histories, those who had increased proliferation at one lung site tended to have increased proliferation at other sites. These findings suggest that, although individuals may differ in their response to carcinogenic insult, the impact of the carcinogenic exposure can be detected throughout the lung, in any area from which a biopsy specimen can be taken. This observation provides direct support for the field-cancerization hypothesis. While the basis for interindividual susceptibility is not well understood, molecular epidemiologic studies *(33,34)* have identified several genetic and physiologic factors (i.e., cytochrome P450 and glutathione *S*-transferase polymorphisms, chromosome damage by mutagens, and DNA repair activities) that distinguish individuals with and without lung cancer. It is, therefore, of considerable interest to examine the relationship between the various intrinsic susceptibility factors, smoking histories, and proliferative activity in the lungs of smokers. Although the relationship between Ki-67-labeling indices and lung cancer risk is not yet known, it is intriguing that some individuals with a history of smoking-associated cancer had higher levels of proliferative activity in their bronchial epithelium, perhaps reflecting their known increased risk for second primary cancers.

Proliferative activity in the bronchial epithelium decreased with time in former smokers following smoking cessation. However, this study was cross-sectional in nature, with the conclusions based on single measurements of different individuals at different stages in their smoking history. Nevertheless, a statistically significant drop in proliferation within the first year of quitting smoking supports our previous longitudinal observations that used the proliferation marker PCNA *(20)*. The kinetics of the reduction in the Ki-67 PLI was slower than in the Ki-67 BLI. This suggests that the factors that control epithelial proliferation may be different between the basal layer, thought to harbor the reserve progenitor cells for tissue renewal, and the parabasal layer, thought to harbor the expanding pool of committed cells. Previously, we noted an association between PCNA expression and epidermal growth factor receptor expression in bronchial biopsy specimens from both active smokers and those who quit smoking dur-

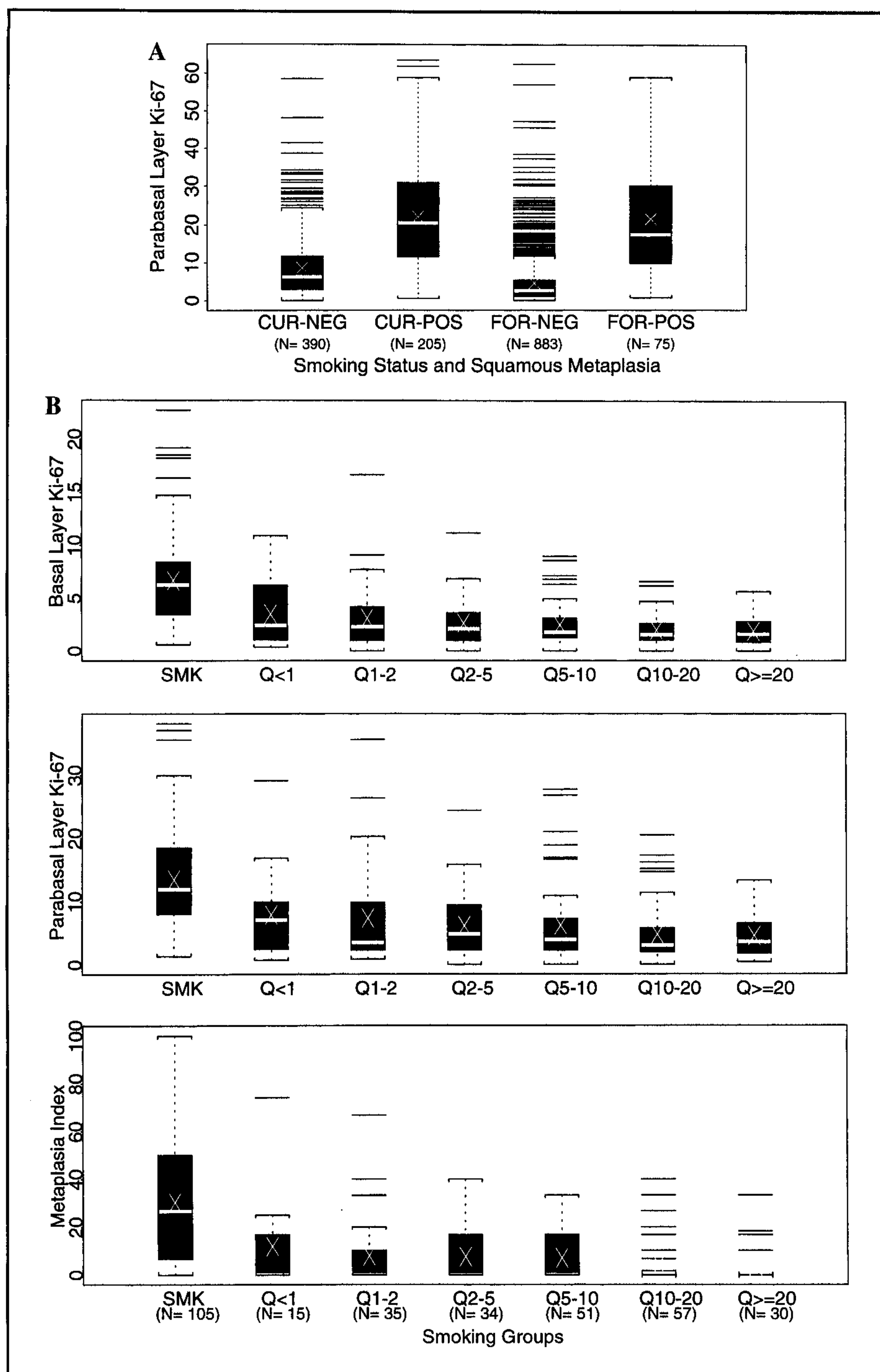

Fig. 3. Panel A: box plots for the parabasal-layer Ki-67-labeling index by smoking status and squamous metaplasia. The biopsy specimen site was used as the analysis unit. The *y-axis* represents the parabasal-layer Ki-67-labeling index expressed as the percentage of positively stained cells. The number of biopsy specimens (N) with adequate staining in each group is shown. CUR-NEG = current smokers without squamous metaplasia; CUR-POS = current smokers with squamous metaplasia; FOR-NEG = former smokers without squamous metaplasia, and FOR-POS = former smokers with squamous metaplasia. **Panel B: box plots** for the basal-layer Ki-67-labeling index, parabasal-layer Ki-67-labeling index, and metaplasia index by groups of smokers as a function of time after quitting smoking. For the **upper** and **middle panels,** the *y-axis* represents the Ki-67-labeling indices expressed as the percentage of positively stained cells. For the **lower panel,** the *y-axis* represents the metaplasia index expressed as the percentage of biopsy specimens with squamous metaplasia of the total number of sections examined. The number of subjects (N) with evaluable biopsy specimens in each group is shown. SMK = active smokers; Q (quit)<1 = quit smoking within 1 year of registration; Q1–2 = quit smoking from 1 to 2 years of registration, etc. For **panels A** and **B,** the **solid box** indicates the location of the middle 50% of the data (1st to 3rd quartile), with the median marked in the center as a **white stripe.** The location of the mean is identified by an **X.** The **brackets** indicate the data range from the end of the **middle box** up to 1.5 times the interquartile range. Data outside the brackets are marked with **individual bars.**

ing the first 6 months of chemopreventive intervention *(20).* We and others *(35,36)* have detected clonal genetic changes in these same biopsy specimens that might impact cell cycle regulation (i.e., loss of heterozygosity at chromosomes 9p and 3p involving genes for p16, fragile histidine triad, and other putative tumor suppressor genes). These biopsy specimens thus provide a unique opportunity to better understand the molecular forces driving proliferative clonal outgrowth in the lungs of current and former smokers.

Our results have several implications for lung cancer prevention. Primary chemoprevention trials that rely on the development of cancer as a primary endpoint are difficult to accomplish because of the large numbers of subjects and long follow-up times required. It is, therefore, important to identify surrogate endpoint biomarkers *(37,38).* Although histologic changes reflect abnormal regulation of the lung epithelia, they have some drawbacks as biomarkers. For example, squamous metaplasia can reflect a reactive condition and may rapidly disappear in individuals who quit smoking. This can create problems interpreting chemopreventive responses in former smokers where bronchial squamous metaplasia is an infrequent event. Moreover, in chemoprevention trials where the incidence of squamous metaplasia varies widely among individuals, large sample sizes would be required for testing treatment effects. Furthermore, the MI is a qualitative endpoint, and its subjectivity makes it prone to interobserver variations.

By contrast, Ki-67-labeling indices possess several desirable properties for their use as biomarkers in chemoprevention trials. First, Ki-67-labeling indices can be measured in former smokers long after they have quit smoking. Second, Ki-67-labeling indices can be potentially modulated by chemopreventive interventions. Third, Ki-67-labeling indices correlate with the MI so that elevated levels can be detected even in bronchial sites that lack histologic evidence of squamous metaplasia. Thus, Ki-67-labeling index at one site in the lung may reflect more global changes occurring throughout the lung. However, variance-component analysis suggests that multiple biopsy specimens are required to gain an adequate estimate for the whole lung.

Ki-67-labeling indices may also be useful markers for lung cancer risk. There was considerable interindividual variation

in the proliferative activity between those with comparable smoking histories, suggesting that Ki-67-labeling indices may reflect differential individual downstream consequences of both carcinogen exposure and intrinsic susceptibility factors. Moreover, subjects with a history of smoking-related cancer, known to be at an increased lung cancer risk, had elevated Ki-67 BLI. It is, therefore, intriguing that some individuals continued to have relatively high proliferative levels in their lung epithelium for many years after they quit smoking. Previously, we showed that normal and premalignant epithelia adjacent to head and neck cancers (i.e., epithelium in a tissue field with a 100% chance of developing a tumor) had increased proliferation levels, suggesting that the degree of abnormal epithelial proliferation may be related to long-term cancer risk *(39)*. Continued follow-up of the individuals participating in our chemoprevention trials will provide insight into the role of Ki-67 labeling as a marker of lung cancer risk.

Although dysregulated proliferation is a hallmark in tumorigenesis, it is unclear whether it is important in the evolution of a tumor. There is some evidence *(40)* to suggest that the loss of cell cycle control can lead to increased genetic instability, thus accelerating the accumulation of genetic changes important for tumor development. Similarly, dysregulated epithelial proliferation associated with altered epithelial–stromal interactions has also been associated with increased genetic instability and subsequent tumor development *(41)*. The same biopsy specimens examined in this study are also being examined for chromosome instability *(42)*, which will provide a unique opportunity to explore the relationship between altered epithelial proliferation and genetic instability. Several chemoprevention candidate agents are hypothesized to directly or indirectly target proliferative pathways *(14)*. The assessment of Ki-67 labeling as a biomarker should prove to be highly useful in chemoprevention studies, especially when considered with measurements of other markers known to influence tumorigenesis.

References

(1) Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics, 2001. CA Cancer J Clin 2001;51:15–36.

(2) Johnson BE, Cortazar P, Chute JP. Second lung cancers in patients successfully treated for lung cancer. Semin Oncol 1997;24:492–9.

(3) Lippman SM, Lee JJ, Karp DD, Vokes EE, Benner SE, Goodman GE, et al. Randomized Phase III Intergroup Trial of Isotretinoin to Prevent Second Primary Tumors in Stage I Non-Small-Cell Lung Cancer. J Natl Cancer Inst 2001;93:605–18.

(4) Peto R, Darby S, Deo H, Silcocks P, Whitley E, Doll R. Smoking, smoking cessation, and lung cancer in the UK since 1950: combination of national statistics with two case–control studies. BMJ 2000;321:323–9.

(5) Smith RA, Glynn TJ. Epidemiology of lung cancer. Radiol Clin North Am 2000;38: 453–70.

(6) Wei Q, Cheng L, Amos CI, Wang LE, Guo Z, Hong WK, et al. Repair of tobacco carcinogen-induced DNA adducts and lung cancer risk: a molecular epidemiologic study. J Natl Cancer Inst 2000;92:1764–72.

(7) Haugen A, Ryberg D, Mollerup S, Zienolddiny S, Skaug V, Svendsrud DH. Gene–environment interactions in human lung cancer. Toxicol Lett 2000;112–113:233–7.

(8) Lubin JH, Blot WJ. Lung cancer and smoking cessation: patterns of risk. J Natl Cancer Inst 1993;85:422–3.

(9) Tong L, Spitz MR, Fueger JJ, Amos CA. Lung carcinoma in former smokers. Cancer 1996;78: 1004–10.

(10) The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. N Engl J Med 1994;330:1029–35.

(11) Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, et al. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. N Engl J Med 1996;334:1150–5.

(12) van Zandwijk N, Dalesio O, Pastorino U, de Vries N, van Tinteren H. EUROSCAN, a randomized trial of vitamin A and *N*-acetylcysteine in patients with head and neck cancer or lung cancer. For the European Organization for Research and Treatment of Cancer Head and Neck and Lung Cancer Cooperative Groups. J Natl Cancer Inst 2000;92:977–86.

(13) Steele VE, Boone CW, Lubet RA, Crowell JA, Holmes CA, Sigman CC, et al. Preclinical drug development paradigms for chemopreventives. Hematol Oncol Clin North Am 1998;12: 943–61.

(14) Kelloff GJ, Sigman CC, Greenwald P. Cancer chemoprevention: progress and promise. Eur J Cancer 1999;35:2031–8.

(15) Lippman SM, Lee JJ, Sabichi AL. Cancer chemoprevention: progress and promise. J Natl Cancer Inst 1998;90:1514–28.

(16) Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium: clinical implications of multicentric origin. Cancer 1953;6:963–8.

(17) Auerbach O, Stout AP, Hammond EC, Garfinkel L. Changes in bronchial epithelium in relation to cigarette smoking and in relation to lung cancer. N Engl J Med 1961;265: 253–67.

(18) Hittelman WN. Genetic instability assessments in the lung cancerization field. In: Brambilla C, Brambilla E, editors. Lung tumors: fundamental biology and clinical management. New York (NY): Marcel-Dekker; 1999. p. 255–67.

(19) Lee JS, Lippman SM, Benner SE, Lee JJ, Ro JR, Lukeman JM, et al. Randomized placebo-controlled trial of isotretinoin in chemoprevention of bronchial squamous metaplasia. J Clin Oncol 1994;12:937–45.

(20) Khuri FR, Lee JS, Lippman SM, Lee JJ, Kalapurakal S, Yu R, et al. Modulation of proliferating cell nuclear antigen in the bronchial epithelium of smokers. Cancer Epidemiol Biomarkers Prev 2001;10:311–8.

(21) McCormick D, Hall PA. The complexities of proliferating cell nuclear antigen. Histopathology 1992;21:591–4.

(22) Yu CC, Woods AL, Levison DA. The assessment of cellular proliferation by immunohistochemistry: a review of currently available methods and their applications. Histochem J 1992;24:121–31.

(23) Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000;182:311–22.

(24) Hommura F, Dosaka-Akita H, Mishina T, Nishi M, Kojima T, Hiroumi H, et al. Prognostic significance of p27KIP1 protein and Ki-67 growth fraction in non-small cell lung cancers. Clin Cancer Res 2000;6:4073–81.

(25) Nguyen VN, Mirejovsky P, Mirejovsky T, Melinova L, Mandys V. Expression of cyclin D1, Ki-67 and PCNA in non-small cell lung cancer: prognostic significance and comparison with p53 and bcl-2. Acta Histochem 2000; 102:323–38.

(26) van Oijen MG, Gilsing MM, Rijksen G, Hordijk GJ, Slootweg PJ. Increased number of proliferating cells in oral epithelium from smokers and ex-smokers. Oral Oncol 1998;34: 297–303.

(27) Barsky SH, Roth MD, Kleerup EC, Siimmons M, Tashkin DP. Histopathologic and molecular alterations in bronchial epithelium in habitual smokers of marijuana, cocaine, and/or tobacco. J Natl Cancer Inst 1998;90:1198–205.

(28) Kurie JM, Lee JS, Khuri FR, Mao L, Morice RC, Lee, JJ, et al. *N*-(4-Hydroxyphenyl)retinamide in the chemoprevention of squamous metaplasia and dysplasia of the bronchial epithelium. Clin Cancer Res 2000;6:2973–9.

(29) SAS user guide. Cary (NC): SAS Institute Inc.; 1998.

(30) S-PLUS 2000 guide to statistics. Seattle (WA): MathSoft, Inc., 2000.

(31) Perera FP. Molecular epidemiology: insights into cancer susceptibility, risk assessment, and prevention. J Natl Cancer Inst 1996;88: 496–509.

(32) Hussain SP, Harris CC. Molecular epidemiology and carcinogenesis: endogenous and exogenous carcinogens. Mutat Res 2000;462: 311–22.

(33) Wu X, Shi H, Jiang H, Kemp B, Hong WK, Delclos GL, et al. Associations between cytochrome P4502E1 genotype, mutagen sensitivity, cigarette smoking and susceptibility to lung cancer. Carcinogenesis 1997;18:967–73.

(34) Wei Q, Spitz MR. The role of DNA repair capacity in susceptibility to lung cancer: a review. Cancer Metastasis Rev 1997;16: 295–307.

(35) Mao L, Lee JS, Kurie JM, Fan YH, Lippman SM, Lee JJ, et al. Clonal genetic alterations in the lungs of current and former smokers. J Natl Cancer Inst 1997;89:857–62.
(36) Wistuba II, Lam S, Behrens C, Virmani AK, Fong KM, LeRiche J, et al. Molecular damage in the bronchial epithelium of current and former smokers. J Natl Cancer Inst 1997; 89: 1366–73.
(37) Misset JL, Mathe G, Santelli G, Gouveia J, Homasson JP, Sudre MC, et al. Regression of bronchial epidermoid metaplasia in heavy smokers with etretinate treatment. Cancer Detect Prev 1986;9:167–70.
(38) Franklin WA. Diagnosis of lung cancer: pathology of invasive and preinvasive neoplasia. Chest 2000;117(4 Suppl 1):80S–89S.
(39) Shin DM, Voravud N, Ro JY, Lee JS, Hong WK, Hittelman WN. Sequential increases in proliferating cell nuclear antigen expression in head and neck tumorigenesis: a potential biomarker. J Natl Cancer Inst 1993;85:971–8.
(40) Tlsty TD. Genomic instability and its role in neoplasia. Curr Top Microbiol Immunol 1997; 221:37–46.
(41) Sternlicht MD, Lochter A, Sympson CJ, Huey B, Rougier JP, Gray JW, et al. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell 1999;98: 137–46.
(42) Hittelman WN, Lee JJ, Lee JS, Yu R, Kurie J, Mao L, et al. Persistent genetic instability despite decreased proliferation in human lung tissue following smoking cessation [abstract]. Proc Am Assoc Cancer Res 1998;39:336.

Notes

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Erythrocyte Membrane Fatty Acids and Subsequent Breast Cancer: a Prospective Italian Study

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Background: **The relationship between erythrocyte membrane fatty acids and postmenopausal breast cancer risk was analyzed previously only by retrospective studies, which suggested a protective effect of increased saturation index (SI), i.e., the ratio of membrane stearic to oleic acid. We investigated the relationships in a prospective study of hormones, diet, and prediagnostic breast cancer (the ORDET study) conducted in northern Italy.** *Methods:* **A total of 4052 postmenopausal women were followed for an average of 5.5 years; 71 cases of invasive breast cancer were identified. For each case subject, two matched control subjects were chosen randomly from among cohort members. The various fatty acids in erythrocyte membranes were measured as a percentage of total fatty acids. Conditional logistic regression analysis evaluated the association between membrane fatty acid composition and breast cancer risk. The SI, which is influenced by the activity of the enzyme delta 9 desaturase (Δ9-d), was also investigated. All statistical tests were two-sided.** *Results:* **Oleic (highest versus lowest tertile of percentage of total fatty acids, odds ratio [OR] = 2.79; 95% confidence interval [CI] = 1.24 to 6.28) and monounsaturated fatty acids (highest to lowest tertile, OR = 5.21; 95% CI = 1.95 to 13.91) were positively associated with breast cancer risk. The SI (highest to lowest tertile, OR = 0.29; 95% CI = 0.13 to 0.64) was inversely associated with breast cancer risk. The analysis suggested an inverse association between total polyunsaturated fatty acids and breast cancer risk, but individual polyunsaturated fatty acids behaved differently. There was no association between saturated fatty acids and breast cancer risk.** *Conclusions:* **We have found that monounsaturated fats and SI in erythrocyte membranes are predictors of postmenopausal breast cancer. Both of these variables depend on the activity of the enzyme Δ9-d. The dietary, metabolic, and hormonal factors acting on Δ9-d expression and activity and, therefore, on patterns of fatty acid metabolism, should be further investigated as possible determinants of breast cancer. [J Natl Cancer Inst 2001;93:1088–95]**

That dietary factors contribute to the etiology of human breast cancer has not been established unequivocally *(1)*; however, variations in fat consumption and metabolism are suspected to contribute to the marked regional differences in the incidence rates of breast cancer *(2,3)* and to the increases in risk that follow migration from a low-incidence to a high-incidence area *(4–6)*.

The fatty acid composition of the erythrocyte membrane, more than a mere biomarker of dietary fat intake, is the integrated result of interactions between dietary fatty acid intake, other dietary factors, and endocrine changes *(7–9)*, in part because of the long half-life (120 days) of erythrocytes. The parameter may, therefore, be an appropriate biomarker for investigating not only dietary intake but also the relations of the patterns of fatty acid metabolism to breast cancer risk.

The saturation index (SI) in red blood cell membranes is the ratio of stearic acid (the most common saturated fatty acid [SFA]) to oleic acid (the most common monounsaturated fatty acid [MUFA]). A reduction in SI was observed in patients with breast and other cancers in several *(10,11)* but not in all *(12,13)* case–control studies. Furthermore, in a recent prospec-

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